COMBINED APPLICATION OF TARGETED THERAPY AND IMMUNOTHERAPY IN CHRONIC MYELOID LEUKAEMIA

BY

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DECLARATION OF ORIGINALITY

This thesis is the result of full time study during the years 2008 to 2011. The work is my own and has not been submitted for the award of any degree at any other university. Where colleagues have made valuable contributions they have been acknowledged.

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Hugues de Lavallade 29th November, 2012

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ABSTRACT

Combining vaccination against leukaemia-derived antigens and treatment with tyrosine kinase inhibitors (TKI) in chronic phase CML is potentially a promising strategy to eradicate a reservoir of TKI resistant leukaemic cells. Previous studies have documented conflicting effects of TKIs on the immune response. I aimed to determine the in vivo immunomodulatory effects of TKIs on T and B-cell immune responses to antigens in patients with CML on TKIs.

I first demonstrated that that the B-cell response to H1N1 influenza vaccine was significantly better in patients with CML compared to patients with other haematological malignancies.

I then performed a more comprehensive analysis of T and B cell responses to a viral (seasonal influenza) and bacterial (pneumococcus) vaccine. I did not find a significant quantitative or qualitative difference in T cell responses to influenza vaccine in patients with CML on TKI compared to controls. However, I demonstrated that CML patients on TKIs have impaired IgM responses to pneumococcal vaccine, associated with lower frequencies of IgM memory B cells. Moreover, treatment with imatinib was associated with a significant reduction in IgM memory B cells. In vitro co-incubation of B-cells with plasma from CML patients on TKI or directly with imatinib, dasatinib or nilotinib, induced a dose-dependent inhibition of Bruton's tyrosine kinase, a tyrosine kinase essential for B cell signalling and survival. These data suggest that the loss of memory B-cell subsets and impaired humoral immune responses may be driven by the off-target kinase inhibitory activity of TKIs.

I further explored the implications of Philadelphia positive (Ph+) lymphopoiesis on B cell function. I found that nearly 50% of CML patients at diagnosis have evidence of

Ph+ B lymphopoiesis. Interestingly, the presence of Ph+ B cells predicted for worse prognosis, suggesting the involvement for a more committed progenitor with biphenotypic self-renewal capacity.

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ABBREVIATIONS

Allo-SCT	Allogeneic haematopoietic stem cell transplantation
APC	Antigen presenting cell
-APC	allophycocyanin
-APC-H7	allophycocyanin H7
BCR	B cell receptor
BFA	Brefeldine
Btk	Bruton's tyrosine kinase
CCyR q	complete cytogenetic remission
CD	cluster of Differentiation
CLL	Chronic lymphocytic leukaemia
CML	Chronic Myeloid Leukaemia
CML-CP	CML chronic phase
CMV	Cytomegalovirus
CTL	cytotoxic T-cell
DC	dendritic cell
DLI	Donor lymphocyte infusion
DMSO	DimethylSufoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EFS	event-free survival
FCS	foetal calf serum
FISH	fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate

FSC	Forward scatter
GMT	geometric mean titres
GVHD	Graft versus host disease
GVL	Graft versus leukaemia
HL	Hodgkin's lymphoma
HLA	Human leucocyte antigen
INF-α	Interferon-alpha
IFN-γ	Interferon-gamma
IL-	Interleukin
LAA	leukaemia associated antigens
LP	Leukapheresis
M	Molar
mAb	monoclonal antibodies
MCyR	major cytogenetic response
MNCs	Mononuclear cells
MRD	minimal residual disease
NHL	non-Hodgkin lymphoma
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer serum
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	phycoerythrin-Cyanine-7
PerCP	Peridinin chlorophyll protein
PerCp Cy5.5	peridinin chlorophyll protein Cyanine 5.5

Ph	Philadelphia
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PLCγ2	phospholipaseCγ2
PPS	pneumococcal polysaccharide
PR3	Proteinase 3
PRAME	Preferentially expressed antigen of melanoma
Qdot605	quantum dot 605
RQ-PCR	Real time quantitative PCR
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SFK	Src family kinase
TBI	Total body irradiation
TCR	T cell receptor
Th	T helper cells
TNF	Tumour necrosis factor
Treg	regulatory T-cell
U	Unit
WT1	Wilms tumour 1
TKI	Tyrosine kinase inhibitor

CHAPTER 1 INTRODUCTION

I.1 CHRONIC MYELOID LEUKAEMIA

Chronic Myeloid Leukaemia (CML) is a clonal myeloproliferative neoplasms originating from a single pluripotent hematopoietic stem cells in which cells of the myeloid lineage undergo inappropriate clonal expansion caused by a molecular lesion. The characteristic genetic abnormality of CML, the Philadelphia chromosome, results from a reciprocal translocation of genetic material on the long arms of one chromosome 9 and one chromosome 22 (Figure I.1). The molecular consequence of this translocation is the generation of a gene encoding the fusion protein BCR-ABL1, a constitutively activated tyrosine kinase (Figure I.2).

Figure I.1: The t(9;22) translocation and resulting mRNA and oncoprotein (Goldman & Melo, 2003)

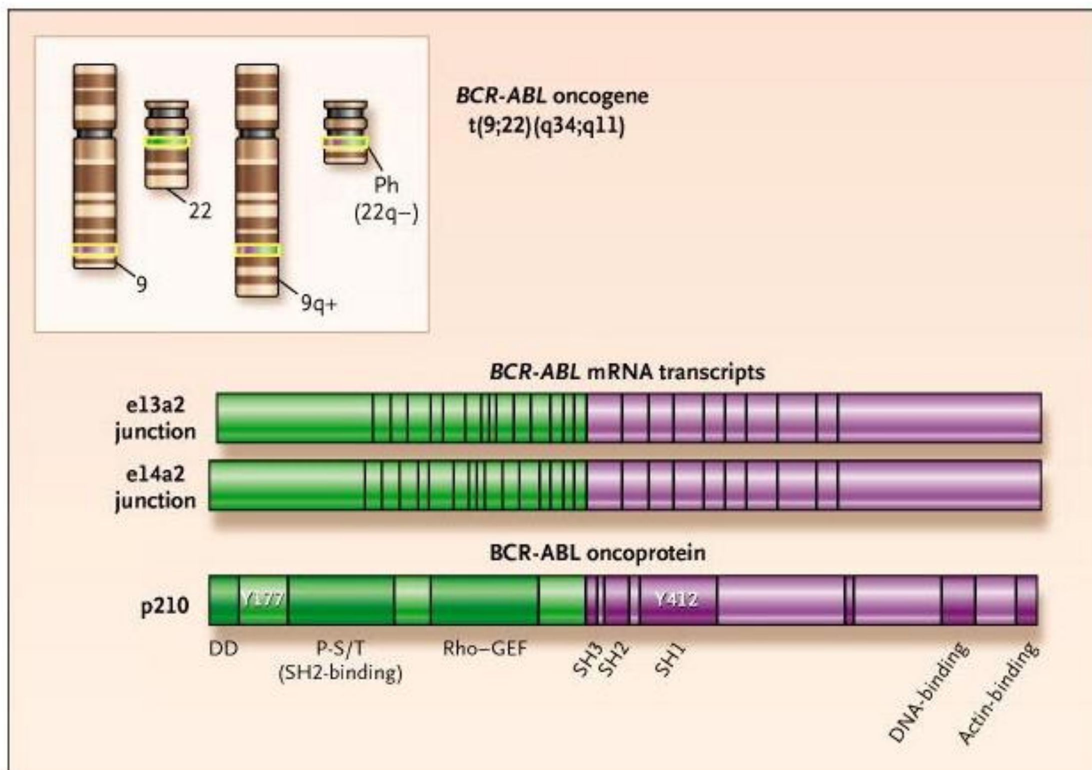
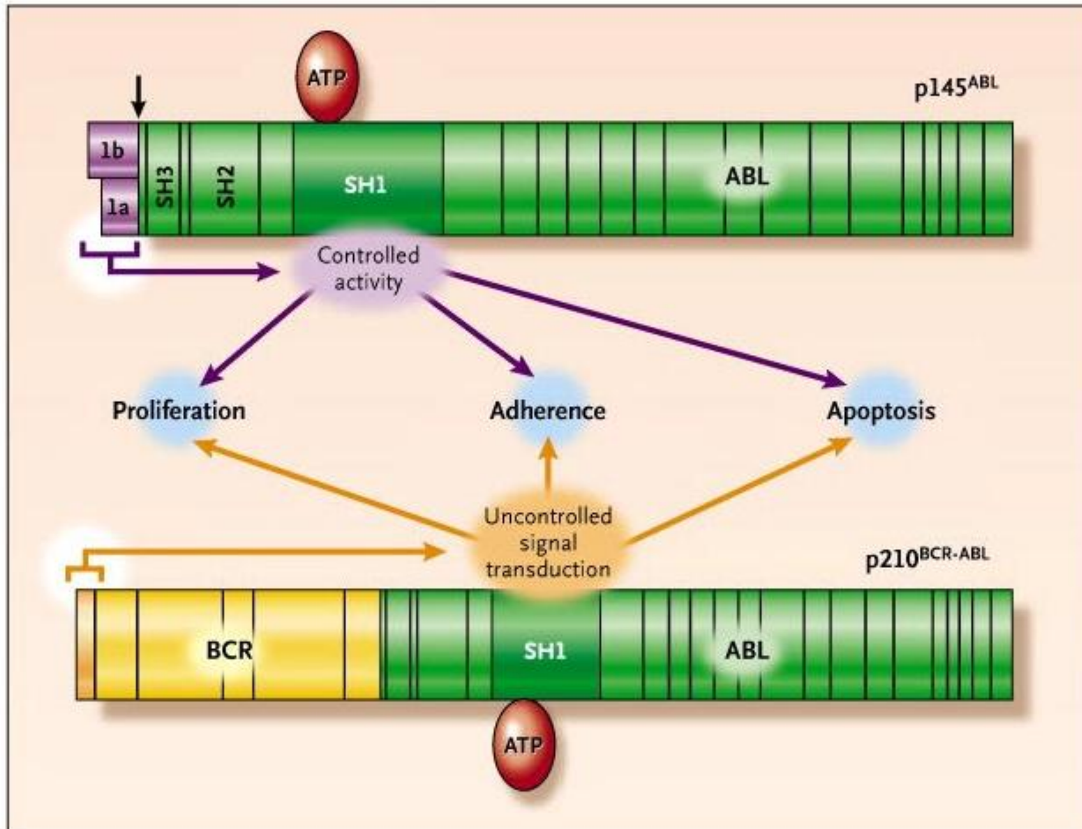


Figure I.2: Physiologic regulation by the normal ABL protein and deregulation by BCR-ABL of key cellular processes(Goldman & Melo, 2003)



This leads to eventual replacement of all myeloid tissue by normally differentiating leukaemia cells. The disease typically progresses through three distinct phases - chronic phase, accelerated phase, and blast crisis - during which the leukaemic clone progressively loses its ability to differentiate(Faderl *et al*, 1999). Definitions of CML chronic phase (CP), accelerated phase, and blast crisis are summarised in Table I.1.

TABLE I.1: Definitions of chronic, accelerated and blastic phase

Kantarjian criteria (Kantarjian *et al*, 1988)
Chronic phase
•none of the criteria for accelerated phase or blastic phase
Accelerated phase (one of the following)
15-29% blasts in peripheral blood or bone marrow
>30% blasts plus promyelocytes in peripheral blood or bone marrow
>20% basophils in peripheral blood or bone marrow
Platelets < 100 × 10^9/L unrelated to therapy
Blastic phase (one of the following)
Blasts in peripheral blood or bone marrow ≥30%
Presence of extramedullary blastic disease
WHO criteria
Accelerated phase
Blasts 10-19% in bone marrow or peripheral blood
Peripheral blood basophils ≥ 20%
Persistent thrombocytopenia (<100 × 109/L) unrelated to therapy, or
persistent thrombocytosis (>1,000 × 109/L) unresponsive to therapy
Increasing spleen size and increasing WBC count unresponsive to therapy
Cytogenetic evidence of clonal evolution
Severe bone marrow fibrosis
Megakaryocytic proliferation?
Blastic phase
Blasts in peripheral blood or bone marrow ≥20%
Presence of extramedullary blastic disease
Large foci or clusters of blasts in the bone marrow biopsy

The worldwide annual incidence of CML is 1 to 1.5 cases per 100,000 population and accounts for 15% to 20% of leukaemia cases in adults. Although the disease may occur at any age, the median age at presentation is between 50 and 60 years. A higher incidence of CML is noted among persons with heavy radiation exposure, and those who have received therapeutic irradiation for malignancy (e.g. lymphoma, breast cancer) have a small but significantly increased risk. There is no recognized familial influence, and no causal association between CML and industrial chemicals or alkylating agents has been demonstrated.

The t(9;22)(q34;q11) translocation juxtaposes the 3' segment of the c-ABL oncogene (normally encoding the Abelson Tyrosine kinase) from the long arm of chromosome 9 to the 5' part of the breakpoint cluster region (BCR) gene on the long arm of chromosome 22, resulting in one shortened chromosome 22 (22q–) (the Ph chromosome, (Rowley, 1973)) and one elongated chromosome 9 (9q+) (Figure I.1).

At diagnosis, the Philadelphia chromosome (Ph) is present in approximately 95% of CML cases. The remaining cases have either variant translocations involving a third and, sometimes, fourth chromosome or cryptic translocations. In these cases, routine cytogenetic analysis is unable to detect the Ph chromosome, and the diagnosis relies on demonstration of the fusion transcript by either fluorescence in situ hybridization (FISH) or real time quantitative polymerase chain reaction (RQ-PCR). The molecular consequence of t(9;22)(q34;q11) translocation is the generation of a gene that is expressed as a BCR-ABL1 RNA transcript translated into a 210-kd protein known as p210BCR-ABL. The p210BCR-ABL oncoprotein

functions as a constitutively active tyrosine kinase that can phosphorylate a number of cytoplasmic substrates with other activities leading to alterations in cell proliferation, differentiation, adhesion, and survival (Goldman & Melo, 2003;O'Hare *et al*, 2012).

The leukaemic clone in CML has a tendency to acquire additional oncogenic mutations over time, usually associated with progression to accelerated phases of disease or resistance to tyrosine kinase inhibitors (TKIs). At the chromosomal level, changes include amplification of t(9;22), trisomy 8, trisomy 19, and abnormalities of chromosome 17. At the molecular level, mutations in the kinase domain of BCR-ABL account for about 50% of imatinib resistance in patients with CML in chronic phase and 80% of advanced phases cases (O'Hare *et al*, 2012).

In vitro studies and studies in animal models have established that BCR-ABL gene alone is sufficient to cause CML, and that the enhanced tyrosine kinase activity of the p210BCR-ABL oncoprotein is required for its oncogenic activity (Heisterkamp *et al*, 1990;Daley *et al*, 1990;Kelliher *et al*, 1990;Lugo *et al*, 1990;Goldman & Melo, 2003).

I.2 TREATMENT OPTIONS IN CML

I.2.1 Tyrosine kinase inhibitors

Imatinib mesylate (imatinib, Gleevec, Novartis) is a an Abl kinase inhibitor (Druker *et al*, 1996), first used in 1998 to treat patients who were judged to have disease refractory to treatment with IFN-α or who could not tolerate IFN-α (Savage & Antman, 2002). Imatinib has produced impressive results in treatment of patients with CML in chronic phase, and the median survival for previously untreated

patients is now likely to be in excess of 10 years. Initial treatment with tyrosine kinase inhibitor (TKI) has become the gold standard for patients who present in chronic phase, and a complete cytogenetic response (CCyR, Table I.2) is considered as the minimum acceptable response, since it translates into improved transformation-free survival (TFS). Around 70% of patients achieve CCyR after frontline treatment with imatinib (de Lavallade H. *et al*, 2008;Druker *et al*, 2006), and the 8-year probability of being in continuing CCyR while still on imatinib or on second line treatment with second generation TKI is at 77% (Marin *et al*, 2012).

TABLE I.2: Conventional definitions of cytogenetic responses to treatment for chronic myeloid leukemia

Ph-positive marrow metaphases (%)	Designation
0	Complete cytogenetic response (CCR)
1-35	Partial cytogenetic response (PCyR)
36-95	Minor cytogenetic response
>95	None

Percentages cited above are based on a minimum of 20 analyzable metaphases.

Complete and partial responses are often grouped together as 'Major cytogenetic responses' (MCyR).

Imatinib has also proved active in patients with accelerated phase (AP) and blastic phase (BP) disease, but in most of these cases the benefits have been transient. Although 70% of imatinib-treated patients with CP CML achieve a CCyR, the majority of patients have persisting molecular disease as assessed by q-PCR for BCR-ABL transcripts and 60% of them will relapse following imatinib withdrawal

(Mahon *et al*, 2010). Functional leukaemic CD34+ progenitor cells have been identified in such patients in CCyR, suggesting the presence of a reservoir of leukaemic primary quiescent CML stem cells that are insensitive to imatinib and persist and accumulate following imatinib exposure (Graham *et al*, 2002) suggesting that imatinib is more cytostatic than pro-apoptotic in this subpopulation. In vivo studies in CML patients have demonstrated persistence of BCR-ABL positive progenitors despite continuing responses to imatinib therapy (Bhatia *et al*, 2003). This suggests that imatinib does not eliminate malignant primitive progenitors in CML patients and these cells provide a reservoir for disease relapse.

I.2.2 Second generation TKI, dasatinib,nilotinib and bosutinib

These drugs have been shown to induce responses in patients who have failed to achieve a cytogenetic response on imatinib (primary resistance) or have lost the response or progressed to advanced phase (secondary resistance) on imatinib therapy. Approximately 50% of patients who fail to obtain a cytogenetic response on imatinib achieve a CCyR on second line therapy, although the durability of these responses has not yet been established. Second generation TKI dasatinib (Sprycel; Bristol-Myers Squibb) and nilotinib (Tasigna; Novartis) have also been used as first line treatment in CML. In a randomised trial higher rates of CCyR have been reported in patients treated with first line nilotinib compared to imatinib (80% vs 65% at 12 months) with significantly lower rate of transformation to AP or BP with nilotinib (2.1%-3.2% vs 6.7%, respectively) (Kantarjian *et al*, 2011). However the benefit of each drug has been considered in isolation, not accounting for the effect of subsequent therapy. To date the only proven cure for CML remains allogeneic haematopoietic stem cell transplantation.

I.3 THE ROLE OF IMMUNOTHERAPY IN CML

1.3.1 Immunomodulatory drugs

Interferon-alfa (IFN-α) (Silver *et al*, 1999) is an immunomodulatory agent, shown to prolong overall survival. The drug may cause a wide range of side effects, especially in older persons, but it induces complete or nearly complete cytogenetic responses in 10-30% of patients and probably prolongs survival to a greater extent than hydroxyurea (1997). IFNα enhances the functional activity of T cells, as well as macrophages and NK cells, and induces dendritic cell (DC) differentiation of CML mononuclear cells. DC serve as antigen-presenting cells for CML-specific peptides (Kanodia *et al*, 2010). On the other hand treatment with IFNα increases the expression of tumor-associated antigens and major histocompatibility complex antigens. It induces Proteinase 3 expression by mononuclear cells, and increased Proteinase 3 expression levels in CML cells has been shown to be associated with a favourable prognosis for patients. Nearly all CML patients who respond to IFNα therapy have cytotoxic T-cells (CTLs) specific for PR1 (an HLA2.1-restricted peptide derived from Proteinase 3). Kanodia and colleagues have reported that the numbers of PR1-CTLs are increased in CML patients who remain in CCyR after IFNα therapy is discontinued (Kanodia *et al*, 2010). In line with those findings Burchert and colleagues have shown that treatment IFNα enables discontinuation of imatinib in CML patients, in relation with the induction of antileukaemic PR1-CTLs (Burchert *et al*, 2010).

1.3.2 Stem cell transplantation (SCT)

Allogeneic haematopoietic stem cell transplantation (allo-SCT) and donor lymphocyte infusions (DLI) offer the possibility of cure for CML and amply demonstrate the potential of immunotherapy in this disease and in other malignancies. However, allo-SCT is associated with an appreciable risk of morbidity and mortality and is only available to a minority of patients.

1.3.3 Immunotherapy

The immunological graft-versus-leukaemia (GVL) effect seen after allo-SCT suggests that stimulating the patient's own T cell responses to CML with a vaccine might retard disease progression and even achieve complete molecular response. In recent years, several classes of human leukaemia-associated antigens (LAA) that may be targets of the GVL effect have been identified and investigated for exploitation of immunotherapy (Bocchia *et al*, 2005;Rojas *et al*, 2007).

I.4 TYROSINE KYNASE INHIBITORS AND THEIR IMMUNOMODULATORY EFFECTS ON T-CELL

I.4.1 Tyrosine Kinases and T-cell receptor (TCR) activation

A number of tyrosine kinases (TK) play a critical role in TCR primary transduction pathways, such as Abl, Src family kinases (SFK) Lck and Fyn, Zap-70 and Tec.

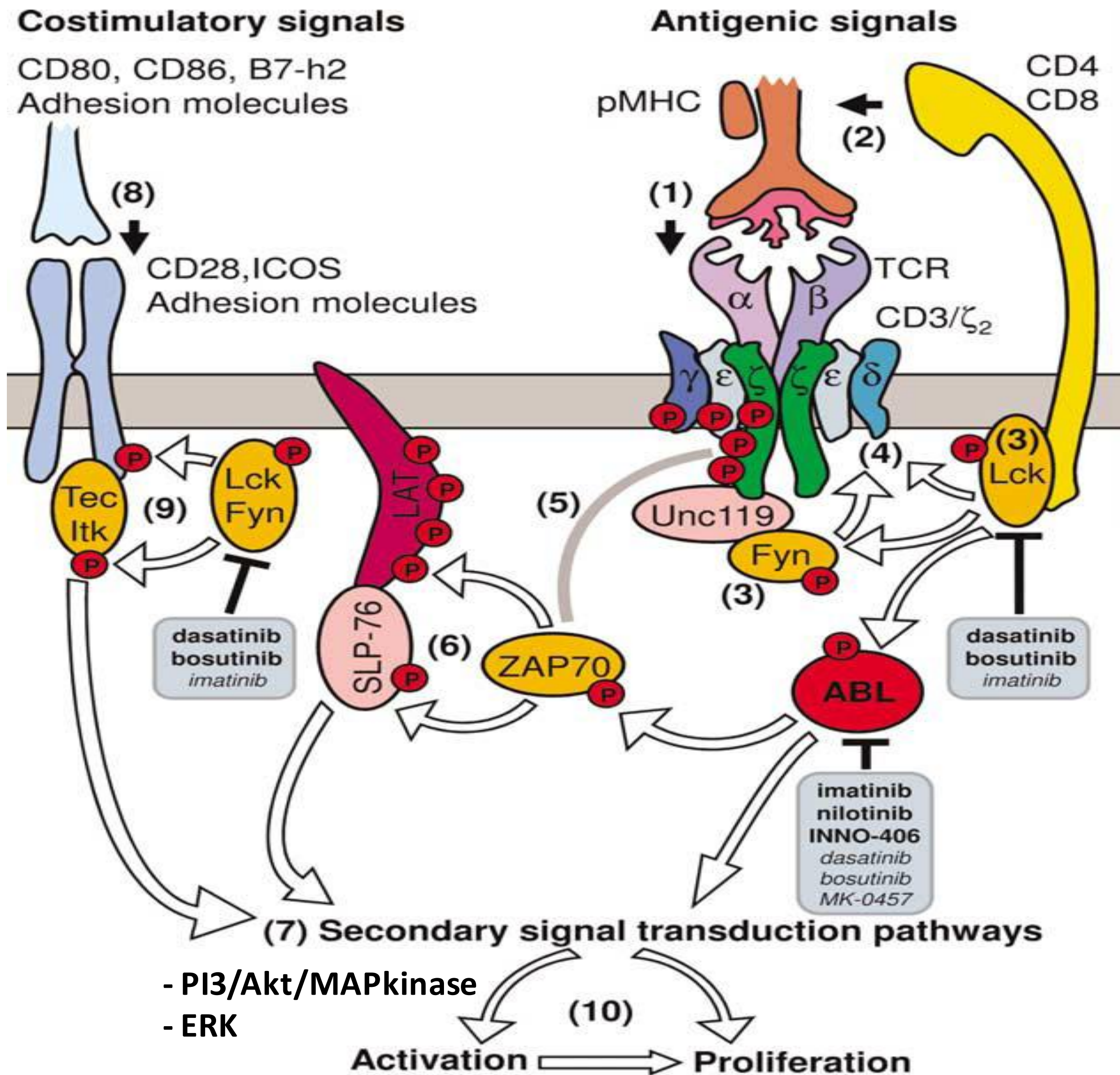
The proximal signalling events of T cell activation through the TCR complex includes the following steps (depicted in Figure I.3): after engagement of the TCR with peptide–MHC and recruitment of the CD4/CD8 co-receptor, the Src tyrosine kinases Lck and Fyn are activated and recruited to the TCR. Of note Lck and Fyn

have been shown to play a role at every stage of T-cell development (Palacios & Weiss, 2004). Activated Lck and Fyn then phosphorylate the tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAM) of the TCR complex which leads to ZAP-70 recruitment followed by LAT and SLP-76 phosphorylation. SLP-76 and LAT induce activation of secondary signal transduction pathways. Co-stimulatory signals mediated by ligation of CD28, ICOS and adhesion molecules are also necessary for complete T-cell activation, and signalling through CD28 has been shown to involve tyrosine phosphorylation by Lck and/or Fyn (Acuto & Michel, 2003).

The tyrosine kinase Abl also plays a significant role in the TCR signalling pathway as shown by Zipfel and colleagues (Zipfel *et al*, 2004): Following TCR engagement, c-Abl is activated through phosphorylation by Lck and subsequently activates ZAP-70.

Figure I.3: T-cell activation via the CD3-TCR complex, (Seggewiss *et al*, 2008)

TKI (boxed) may interfere with this signalling cascade by blocking specific tyrosine kinases as shown. Primary TK inhibitor actions are shown in bold, with secondary inhibition shown in italics.



I.4.2 Immunomodulatory effect of tyrosine kinase inhibitors on T-cells

Over the last few years, a number of studies have documented seemingly contradictory effects of the TKI imatinib on the immune response, ranging from impaired antigen-specific T-cell responses (Mumprecht *et al*, 2006;Seggewiss *et al*, 2005;Cwynarski *et al*, 2004) to enhanced stimulation of tolerant CD4+ T-cells (Wang *et al*, 2005) or enhanced active immunotherapy against BCR-ABL-expressing tumours (Larmonier *et al*, 2008). However, to date few studies have been performed to assess the potential immunomodulatory effects of the second-generation tyrosine inhibitors dasatinib and little data are available for nilotinib. It is possible that these three increasingly used agents differ in their effects on the immune response and as such may have differing implications for patient management. Information on any positive or negative effects of these drugs on the immune system will have significant implications for long-term immune surveillance against infectious agents and leukaemia. Such data will be clinically very useful as it may help in the choice of drug for therapy, especially for patients susceptible to haematological complications such as lymphopaenia or neutropaenia and as such at increased risk of infectious complications.

Furthermore a better understanding of potential immunomodulatory effects of TKIs is of particular importance in the design of immunotherapeutic strategies, especially in view of fact that dasatinib was first developed as a potential immunosuppressive drug. Indeed in vitro data support a global immunosuppressive role for dasatinib, including suppression of NK cell cytotoxicity (Blake *et al*, 2008b), inhibition of T-cell activation and proliferation (Schade *et al*, 2008), suppression of CD4+CD25+

regulatory T cells (Fei *et al*, 2009), and antigen-specific proliferation of murine and human T cells(Fraser *et al*, 2009a;Weichsel *et al*, 2008).

Seminal work by Rix and colleagues using chemical proteomic profiles of imatinib, dasatinib and nilotinib identified a number of kinases including major regulators of T-cell and B-cell signalling pathways that are substrates of off-target inhibition by TKI, as shown in Figure I.4 (Rix *et al*, 2007).

Figure I.4: A list of target kinases inhibited by dasatinib, nilotinib and imatinib (Rix *et al*, 2007)

Unique peptides no: number of all unique peptides observed for a particular protein. The listed sequence coverage (SC) is based on these unique peptides. IPI-ID indicates IPI protein database entries to which identified peptides were assigned; SC, sequence coverage; and —, no entry.

Gene name	Description	IPI-ID	Dasatinib		Nilotinib		Imatinib	
			Unique peptides, no.	SC, %	Unique peptides, no.	SC, %	Unique peptides, no.	SC, %
BCR-ABL	BCR-ABL fusion protein	—	57	31.9	15	6.8	9	4.2
-BCR-ABL	BCR/ABL1 fusion protein (fragment)	IPI00784719	2	16.9	—	—	—	—
-BCR	Breakpoint cluster region	IPI00472302	31	29.8	5	4.7	6	5.2
-ABL1	Abelson tyrosine kinase	IPI00221171	27	28.5	10	9.3	3	2.3
BTK	Tyrosine-protein kinase BTK	IPI00029132	41/20	70.5/36.9	—	—	—	—
CSK	Tyrosine-protein kinase CSK	IPI00013212	26/21	59.3/55.1	—	—	—	—
MAP3K4	Mitogen-activated protein kinase kinase kinase 4	IPI00186536	24	16.9	—	—	—	—
DDR1	Discoidin domain receptor 1	IPI00219996	23	27.3	15	17.0	—	—
GAK	Cyclin G–associated kinase	IPI00298949	22	19.5	—	—	—	—
TEC	Tyrosine-protein kinase TEC	IPI00000878	21	32.3	—	—	—	—
ABL2	Tyrosine-protein kinase ABL2	IPI00329488	21	19.3	13	11.3	—	—
LYN	Tyrosine-protein kinase LYN	IPI00298625	14/20	33.0/46.9	—	—	—	—
MAPK14	p38α	IPI00002857	14/15	43.1/43.6	—	—	—	—
EPHB6	Ephrin type-B receptor 6 precursor	IPI00005222	14	17.7	—	—	—	—
YES1	Proto-oncogene tyrosine-protein kinase YES	IPI00013981	13/10	23.4/17.5	—	—	—	—
EPHB4	Ephrin type-B receptor 4 precursor	IPI00289342	12/5	13.3/5.0	—	—	—	—
ZAK	Mitogen-activated protein kinase kinase kinase MLT	IPI00029643	11	29.7	—	—	—	—
SNF1LK2	Serine/threonine-protein kinase SNF1-like kinase 2 (QIK)	IPI00465291	10	10.7	—	—	—	—
FYN	Tyrosine-protein kinase FYN	IPI00640091	9/7	15.9/13.3	—	—	—	—
SRC	Proto-oncogene tyrosine-protein kinase SRC	IPI00328867	8/18	14.0/40.4	—	—	—	—
RIPK2	Receptor-interacting serine/threonine-protein kinase 2	IPI00021917	5/2	10.6/4.3	—	—	—	—
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	IPI00012318	5	3.8	—	—	—	—
FRK	Tyrosine-protein kinase FRK	IPI00000885	4/2	7.3/3.2	—	—	—	—
EPHB2	Ephrin type-B receptor 2 precursor	IPI00219421	4	4.5	—	—	—	—
FGR	Proto-oncogene tyrosine-protein kinase FGR	IPI00016871	3/24	4.5/50.5	—	—	—	—
TYK2	Nonreceptor tyrosine-protein kinase TYK2	IPI00022353	3	2.5	—	—	—	—
HCK	Tyrosine-protein kinase HCK	IPI00029769	—/23	—/56.0	—	—	—	—
BMX	Tyrosine-protein kinase BMX	IPI00020899	—/11	—/20.1	—	—	—	—
ILK	Integrin-linked protein kinase	IPI00013219	—/9	—/23.0	—	—	—	—
MAP3K3	Mitogen-activated protein kinase kinase kinase 3	IPI00181703	—/5	—/11.4	—	—	—	—
LCK	Proto-oncogene tyrosine-protein kinase LCK	IPI00555672	—/3	—/5.6	—	—	—	—
LIMK2	LIM domain kinase 2	IPI00100853	—/2	—/4.1	—	—	—	—
SYK	Spleen tyrosine kinase	IPI00018597	—/2	—/3.3	—	—	—	—

Indeed a number of tyrosine kinases which play a critical role in TCR primary transduction pathways are potentially targeted by the TKIs imatinib, nilotinib dasatinib and bosutinib (Bosulif, Pfizer). Figure I.3 shows where TKI interfere with the TCR signalling cascade by blocking specific tyrosine kinases.

Imatinib

Cwynarski and colleagues were the first to demonstrate that imatinib inhibits PHA-induced proliferation of normal peripheral blood mononuclear cells (PBMCs) and CD3/CD28-induced T-cell stimulation at pharmacological concentrations (5 to 7.5 μM) of imatinib (Cwynarski *et al*, 2004). Dose-dependent inhibition of the proliferative response of purified CD8+ and CD4+ T cells to anti-CD3/CD28 was also observed and associated with reduction in IFN-gamma production, while inhibition of T-cell proliferation was reversible after removal of the drug from the cultures. Seggewiss and colleagues further investigated the effect of imatinib on TCR-mediated activation. In line with the results from Cwynarski and colleagues they showed that proliferation of activated T-cells in response to stimulation with anti-CD3 antibody was almost completely inhibited by imatinib. Furthermore antigen-triggered expansion of CD8+ T cells in response to cytomegalovirus (CMV) and Epstein-Barr virus (EBV) peptides was significantly reduced. Finally imatinib reduced tyrosine phosphorylation of ZAP70 and LAT in response to activation through the TCR; while in an in vitro tyrosine kinase assay they showed Lck inhibition by imatinib. This finding was later confirmed by the work from Lee and colleagues (Lee *et al*, 2010) who demonstrated that imatinib inhibits Lck phosphorylation during human T-cell activation, although at relatively higher

concentrations of imatinib (25 μM).

Further to the work by Seggewiss and colleagues, Mumprecht and colleagues showed in a murine model that imatinib selectively impairs expansion of lymphocytic choriomeningitis virus (LCMV)-memory cytotoxic T-cell (CTL) without affecting the control of primary infections (Mumprecht *et al*, 2006). Furthermore, imatinib delayed the onset of CTL-induced diabetes in mice. These observations suggest a reduced secondary expansion of antigen-specific memory CTL in the presence of imatinib, resulting in impaired protection against reinfection.

In another murine model Wang and colleagues described imatinib-induced enhancement of APC function that overcame tumor-induced T-cell tolerance: in vitro treatment of APCs with imatinib enhanced the activation of naive antigen-specific T-cells and restored the responsiveness of tolerant T-cells from tumour-bearing hosts (Wang *et al*, 2005). Furthermore, in vivo treatment with imatinib not only prevented the induction of tolerance in tumour-specific CD4+ T-cells, preserving their responsiveness to a subsequent immunization, but also resulted in enhanced vaccine efficacy. Further to this study Larmonier and colleagues investigated the influence of imatinib on CD4+CD25+FoxP3+ regulatory T-cells (Treg) (Larmonier *et al*, 2008). They found that imatinib impaired Treg immunosuppressive function and FoxP3 expression associated with a reduction of STAT3 and STAT5 activation, two transcription factors which are required for FoxP3 up-regulation in Tregs. Importantly CD28-induced Lck activation is critical for STAT3 induction in FoxP3+ Tregs. As Lck has been shown to be a possible target of imatinib (Seggewiss *et al*, 2005) the authors showed that imatinib inhibited phosphorylation of ZAP70 and LAT, both located immediately downstream Lck in

the signal transduction cascade. These observations provide a possible molecular basis for the attenuation of Treg suppressive activity by imatinib, although in contrast to the well-characterized intracellular events leading to conventional T-cell activation, the signalling pathways regulating Treg activity remain more elusive. Furthermore, in a murine model, imatinib treatment for 7 days decreased Treg frequency and impaired their immunosuppressive function in vivo. Imatinib significantly enhanced antitumor immune responses to dendritic cell-based immunization against lymphoma, providing the proof of principle that imatinib can be successfully incorporated in immunotherapy protocols. In line with this murine model, Bocchia and colleagues reported that CML patients undergoing prolonged imatinib therapy mount similar CD4+ T-cell responses against a poorly immunogenic tumour peptide Ag, BCR-ABL, as observed in patients treated with IFN-α (Bocchia *et al*, 2006).

However imatinib has also been shown to have both positive and negative effects on the function and differentiation of antigen-presenting cells in several in vitro studies and in animal models (Appel *et al*, 2004;Taieb *et al*, 2004;Mohty *et al*, 2004).

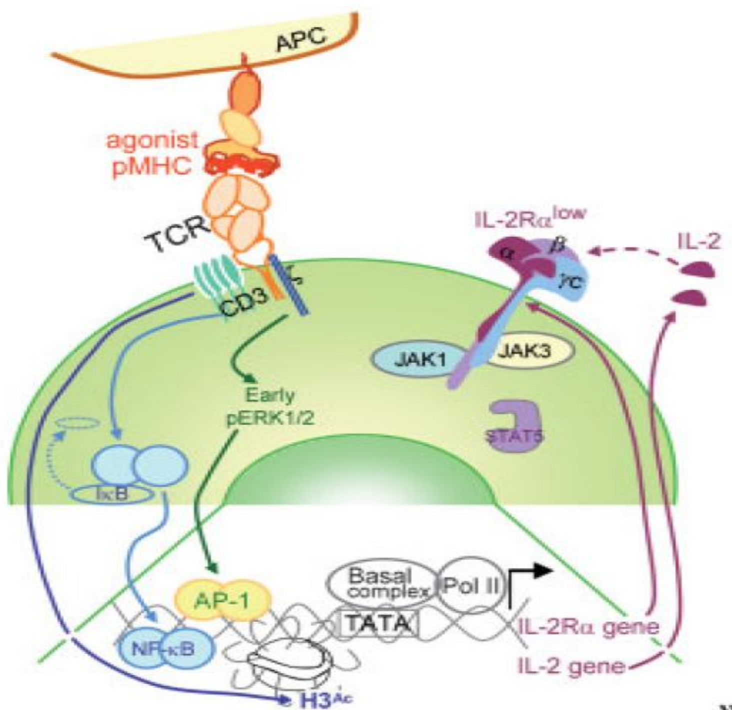
Dasatinib

Dasatinib was first developed as a potential immunosuppressive drug. Indeed its tyrosine kinase activity includes a broader range of protein kinases than imatinib such as the SFKs Lck and Fyn, both of which play an important role in TCR primary signal transduction pathways as shown in Figure I.3 (Section I.4.1).

Schade and colleagues were the first to explore the inhibitory effect of dasatinib on

T-cells through its impact on TCR downstream pathway (Schade *et al*, 2008). They showed that dasatinib inhibits the TCR-mediated signal transduction in vitro, while signal transduction and proliferative responses via IL-2 remained essentially unperturbed: dasatinib was shown to inhibit TCR secondary signal pathway pERK but not the IL2 downstream pathway JAK-STAT (see Figure I.5), thus suggesting that dasatinib displays specificity for TCR signalling.

Figure I.5: Downstream pathways of TCR and IL2 receptor (Verdeil *et al*, 2006)



Moreover they showed that dasatinib inhibitis cellular proliferation, proinflammatory cytokine production, and in vivo T-cell responses. Interestingly dasatinib-mediated inhibition did not induce apoptosis as the effect was reversible. The potent inhibitory effect of low concentrations of dasatinib on Lck was further studied by Lee and colleagues who showed this effect both in cell line and in human T cells (Lee *et al*, 2010). In a similar work, Blake and colleagues confirmed the finding that

dasatinib blocks the function of normal human T-cells in vitro, including proliferation, activation and cytokine production through Lck inhibition. Their findings were in keeping with work from other groups showing suppression of antigen-specific T-cell proliferation both in murine and human studies (Fraser *et al*, 2009a;Weichsel *et al*, 2008). Weichsel and colleagues compared the effect of dasatinib and the promiscuous TK inhibitor staurosporine on T-cells and showed that naive T-cells were more sensitive to dasatinib than memory T-cells; in addition virus specific CD8+ T-cells responses were suppressed by dasatinib in a dose-dependent manner (Weichsel *et al*, 2008).

The effect of dasatinib on Tregs was studied by Fei and colleagues who suggested that dasatinib inhibits proliferation of Tregs in a dose-dependent manner, associated with reduced IL-10 production and inhibition of Treg suppressive capacity. Further in vitro data also supported an immunosuppressive role for dasatinib on NK cells showing suppression of NK cell cytotoxicity (Blake *et al*, 2008b).

Nilotinib

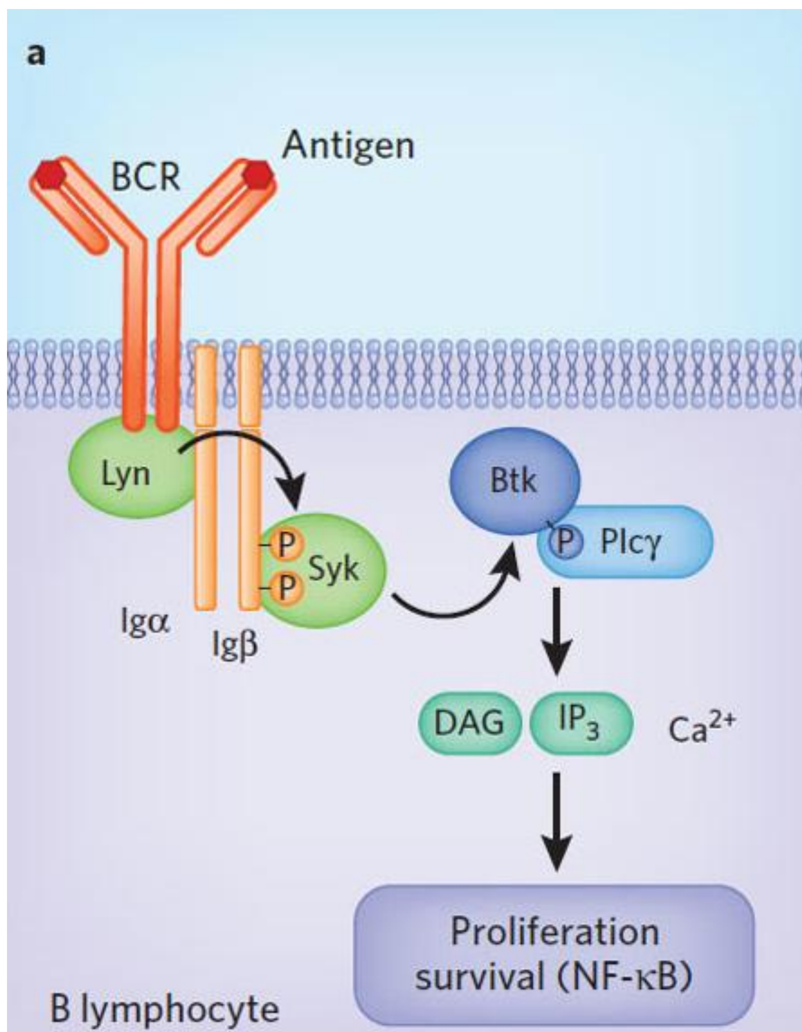
Similarly, nilotinib has also been shown to inhibit the proliferation and function of CD8+ T-cell in vitro (Chen *et al*, 2008) while no effect was seen on Tregs at clinical relevant doses (Fei *et al*, 2009).

I.5 TYROSINE KINASE INHIBITORS AND THEIR IMMUNOMODULATORY EFFECTS ON B-CELLS

I.5.1 Tyrosine kinases and B-cell receptor (BCR) signalling pathway

A number of TKIs could also potentially inhibit the B-cell receptor (BCR) downstream signalling pathways through the inhibition of tyrosine kinases such as Lyn, Syk, or Btk all involved in the BCR signalling cascade as shown in Figure I.6).

Figure I.6: BCR signalling upon antigen binding in B lymphocytes (Hendriks, 2011)

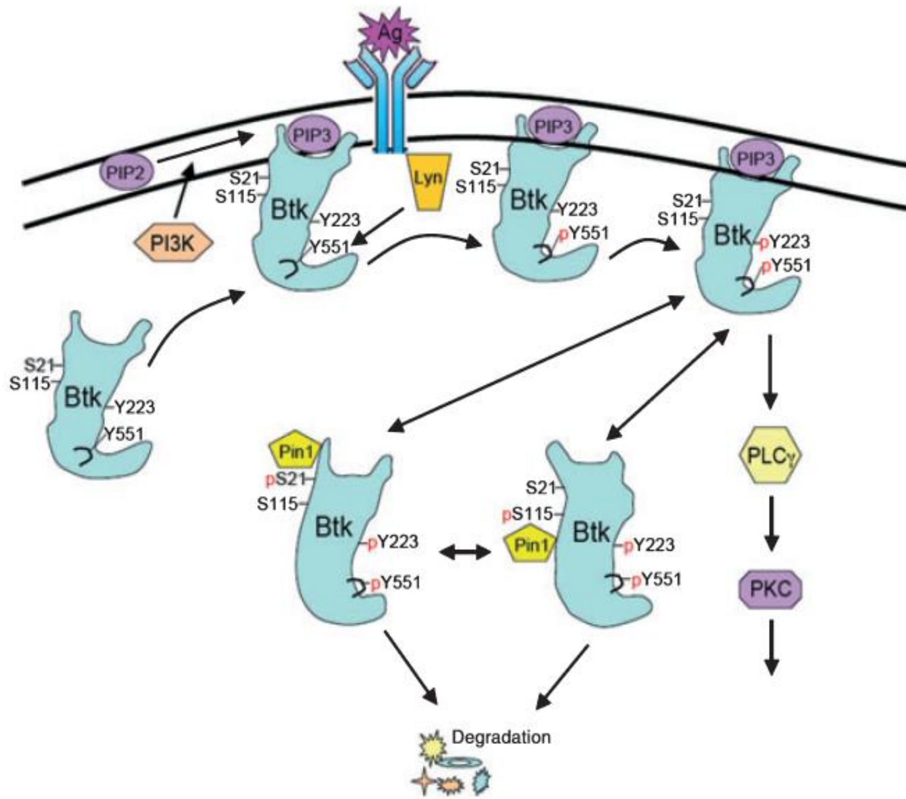


Indeed off-target inhibition of Lyn, Syk and Btk by dasatinib has been shown by the work of Rix and colleagues (summarised in Figure I.4, (Rix *et al*, 2007)). Hantschel and colleagues further confirmed Btk as a major target of dasatinib-induced kinase inhibtion (Hantschel *et al*, 2007).

Using proteomics analysis, Breitkopf and colleagues have reported Btk and Syk as potential imatinib targets, suggesting that imatinib could also potentially inhibit the BCR downstream pathways (Atwell *et al*, 2004;Breitkopf *et al*, 2010).

Btk is a crucial regulator for B-cell development and differentiation, and its expression is believed to be a prerequisite for B-cell proliferation and survival. Btk-deficient B lymphocytes fail to reach the mature state and are likely to undergo premature death. In humans, mutations in the Btk gene cause X-linked agammaglobulinaemia which is an immunodeficiency disease characterized by an almost complete absence of circulating B cells and an inability to generate immunoglobulins of all classes. Therefore patients with mutations in the Btk gene fail to mount humoral immune responses. A schematic model of Btk regulation is shown in Figure I.7: Upon BCR stimulation, phosphatidylinositol-3-kinase (PI3K) is activated which results in the production of the phosphoinositide, phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 generation leads to recruitment of several intracellular signalling proteins including Btk. Once present at the cytoplasmic surface of the plasma membrane, Btk is phosphorylated on its tyrosine 551 (Y551) in the activation loop, followed by the subsequent autophosphorylation of Y223 in the SH3 domain which leads to its fully activated conformation. Functionally competent Btk executes downstream signalling duties, such as phosphorylation of phospholipaseCγ2 (PLCγ2).

Figure I.7: Model for activation of BCR signalling (Mohamed *et al*, 2009)



I.5.2 Putative effect of TKIs on B cells (murine models)

To date only two studies have examined the impact of TKIs on B cells, although hypogammaglobulinaemia has been reported in CML patients treated with imatinib (Steegmann *et al*, 2003). Indeed Steegman and colleagues reported that IgG and IgM hypogammaglobulinaemia developed in 28%, and 22% of CML patients treated with imatinib respectively.

In a murine model of autoimmune arthritis, Paniagua and colleagues reported that imatinib prevents and treats murine collagen-induced arthritis, partly through B-cell inhibition (Paniagua *et al*, 2006). They showed that mouse B cell proliferation

induced by anti-IgM was inhibited by imatinib as well as IgM production by LPS-stimulated B cells.

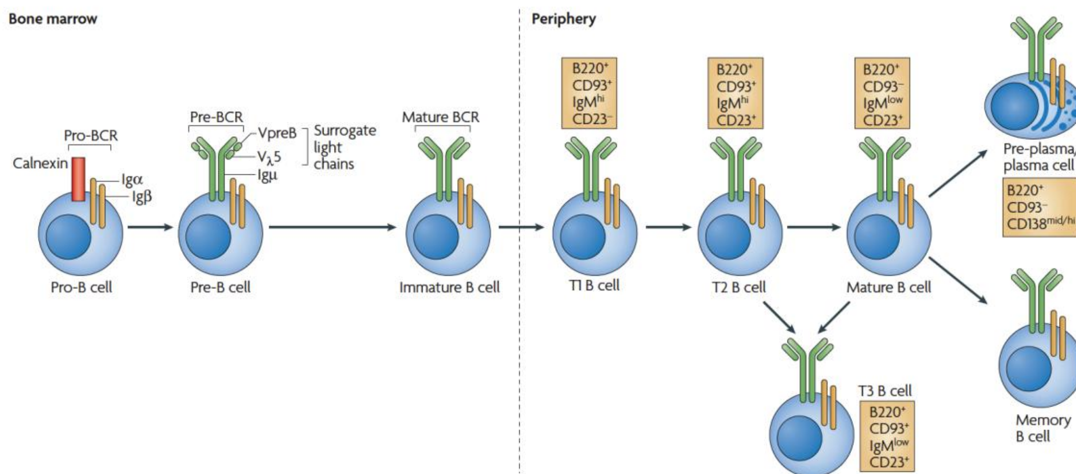
More recently Kawamata and colleagues reported in a murine study that imatinib may directly impair class switch recombination following B cell activation through downregulation of activation-induced cytidine deaminase (AID) known to be essential for class switch recombination (CSR) and somatic hypermutation (Kawamata *et al*, 2012). They first showed that following stimulation with IL-4 and LPS for 72 hours, imatinib decreased the proportion of IgG1-positive B-cells dose-dependently after, associated with AID suppression. In an in vivo murine model they found that AID expression, which is induced in germinal centre-activated B-cells, was barely detectable in the spleens of imatinib-treated mice but was strongly positive in the spleens of nontreated mice, while ectopic expression of AID rescued impairment of CSR in vitro. Finally, they found that expression of E2A, a key transcription factor for AID induction, was markedly suppressed by imatinib, therefore suggesting a possible mechanism for AID inhibiton. They did not investigate the role of Btk or Syk inhibition by imatinib as a possible contributing factor to CSR impairment.

In summary, although there is growing evidence that TKIs inhibit kinases important for BCR downstream signalling and for B-cell development, differentiation and proliferation, there are currently no data to relate this to the hypogammaglobulinaemia seen in CML patients on TKI.

I.5.3 B-cell development and B cell subsets

B-cell development occurs in both the bone marrow and peripheral lymphoid tissues such as the spleen. In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages (Figure I.8).

Figure I.8: Stages of B cell development (Cambier *et al*, 2007)



During B cell maturation and differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor (pre-BCR, which is comprised of an Igμ heavy chain and surrogate light chains) and finally a mature BCR (comprised of rearranged heavy- and light-chain genes) that is capable of binding antigen. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells. Both receptor editing and clonal deletion have a role at this stage. Cells successfully completing this checkpoint leave the bone marrow as transitional B cells, eventually maturing into mature follicular B cells (or marginal-zone B cells). BCR signalling appears to be an important determinant in the transition from T1/T2 to mature B cells. Many mutations that affect BCR signalling pathways also interfere with transition to mature B cells. In the absence of Btk, BLNK or PLCγ2 BCR signalling is insufficient to induce T1/T2 cells to differentiate into mature B

cells.

Transitional B cells represent 2.2 to 7% of circulating human B cells and are defined by high expression of CD24, CD38. They also are IgM+ and are negative for CD27, a marker for memory B cells. As they progress through maturation they express decreasing levels of IgM and CD10. Further maturation of transitional B cells occurs in the spleen to naive B cells bearing functional BCR which can proliferate upon BCR engagement. Naive B cells represent 40 to 60% of total B cells and express $CD24^{int}$, $CD38^{int}$, CD21 and are negative for CD27 and CD10. Following an immune response, antigen-specific B cells develop into either plasma (antibody-secreting) cells or memory B cells.

Memory B cells are antigen experienced cells, that secret Immunoglobulin under stimulation, persist in the absence of the immunizing agents and respond rapidly during a secondary response. CD27+ memory B cells consist of two major groups, the IgM memory B cell (IgM+IgD+CD27+) and the isotype switched memory B cells (IgM-IgD-CD27+), which are the final product of a successful T-cell dependent germinal centre reaction.

I.6 VACCINE STRATEGIES IN CML AND MYELOID LEUKAEMIAS

I.6.1 Leukaemia-associated antigens

In recent years, several classes of human leukaemia associated antigens (LAA) that can be used as targets of immunotherapy have been identified, including minor histocompatibility antigens such as HA-1 (Mutis *et al*, 1999), neoantigens created by chromosomal translocations such as BCR-ABL (Clark & Christmas, 2001), overexpressed self-antigens such as Wilms tumour (WT1) (Gao *et al*, 2000), proteinase 3 (PR1) (Molldrem *et al*, 1997), and cancer testis antigens such as Preferentially Expressed Antigen of Melanoma (PRAME) (Ikeda *et al*, 1997). A number of studies including from our group, have previously demonstrated that low frequencies of T cells recognising self-antigens overexpressed by leukaemia cells such as WT1, PR1 and PRAME exist in healthy individuals and patients with leukaemia (Rezvani *et al*, 2003;Rezvani *et al*, 2005;Rezvani *et al*, 2009). These responses were found to be significantly higher in leukaemia patients compared to healthy donors but the greatest responses occurred after SCT with most patients responding to multiple antigens. These LAA specific CD8+ T cells were found to be of memory phenotype, suggesting ongoing in vivo antigenic stimulation. These findings formed the basis for design of clinical protocols led by Dr. Rezvani to test WT1 and PR1 peptides as leukaemia vaccines at the National Institutes of Health, Bethesda, USA. Patients with myeloid malignancies received a single subcutaneous injection of PR1 and WT1 peptides. The combination of peptide vaccines was safe with minimal toxicity. CD8+ T-cell responses against PR1 or WT1 were associated with a significant but transient reduction in minimal residual disease (MRD) as assessed by WT1 gene expression, suggesting a vaccine-

induced anti-leukaemia response(Rezvani *et al*, 2008).

I.6.2 BCR–ABL vaccine

Four fusion variants of the BCR–ABL protein are known. Of these, the p210 b3a2 variant appears the most promising target for vaccination with immunogenic HLA class I and II binding peptides (Bocchia *et al*, 1996;ten Bosch *et al*, 1999;Mannering *et al*, 1997;Pawelec *et al*, 1996;Clark & Christmas, 2001). A number of studies have shown that b3a2 peptides can induce peptide-specific T cells and that CML cells can present BCR–ABL peptides, but in vitro expanded BCR–ABL specific T cells have not been reproducibly shown to be cytotoxic against CML. The most convincing results come from studies by Clark et al., who were able to generate HLA-restricted CTL responses to CML cells using an HLA A-3 binding BCR-ABL peptide (Clark *et al*, 2001).

Pinilla-Ibarz and colleagues, at the Memorial Sloane Kettering Institute, were the first to develop a BCR–ABL vaccine composed of a pool of six peptide fragments. A safety study showed that the vaccine and QS-21 adjuvant were well tolerated and could elicit T cell immune responses to BCR–ABL, although no direct evidence of cytotoxicity was shown (Pinilla-Ibarz *et al*, 2000). They then performed a phase II study where 14 CML patients in chronic phase received five doses of the vaccine and adjuvant (Cathcart *et al*, 2004). All patients developed delayed-type hypersensitivity or CD4+ proliferative responses to the peptides. IFN-γ-producing CD4 and CD8 T cells developed in 11 and 4 patients, respectively. Reduction in marrow Ph chromosomes occurred in four patients, but three of these continued other treatments with IFN-α or imatinib. Three patients in molecular relapse after

SCT had transient molecular remissions, but two had also been treated with donor lymphocyte infusions. These results, while promising, did not demonstrate convincingly the efficacy of the vaccine. In a subsequent trial using a similar peptide combination, Bocchia et al. studied 16 b3a2 variant CML patients with stable residual disease for at least 6 months, after a minimum of 12 months treatment with imatinib or 24 months with IFN-α (Bocchia *et al*, 2005). Patients received six vaccinations and were assessed for immunological and disease response. It is important to note that patients continued their conventional treatment during and after vaccination. Of 10 patients on imatinib, nine had stable cytogenetic disease – of whom seven were in major cytogenetic response- and one was in complete cytogenetic remission (CCyR) for a median of 10 months. Five achieved complete cytogenetic remission, with a negative polymerase chain reaction (PCR) for BCR–ABL in three. Of six patients stable for a median of 17 months on IFN-α treatment with 13% median Ph+ chromosomes in the marrow, five showed reductions in the percentage of Ph+ chromosomes, with two reaching complete cytogenetic remission. Five of five patients showed specific responses to the peptide with IFN-γ production, 13 of 14 had proliferative responses and delayed-type hypersensitivity to the peptides was seen in 11 patients. Following this pilot study, a phase 2 multicenter study was conducted in 57 chronic phase CML patients who had achieved a CCyR on at least 18 months of IM treatment. The interim analysis presented at the Annual Meeting of the American Society of Hematology (ASH) in 2009 reported that 67% of 43 evaluable patients had a significant in vitro b3a2-peptide-specific CD4+ T cell proliferation (Bocchia *et al*, 2009). Fourteen of 29 evaluable patients who reached the 9th month evaluation had at least a 0.5 log reduction in the BCR-ABL transcript levels.

A similar phase I/II study from the UK using BCR-ABL peptides, linked to the pan DR epitope PADRE, reported no molecular benefit in five patients not in major cytogenetic response (MCyR) at baseline. However, of the 14 patients in MCyR at baseline, 13 developed at least 1 log fall in BCR-ABL transcripts, although this occurred several months after completing vaccination (Rojas *et al*, 2007).

These studies support the immunogenicity and safety of BCR-ABL peptide vaccination in patients with CML. Although it is possible that clinical responses to BCR–ABL peptide vaccination may be induced in patients with CML with low levels of stable disease, the rather modest results raise concerns that the method of vaccine administration or the immune status of the treated patients may be suboptimal, or that BCR–ABL does not induce sufficiently powerful cytolytic T cell responses to CML. Furthermore, the benefit of this approach will remain uncertain without a randomized arm to take into account any effect of imatinib itself on BCR-ABL reduction.

Recently Maslak and colleagues conducted a pilot vaccine study in 13 patients with CML on imatinib with stable complete or major cytogenic response with residual molecular disease (Maslak *et al*, 2008). Patients with the b3a2 or with the b2a2 breakpoint were vaccinated with a heteroclitic class I peptide that bind with high affinity to HLA-0201 and a class II peptide. Vaccination induced a CD8+ T cell response that cross-reacted with native junctional peptides, overcoming the poor immunogenicity of the peptides. However the trial failed to demonstrate a relevant clinical activity, even in the setting of MRD, and underscores the difficulty of making any conclusions on the reduction of BCR-ABL transcripts in the setting of current

treatments with tyrosine kinase inhibitors.

I.6.3 WT-1 vaccine trials

Preclinical studies suggest that Wilms tumour antigen (WT1) is highly immunogenic in man and an interesting vaccine candidate for haematological malignancies and cancer in general. Oka et al. reported the outcome of a phase I clinical study of WT1 peptide-based immunotherapy in patients with breast or lung cancer, MDS or AML (Oka *et al*, 2004;Morita *et al*, 2006). Patients received an HLA-A24 restricted, 9-mer WT1 peptide in Montanide adjuvant at 2-weekly intervals in a dose escalation study. The vaccine was well tolerated and 18 of 26 patients received three or more vaccinations. The only notable side effect was profound leucopaenia in two MDS patients reversed by steroid treatment, which concomitantly abrogated the WT1 T cell response. Twelve of the 20 evaluable patients had clinical responses, including reductions in blood or marrow leukaemic blasts, tumour size or tumour markers. Increases in WT1-specific CTL frequency correlated with a clinical response.

Scheibenbogen and colleagues used a WT1-126 peptide vaccine in combination with KLH and GM-CSF as adjuvants in 17 patients with AML and 2 patients with high risk MDS. Patients received a median of 11 vaccinations (Keilholz *et al*, 2009). WT1-specific T cell responses were detected in 8 of 18 patients in the peripheral blood and 8/17 in the bone marrow by tetramer analysis. Clinical responses were seen in 6 of 10 patients, including four stable disease (SDs) with more than 50% blast reduction and two with haematological improvement. An additional four patients had clinical benefit after initial progression, including one complete

remission and three SDs. WT1 transcripts as molecular disease marker decreased in 35% of patients. These promising results indicate that WT1 vaccination can induce functional CTL responses which may be associated with clinical improvement in some patients.

A recent pilot study was undertaken to assess the immunogenicity of a polyvalent WT1 peptide vaccine previously described to have higher binding and immunogenicity, containing 1 WT1-derived peptide to stimulate CD8+ T-cell responses, 2 WT1 peptides to stimulate CD4+ responses and one modified peptide that could stimulate both CD4 and CD8 T cells (Maslak *et al*, 2010). Ten AML patients in CR but with molecular evidence of WT1 transcript received six vaccinations plus immune adjuvants over 12 weeks. Of the nine evaluable patients, seven completed six vaccinations and WT1-specific T-cell responses were noted in 7 of 8 patients. Three patients who were HLA-A0201-positive showed significant increases in the frequency of WT-1-tetramer-positive CD8 T cells. At the time of publication, the median disease-free survival for the group had not been reached. Although the small number of patients and lack of a control group limit this trial, the results are intriguing enough to warrant further study.

Research into cellular vaccines was re-enthused with techniques to generate DCs from AML and CML cells. Such DC vaccines have the potential to optimally stimulate T cell responses to the leukaemia. A Phase I/II trial investigating the effects of vaccination with autologous monocyte-derived DCs transfected with WT1 mRNA in 10 patients with AML was recently reported (Van, V *et al*, 2010). Successful vaccine production was obtained in all patients from a single

aphaeresis procedure; mRNA-loaded DC vaccines were administered at biweekly intervals and were well tolerated. Two patients in partial remission after chemotherapy were brought into complete remission after vaccination. In these two patients and three other patients who were in complete remission, the AML-associated tumour marker returned to normal after vaccination, compatible with the induction of molecular remission. Interestingly, following the first round of DNA vaccination, WT1 mRNA expression increased on different occasions, compatible with molecular relapse. This was reversed by additional rounds of DC vaccination, further supporting the anti-leukaemia effect of the vaccine. Clinical responses were correlated with vaccine-associated increases in WT1-specific CD8 T cell frequencies, as detected by peptide/HLA-A*0201 tetramer staining, and elevated levels of activated natural killer cells post-vaccination. These promising data support the development of WT1 mRNA-electroporated autologous DC in AML.

I.6.4 PR1 vaccine trials

PR1 is a nine-amino-acid HLA-A*0201 restricted peptide derived from proteinase 3 (PR3), shown to elicit myeloid-leukaemia-specific CTL responses that selectively kill leukaemic CD34+cells (Molldrem *et al*, 1997;Molldrem *et al*, 1996). PR1-specific CD8 T cells with a memory phenotype occur at low frequencies in healthy individuals and at higher frequencies in patients with leukaemia, suggesting that it should be feasible to boost these immune responses with vaccination (Rezvani *et al*, 2003;Rezvani *et al*, 2007). To explore the therapeutic potential of boosting immunity to PR1, Molldrem and colleagues began a pilot study of PR1 peptide vaccination in patients with myeloid malignancies. Highly encouraging preliminary data from this study were first presented at ASH in 2004 and an update presented

at ASH in 2007 (Qazilbash *et al*, 2004). A total of 66 HLA-A0201 patients with AML (42), CML (13) or MDS (11) were treated with PR1 peptide vaccine. Fifty three had active disease and 13 were in remission on entering the trial. The first 54 patients received three vaccinations, and the last 12 patients received 6 vaccinations. Patients received one of three dose levels: 0.25, 0.5 or 1.0 mg per vaccination every 3 weeks, together with Montanide adjuvant and GM-CSF. The vaccine was well-tolerated. Of the 53 with active disease, 25 (47%) had an immune response. Clinical responses ranging from improvements in blood counts to complete cytogenetic remission were observed in 9 out of 25 responders (36%), compared with 3 of the 28 non-responders (10%). Importantly, immune response to the PR1 vaccine was associated with an 8.7 month event-free survival (EFS) compared with 2.4 months for non-responders (p = 0.03), with a trend towards longer OS (Qazilbash *et al*, 2007a). Among the 13 patients in remission at the start of the trial, four remained in remission for a median time of 30.5 months. Analysis of the effectiveness of this approach in a subgroup of 20 patients with myeloid leukaemia vaccinated following SCT showed a PR1 response to the vaccine in 11/20 (55%) patients. Nine of 11 (82%) patients with a PR1 response compared to 1 of 9 (11%) patients who failed to mount an immunological response to the vaccine had clinical responses (P = 0.005). Importantly, a significant PR1 response to the vaccine was associated with significantly better clinical response and longer EFS (Qazilbash *et al*, 2007b). Although the full results of this study are yet to be published, these promising data have led to the initiation of a phase III, multicentre study of PR1 peptide vaccination in myeloid malignancies.

I.6.5 Trial of combination PR1 and WT1 peptide vaccines

A recent Phase I safety study from the National Institutes of Health (Bethesda, USA), reported the results of peptide vaccination with a combination of PR1 and WT1-126 in 8 HLA-A*0201+ patients with low risk myeloid malignancies (Rezvani *et al*, 2008). Five patients with AML in complete remission (1 following a second unrelated donor SCT), 2 patients with MDS (refractory anaemia and refractory anaemia with ring sideroblasts) and 1 patient with CML in chronic phase (BCR-ABL negative on imatinib) were recruited. Patients received a single subcutaneous injection of PR1 (0.5 mg) and WT1-126 (0.2 mg) peptides in Montanide adjuvant, administered concomitantly with GM-CSF (Sargramostim). Patients were reviewed weekly to monitor toxicity and immunological responses. Toxicity was limited to grade 1-2. CD8 T-cell responses against PR1 were detected in 7/8 and against WT1-126 in 5/8 vaccinated patients. Vaccine-induced T cell responses were detected as early as 1-2 weeks post-vaccination in most patients, however these responses were short-lived. Post-vaccination, the emergence of PR1 or WT1-specific CD8 T cells was associated with a significant but transient reduction in MRD as assessed by WT1 gene expression, suggesting a vaccine-induced anti-leukaemia response. These results suggested that the maintenance of sustained or at least repetitive response may require frequent boost injections. This led to the initiation of a phase 2 study of biweekly injections of PR1 and WT1 peptide vaccines in patients with myeloid malignancies. Again, the combination of PR1 and WT1 peptide vaccines was found to be immunogenic in all patients. However, repeated vaccination led to selective deletion of high avidity vaccine-induced T cells and additional boosting did not increase the frequency of PR-1 or WT-1-specific

CD8 T-cells further. In four of six patients the vaccine-induced T-cell response was lost after the fourth dose and in all patients after the sixth dose of vaccine (Rezvani *et al*, 2011). Therefore, whilst these results support the immunogenicity of PR1 and WT1 peptide vaccination, novel approaches will be needed to induce long-term immune responses against these leukaemia antigens.

I.6.6 RHAMM vaccine trial

The receptor for hyaluronic acid mediated motility (RHAMM) is an immunogenic antigen that is strongly expressed in several haematological malignancies and has been shown to induce humoral and cellular immune responses (Greiner *et al*, 2002). A phase I/II study investigated the safety and immunogenicity of vaccination using the HLA-A2-restricted RHAMM/CD168-R3-peptide in 10 patients with AML, MDS and multiple myeloma (Schmitt *et al*, 2008). Vaccination with 300 µg of R3 peptide induced immunological responses in 5/10 patients. Clinical responses were observed in 3 patients; 1 patient with AML and 1 with MDS had a significant reduction in blasts in the bone marrow and one patient with MDS no longer required erythrocyte transfusions after four doses of vaccine. The study was therefore extended and further patients were vaccinated with the increased dose of 1000 µg R3 peptide (Greiner *et al*, 2010). Nine patients were vaccinated, of whom 4 had positive immunological responses. However, higher doses of peptide did not improve the frequency and intensity of immune responses in this trial.

I.6.7 Other approaches

The paracrine production of GM-CSF by irradiated tumour cells has been shown to promote the local recruitment of DCs to the vaccine site, which capture liberated tumour antigen and traffic to draining lymph nodes to activate tumour-specific CD4 and CD8 T cells. K562/GM-CSF, a CML cell line genetically engineered to produce GM-CSF, expresses a number of CML-associated antigens and has the potential to generate or augment CML-specific immune responses. A pilot study was conducted at Johns Hopkins Medical Institute (Baltimore, USA), to determine whether K562/GM-CSF immunotherapy (GVAX) could improve molecular responses in patients with chronic phase CML on imatinib. Patients with CML-CP who have achieved at least a major cytogenetic response, but remained with measurable disease despite 1 or more years of imatinib therapy were recruited (Smith *et al*, 2010). Nineteen patients received four K562/GM-CSF vaccinations every 3 weeks. Mean PCR measurements of BCR-ABL for the group declined significantly following the vaccines. Thirteen patients had a progressive decline in disease burden, including seven who became PCR undetectable.

In a phase 2 study, the same group explored the efficacy of the GVAX platform accompanied by infusion of immunotherapy-primed lymphocytes after autologous stem cell transplantation (ASCT) in haematological malignancies. The vaccine consisted of autologous leukaemia cells admixed with GM-CSF-secreting K562 cells. After a single pretransplantation dose of vaccine, the "primed" lymphocytes were collected and reinfused with the stem cell graft as post remission therapy following autologous stem cell transplantation for AML (Borrello *et al*, 2009). Fifty-

four subjects were enrolled; of whom 46 achieved a CR, and 28 received a total of nine vaccinations at 3-weekly intervals post-ASCT. For all patients who achieved CR, the 3-year relapse-free survival (RFS) rate was 47.4% and overall survival was 57.4% compared to 61.8% and 73.4% in the 28 immunotherapy-treated patients. Following post-transplant immunotherapy, immune responses to the vaccine were detected in 100% of patients, including DTH reactions in 7 of 18 patients (39%), T-cell responses (assessed by 7-day Elispot assay) in 15/17 and antibody responses to GVAX in 17/17 (100%). These results suggest that this immunotherapy approach may offer an interesting platform for post-remission maintenance therapy.

I.7 THESIS AIMS

I hypothesise that:

- TKIs may have different immunomodulatory effects on T cells and B cells which may impact on humoral and cellular immune responses to vaccination against infectious agents and leukaemia-specific antigens

- The mechanism for the altered immune responsiveness in patients with CML on TKI could be related to the CML disease itself of a consequence of TKI-mediated inhibition of physiological ABL, SFKs or other off-target kinases activity.

Aims: The aims of this project are:

1. Determine the in vivo cellular and humoral immune responses to 2009 H1N1 vaccine in CML-CP patients on TKI, compared to patients with other haematological malignancies and healthy controls: I aimed to assess the extent of the underlying immunosuppression in patients with CML compared to patients with other haematological malignancies such as non-Hodgkin lymphoma (NHL), chronic lymphocytic leukaemia (CLL), allogeneic stem cell transplant (allo-SCT) recipients and healthy controls. I compared the B and T cell response to the first vaccine and the second booster injection in these patient groups.

2. Determine T-cell and B-cell responses to vaccination against seasonal influenza virus and pneumococcus in CML patients treated with tyrosine kinase inhibitors: I carried a comprehensive immunological analysis following routine vaccination against seasonal influenza virus and streptococcal pneumonia in patients with CML on standard dose imatinib, dasatinib and nilotinib. The aim was to determine the potential impact of treatment with TKI on the efficacy of antigen-specific T-cell (against influenza) and polysaccharide-specific B-cell (against pneumococcus) responses. This comprehensive study will form the basis for an immunotherapy protocol to combine vaccination against LAA with TKI in patients with CML

3. Exploring the immunomodulatory effect of TKIs through their impact on BCR downstream pathway.

   This study focused on evaluating the effect of TKI on BCR downstream signalling pathways and their impact on the B cell functions directly ex vivo. My goal was to explore the possible mechanisms underlying impaired B cell responses to

vaccination in CML patients on TKI.

4. To investigate the possible impact of Ph+ B lymphopoesis on the vaccine-induced B cell responses in CML. The impaired B cell response to vaccination could be related to the CML disease itself or a consequence of TKI-mediated inhibition of multi tyrosine kinases activity. The aim of this work was to further explore the effect of TKI on different B-cell subsets and to look for the presence of, and implications of Philadelphia positive lymphopoiesis on the vaccine-induced humoral response.

CHAPTER 2 LABORATORY AND ANALYTICAL METHODS

II.1 PATIENTS

Informed consent for the use of patient material was obtained from all patients at Imperial NHS Trust, Hammersmith Hospital. The study was approved by the local ethics review board.

TABLE II.1: inclusion of CML patients in studies reported in II.1.1 and II.1.2

Patients 36 to 51 (cf Table IV.2 page 141) were vaccinated first with the seasonal influenza vaccine 2009/2010

Study ‘T cell and B cell responses after vaccination against seasonal influenza and pneumococcus’, n=51

Study ‘Immune response following vaccination against influenza A (H1N1) 2009 virus’, n=32

CML Patients	Seasonal influenza vaccine2008/2009 (CSL biotherapy)	Seasonal influenza vaccine2009/2010 (CSL biotherapy)	H1N1 vaccine 2009/2010 (Pandemrix GSK)
Patient 1 to 35 (cf Table IV.2, page 142)	Yes		
Patients 36 to 51 (n=16) Dasatinib, n=4 Imatinib, n=12		Yes	Yes
Patients included in H1N1 study only N=16 dasatinib, n=5 imatinib, n=11		Yes	Yes

II.1.1 Patient selection for inclusion in the study ‘Immune response following vaccination against influenza A (H1N1) 2009 virus’

Individuals recruited in this study were CML patients, chronic lymphocytic leukaemia patients, Non-Hodgkin lymphoma patients, recipients of allogeneic stem cell transplantation (allo-SCT) and healthy volunteers.

Study design

From 28th October until 18th December 2009, 97 adult patients with haematological malignancies and 25 adult controls were vaccinated in compliance with UK DoH guidelines (Department of Health, 2009). All patients and donors gave informed consent for collection of serum and PBMC and the study protocol was approved by the local research ethics committee. Of the 97 patients, 32 had chronic myeloid leukaemia (CML) in chronic phase (CP) in complete cytogenetic response (CCyR) on the tyrosine kinase inhibitors imatinib or dasatinib, 39 had a B-cell malignancy in complete remission (CR) or untreated, including non-Hodgkin's lymphoma (NHL), Hodgkin's lymphoma (HL) or chronic lymphocytic leukaemia (CLL), and 26 were recipients of allogeneic hematopoietic stem cell transplantation in CR at least 6 months beyond transplant and without evidence of active graft versus host disease (GVHD). Healthy controls were recruited from hospital staff who were offered vaccination as front-line healthcare workers. Patients and controls with a previous exposure to 2009 H1N1 infection, as confirmed by RT-PCR, were excluded from this study.

Vaccine

Consistent with UK DoH guidelines all patients received an inactivated split-virion preparation of the influenza A/California/2009 (H1N1)v-like strain containing 3.75µg of haemagglutinin and AS03 adjuvant (Pandemrix GSK, UK). The vaccine was administered by intramuscular injection into the deltoid muscle of the non-dominant arm by the patient's primary care physician, as per DoH guidelines. Over the same period, 89 of 97 patients and 15 of 25 controls also received one dose of a seasonal influenza vaccine containing 15 µg of haemagglutinin antigens of the

three following strains: A/Brisbane/59/2007 (H1N1)-, A/Brisbane/10/2007 (H3N2)- and B/Florida/30/2008-like strain; in the majority of patients and controls the vaccines were not given concomitantly.

Safety assessments and assessment of influenza-like illness

We solicited reports of local (pain, tenderness, redness, induration and ecchymosis) and systemic (fever, headache, malaise, myalgia, chills and nausea) adverse events by two-weekly phone calls performed by trained medical students, starting one week after the first injection. All solicited local and systemic adverse events within 7 days post vaccine were considered to be related to the vaccines. Symptoms were graded as follows: none, mild if they did not interfere with normal activities, moderate if they resulted in interference with normal activities, and severe if they prevented engagement in daily activities or necessitated medical attention.

An influenza–like illness was defined as an oral temperature of more than 38°C or a history of fever or chills and at least one influenza-like symptom.

Serum and peripheral blood mononuclear cells (PBMC) were collected before vaccination and on days 21 and 49 after the first vaccine dose, and cryopreserved.

II.1.2 Patient selection for inclusion in the study ‘T cell and B cell responses after vaccination against seasonal influenza and pneumococcus’

Individuals recruited in this study were healthy volunteers and CML patients only.

Fifty one patients with CML-CP patients in complete cytogenetic response (CCyR) on standard dose imatinib (n=26), dasatinib (n=13) or nilotinib (n=12) and 24 adult

controls were recruited in this study during two influenza seasons (2008 and 2009). Patient characteristics are summarized in Table IV.1. Healthy controls were recruited amongst hospital staff. The median age for CML patients was 52 years and for healthy controls 41 years (p=0.10). All patients and controls were vaccinated against influenza (Influenza vaccine Ph. Eur. 2008/2009, or Influenza vaccine Ph. Eur. 2009/2010, CSL biotherapies, Marburg Germany) and the pandemic influenza A(H1N1) in 2009 (de Lavallade H. *et al*, 2011). Forty five patients with CML and 12 healthy controls were concomitantly immunized with the 23-valent polysaccharide pneumococcal vaccine (Pneumovax II; Sanofi Pasteur MSD, Maidenhead, UK). Only patients and controls who had not received a pneumococcal vaccine within the previous 5 years were re-immunized.

Peripheral blood mononuclear cells (PBMCs) and serum samples were collected from all patients and donors prior to vaccination and responses were assessed at 4 weeks and at 2–3 months post-immunization. All patients and adult controls gave informed written consent and the local institutional ethics board approved the study protocol.

II.1.3 Patient selection for the study 'effect of imatinib on B cell subsets in CML patients and impact of Philadelphia positive B cell subsets'

In this study the samples were directly requested from the John Goldman Centre for Cellular Therapy (JGCCT) in the department of Haematology at Hammersmith Hospital. The samples were apheresis-derived mononuclear cells (MNCs) obtained from newly diagnosed CML patients using a COBE® spectra Apheresis system. Patients were harvested before starting on imatinib and, where applicable, once they achieved CCyR on this treatment.

Selection of samples was done according to the following inclusion criteria:

- All samples at diagnosis were from previously untreated CML patients in chronic phase
- Samples harvested at the time of CCyR on imatinib were only requested if a paired diagnostic sample was available ('responder group')
- Diagnostic sample from patients who did not respond to imatinib were selected only if they had not achieved a major cytogenetic response (MCyR) at 12 months ('non-responder' group)
- Diagnostic samples from CML patients in chronic phase whose disease secondarily transformed to lymphoid or myeloid blast crisis while on imatinib were also selected and included in the 'non responder' group

Thirty nine chronic phase CML patients were included in this retrospective study. Paired samples from 25 CML patients who responded to imatinib ('responder' group) were studied. We also analysed samples collected at presentation from 14 patients who subsequently failed imatinib therapy ('non responder' group, including 10 patients who subsequently developed blast crisis). All 25 patients in the 'responder' group achieved complete cytogenetic response and diagnostic samples were taken at presentation while remission samples were taken at a median time of 25 months (range, 15 to 56 months) from presentation sample once patients had achieved CCyR. Samples used in this experiment were apheresis-derived MNCs obtained from leukapheresis products and subsequently frozen.

II.2 PROCESSING, FREEZING AND THAWING OF PBMC

II.2.1 Isolation of PBMC

A maximum of 20-30ml of whole blood was collected from patients and healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient separation technique (Lymphoprep). Briefly, 15 ml heparinised whole blood was diluted 1:1 with RPMI 1640 media (GIBCO / Invitrogen) in a 50ml falcon tube and 10ml of lymphoprep (Axis Shield, UK) was gently layered under the diluted blood. After centrifugation at 1800 rpm for 30 min, the interface layer between plasma and lymphoprep, which contains mononuclear cells, was collected and re-suspended in fresh RPMI 1640. Cells were washed twice with RPMI 1640 media at 1200 rpm for 10 min. PBMC were resuspended in 10% fetal calf serum (FCS)/ RPMI, counted using Trypan Blue (TB) for cell viability assessment.

II.2.2 Cell Freezing

PBMCs were suspended in freezing media (RPMI-CM with 20% foetal calf serum (FCS) and 20% DimethylSufoxide (DMSO) (Sigma-Aldrich, UK)) on ice and aliquoted into cryovials and stored in -80 (max 1 week) before transfer to liquid nitrogen.

II.2.3 Cell Thawing

Cryovials of PBMCs were transferred from liquid nitrogen on dry ice. Each vial was thawed in a water bath at 37 ºC. Once thawed, PBMCs were transferred immediately to a 15 ml falcon tube containing 10 ml thawing media (RPMI 20% FCS containing 50,000 unit DNase). PBMCs were centrifuged at 1200 rpm for 10

min. PBMCs were then re-suspended in 1 ml media and cell count and viability were assessed.

Following freeze-thaw procedure, the average yield of live cells was 90% (range, 80 to 95%).

II.2.4 Cell count and viability

Cell counts were performed by trypan blue exclusion. Cell volume was diluted 1:1 with trypan blue and a viable cell count was performed on a standard haemocytometer.

II.2.5 Apheresis-derived MNCs: freezing

The MNCs obtained from patients' apheresis were transferred to the JGCCT (Department of Haematology, Hammersmith Hospital), frozen and stored according to the Good Manufacturing Practice (GMP)' and Human Tissue Authority (HTA) approved Standard Operating Procedures (SOPs) of the JGCCT.

II.2.6 Apheresis-derived MNCs: thawing

Thawing of apheresis-derived MNCs material was done using the following protocol:

- Prepare 50 mL of 5x thawing solution (RPMI 1640 + 50% FCS + 50 000 U DNAse + 500 U preservative free heparin + 2% glutamine + 2 % pen/strep)
- Prepare buffer for cell sorting using 500 mls CliniMACS® PBS/EDTA Buffer (Myltenyi Biotec GmbH, Germany) to which 50mls of Albumin 5% is added to obtain a 0.5% albumin MACS buffer
- Thaw the cryovial in watherbath at 37º, transfer the cells in a universal tube

and add dropwise 10 mL of pre-warmed 5x thawing solution

- Spin + discard supernatant and add 200 000 UI DNAse directly to the pellet for 2 minutes
- Wash and resuspend in 10 mL RPMI 1640
- Add 15 mL lymphoprep in a universal tube and layer the 10 mL cells in suspension; spin for 30 min
- Take layer, resuspend in MACS buffer and count cells
- Label cells with CD34+ beads (Mylteni) and incubate for 30 min
- Wash and apply cells to an LS column; collect unlabelled cells that pass through
- wash unlabelled cells in 10 ml MACS buffer and count cells
- Take an aliquot of 10 x 10^6 cells for staining with the extended B cell panel (see II.9.5) and use the remains of the cells for B cell enrichment (see II.9.2).

II-3 FLOW CYTOMETRY MATERIALS

II-3.1 Antibodies

Fluorescent-conjugated monoclonal antibodies (mAb) were used for phenotypic determination of cell subsets. A list of mAbs used in this project is shown in table II-1. Antibodies were purchased from BD Biosciences UK, Beckmann Coulter UK, DakoCytomation, invitrogen and eBioscience. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC) were used as fluorophores for intra-cellular staining experiments and some surface staining experiments, while phycoerythrin-Cyanine-7 (PE-Cy7), peridinin chlorophyll protein Cyanine 5.5 (PerCp Cy5.5), allophycocyanin H7 (APC-H7)

quantum dot 605 (Qdot605).

TABLE II.2: The panel and clones of monoclonal antibodies used for surface staining and intra-cellular staining experiments. Figures in bold denote the antibody manufacturer (BD: BD Biosciences UK; BC: Beckmann Coulter UK,)

Antibody	**Manufacturer (Clone)**	**Description/function**
CD3	BD (SK7)	Pan T cells marker
CD4	BD (RPA-T4)	T-helper marker
CD8	BD (HIT8a)	T-cytotoxic marker
CD19	Beckman Coulter /Immunotech (J3-119)	Pan B-cell marker
CD27	DakoCytomation (M-T271)	Memory marker
IgD	SouthernBiotech (IADB6)	Immunoglobulin D marker
IgM	Beckman Coulter (SA-DA4)	Immunoglobulin M marker
CD107a	BD (H4A3)	Degranulation marker
CD154	BD (TRAP1)	CD40 ligand
IFN-γ	BD (25723.11)	Cytokine,Th1 marker
TNF-α	BD (MAb11)	Cytokine,Th1 marker
IL-2	BD (5344.111)	Cytokine,Th1 marker
CD24	BD (ML5)	B cell proliferation and maturation
CD27	BD (M-T271)	TNF-receptor super-family Regulation of B-cell activation and immunoglobulin synthesis
CD38	eBioscience (HIT2)	Ectoenzyme (calcium signalling)
IgM	BD (G20-127)	Surface antibody

Antibody	Manufacturer (Clone)	Description/function
CD21	eBioscience (HB5)	Complement component receptor 2
CD19	BD (SJ25C1)	Pan B-cell marker
CD10	Invitrogen (MEM-78)	Membrane metallo-endopeptidase
IgD	BD (IA6-2)	Surface antibody
Btk (pY551)	BD (24a)	Tyrosine kinase BCR signal transduction molecule
PLCγ2 (pY759)	BD (K86-689.37)	Superfamily of Phospholipase C Lipid signalling pathways BCR signal transduction molecule

II-3.2 Other materials

Brefeldin A and paraformaldehyde were purchased from Sigma. CD28/49d co-stimulatory reagent, monensin, BD FACS Permeabilizing Solution 2 and BD FACS Lysing Solution were purchased from BD Biosciences UK. Two different live/dead markers were used:

- LIVE/DEAD Fixable Aqua Dead cell stain Kit (invitrogen)
- propidium iodide 1.0 mg⁄mL (invitrogen, P3566)

II-3.3 Antibody titration

The volume of monoclonal antibody (Mab) was determined in dose titration experiments. The volume which resulted in maximal mean fluorescence intensity (MFI) and maximal percentage of positively staining cells with minimal non-specific staining was selected. An example of antibody titration is shown in Figure II.4 (page 84).

II-3.4 Immunophenotyping

Direct cell surface phenotyping was performed as follows; thawed cells were washed in 2 ml of PBS (pH 7.2). Samples were incubated at 4ºC for 20 minutes in the dark with directly conjugated surface antibodies (Table II-1). Cells were then washed in 2ml PBS buffer and re-suspended in 100µl prior to analysis. Data acquisition was performed with FACSCalibur™ (BD/Pharmingen, San Jose, CA). Data were analysed using CellQuest™ and FlowJo™ software (TreeStar, San Carlos, CA).

II-4 DETECTION OF INFLUENZA-SPECIFIC CD8+ T CELLS USING PENTAMER

II-4.1 HLA tissue typing

High resolution HLA class I genotyping was performed by sequence-specific PCR using genomic DNA in the HLA Laboratory, Department of Immunology at Hammersmith Hospital.

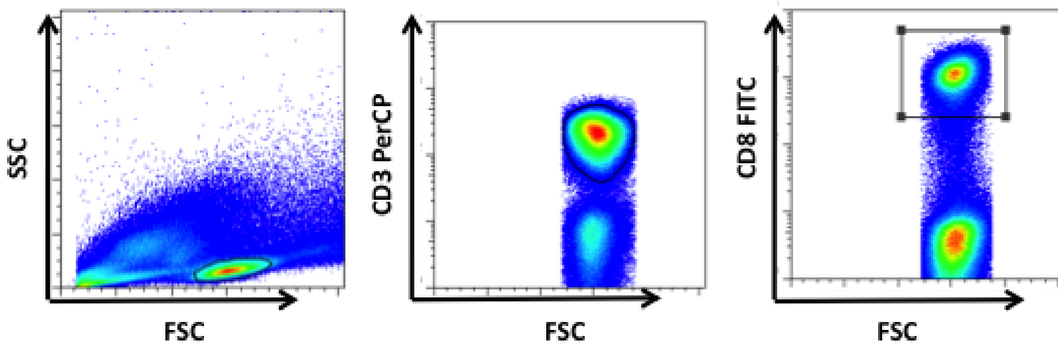
II-4.2 MHC class I pentamer staining

To assess the percentage of Influenza specific CD8+ T cells, PBMCs from HLA A0201 patients were stained with HLA-A*0201/GILGFVFTL (FluMP) Pro5™ MHC I Pentamer (Pro-immune) conjugated to APC. PBMC were stained according to the manufacturer's protocol. MHC Class I Pentamer was centrifuged at 14,000 × g for 5 minutes in a chilled microcentrifuge to remove protein aggregates that contribute to non-specific staining; PBMC were washed and resuspended in 50 µL PBS at a concentration of 1 x 10^6 and incubated with 10µl of APC-labeled Class I Pentamer

for 10 minutes at room temperature (22ºC) in the dark. Cells were then washed and resuspended in ~ 50µl PBS and incubated on ice for 20 minutes with CD8-FITC (Pro-immune 1 µl), and CD3-PerCp (5 µl, BD). Cells were washed twice in PBS and processed on FACSCalibur™ immediately. Data were analysed using FlowJo software (TreeStar, San Carlos, CA). The volume of pentamer to be used in experiments was determined in dose titration experiments. The volume which resulted in maximal mean fluorescence intensity (MFI) and maximal percentage of positively staining cells with minimal non-specific staining was selected.

Figure II.1: Gating strategy to characterize HLA A0201 restricted Flu-specific CD8+ T cells

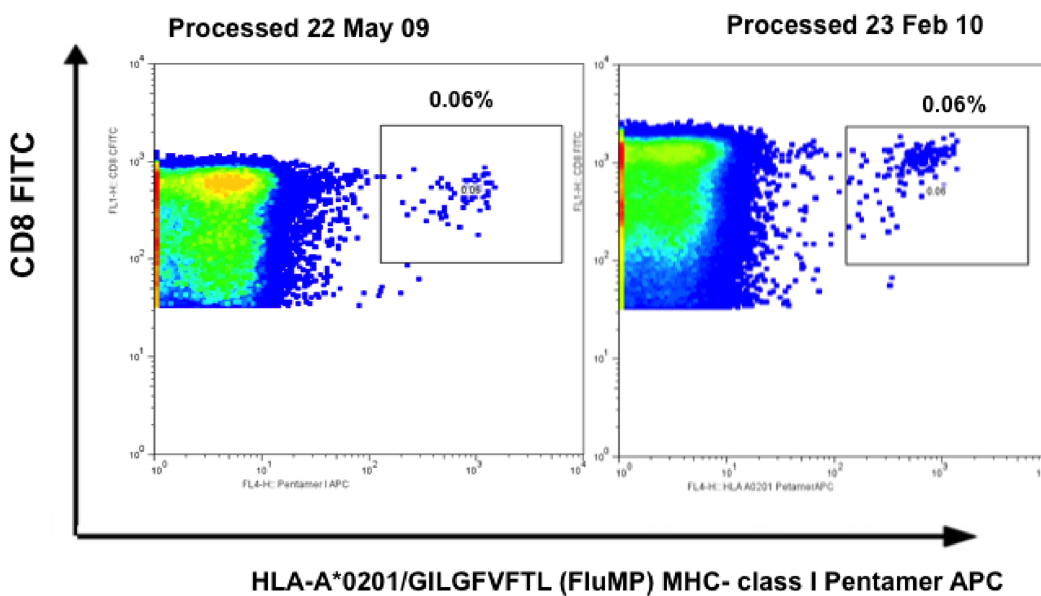
Cells were first gated on lymphocytes (black round gate, left panel), of which CD3+ cells were selected (black round gate middle panel). CD8+ were finally gated from CD3+ cells (black square gate, right panel)



When a new batch of pentamer was purchased, reproducibility of the assay was assessed by comparing the staining of the same sample, with the two different batches (Figure II-2).

Figure II.2: example of reproducibility of results showing an identical sample (CML patient HLA -A0201, 4 months post influenza vaccination) thawed and stained on two different days, 9 months apart, with two different batches of Pentamer.

CD8+ cells were gated from CD8+ CD3+ lymphocytes, as shown in Figure II.1.



II-5 DETECTION OF POLYFUNCTIONAL CD8+ AND CD4+ ANTIGEN SPECIFIC T CELLS: OPTIMIZATION AND STANDARDISATION

II.5.1 Intracellular cytokine assay and T cells cytotoxicity assay

Frequency of antigen-specific CD8+ and CD4+ T cells was assessed by production of cytokines in response to antigenic stimulation. Production of TNFα, INFγ and IL-2 were assessed to study CD4+ Th1 and CD8+ cytotoxic T cell responses. T-cell cytotoxicity was studied after incubation with relevant antigens and measuring CD107a production, that is a sensitive surrogate marker for T cell degranulation (Lamoreaux *et al*, 2006). Surface expression of CD107a is found only in cells that have degranulated. Upon stimulation of PBMCs, cytotoxic granules that contain CD107a and CD107b in their membranes migrate toward the cell membrane and the CD107a and CD107b are transiently exposed on the cell surface. At this point, CD107a and CD107b are available for binding to fluorescence-tagged antibodies before being reinternalized into the endosomal spaces.

PBMC were resuspended at a concentration of 1 x 10^6 PBMC/ 200 µL and plated in flat-bottom 96 well plates. PBMC were stimulated using 3 different conditions:

1- No stimulation (negative control),

2- PMA (50ng/ml)/Ionomycin (2ug/ml) (Sigma Aldrich), (positive control)

3- Flu antigens (see II.5.2)

The choice of cytokine secretion inhibitor was based on its potential to trap cytokine in the most effective manner. Monensin fails to optimally inhibit TNFα secretion from the cell while Brefeldine A (BFA) (Sigma- Aldrich) successfully blocks it

(Lamoreaux *et al*, 2006;O'Neil-Andersen & Lawrence, 2002).

BFA was therefore used for TNFα detection. For CD107a degranulation assay monensin was used in addition to BFA because it is required to prevent acidification of lysosomes and endosomes when reinternalization of CD107a occurs during T-cell stimulation.

Cells were incubated with either 10 µl Brefeldin A (BFA, 0.5mg/ml) alone (for TNFα and IFNγ intracellular staining), or both BFA and 5µl monensin along with 10µl CD107a-FITC. For the study on immune responses to 2009 H1N1 vaccine, only TNFα and IFNγ production were evaluated. Ten microlitres of 1ug/ml PMA (ie 50ng/ml) and 8 µl of 50ug/ml ionomycin (ie 2ug/ml) were added as positive control at the same time as BFA or monensin.

After 5 hours incubation, cells were washed and surface stained with 5 µl CD3-PerCp and 5 µl CD8-PE (BD biosciences, UK) (as described in II.3.4). The cells were then fixed in 2 mL FACS lysing solution (BD biosciences, UK) for 10 minutes in the dark, centrifuged and then permeabilized using 0.5 mL FACS permeabilizing solution (BD biosciences, UK) for 10 minutes in t **DECLARATION** he dark. After a further wash, intracellular staining was performed using either 5 µl IFNγ-APC and 0.5 µl TNFα-FITC or 15 µl IL2-APC. For the study on immune responses to 2009 H1N1 vaccine, 5 µl IFNγ-APC and 1 µl TNFα-APC were used in order to capture T-cells producing effector cytokines.

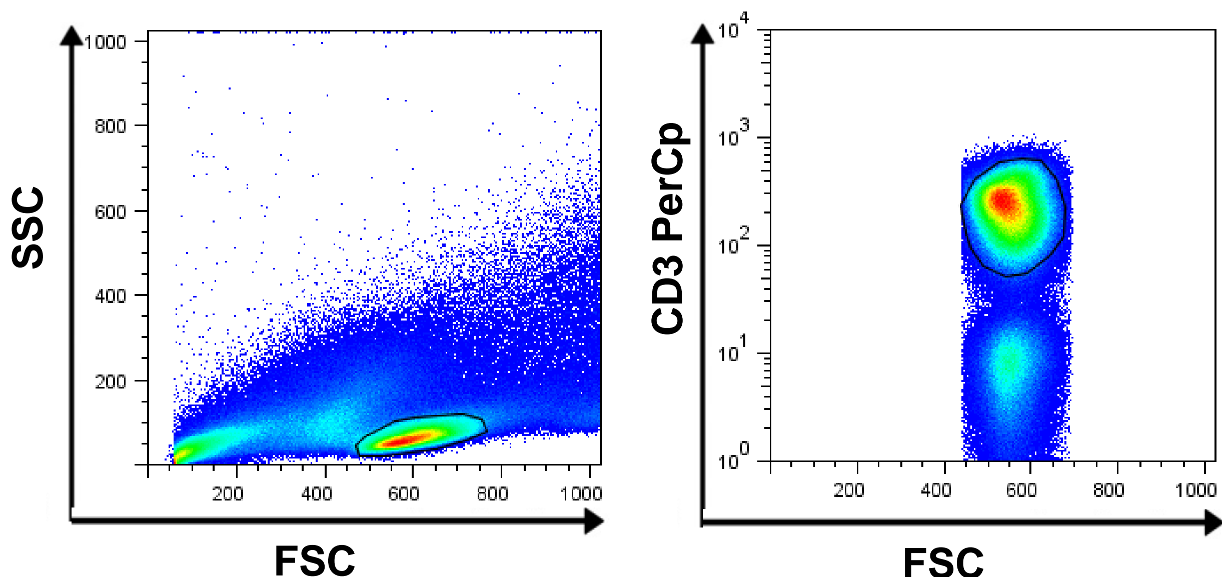
Cells were incubated for 30 minutes in the dark, fixed in 1% paraformaldehye (PFA, Sigma) and refrigerated until acquisition. Data acquisition was performed using FACSCalibur™ and a minimum of 300 000 events were acquired. Data were

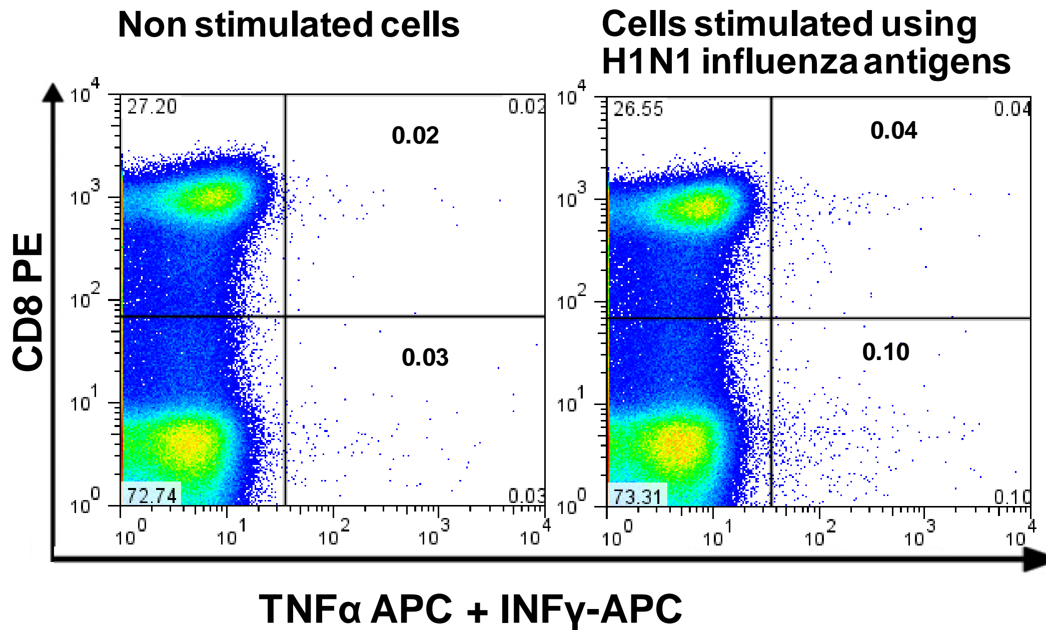
analysed using CellQuest™ and FlowJo™ software (TreeStar, San Carlos, CA).

The gating strategy was performed as shown on Figure II-3; the threshold of positivity for cytokines and CD107a was set in order to minimize non-specific staining in non-stimulated cells (negative control) as shown on Figure II-3. The results were evaluated by subtracting the background positivity, and by comparing the fold increase in frequency of cytokine positive CD8+ and CD4+ T-cells (ie stimulated cells/non-stimulated cells).

Protocol optimization included PMA titration, ionomycin titration, antibody titration (CD107a, IL2 and TNFα), BFA titration, monensin titration and comparison between BFA alone versus monensin alone versus BFA + monensin.

Figure II.3: Example of gating strategy for intracellular cytokine assay. The threshold was set based on the isotype and negative control (unstimulated).





II.5.2 Protocol optimization for antigenic stimulation

Stimulation using seasonal influenza vaccine solution

Protocol optimization was first performed on fresh PBMCs from adult controls that had been vaccinated with seasonal influenza vaccine. Briefly fresh PBMCs were plated at $2x10^6$ cells/ 200 µL RPMI supplemented with 10% FCS per well of a 96-well plate (BD FalconTM, Oxford, UK) and rested for 4 hours. To determine the optimal dose for the vaccine to induce CD8+ or CD4+ T-cells, PBMC were incubated with increasing dose of the seasonal influenza vaccine solution (0.5 µl, 1µl, 5µl, 10 µl or 50 µl) of the 0.5 ml (CSL Biotherapy) containing 15 µg of Haemagglutinin antigens of the three following strains: A/Brisbane/59/2007 (H1N1)-, A/Brisbane/10/2007 (H3N2)- and B/Florida/30/2008-like strain and 1 µl CD28/49d co-stimulatory reagent (BD biosciences, UK) for 12 hours. The optimal CD8+ and CD4+ T-cell response to the influenza vaccine was achieved using 10µl of the vaccine solution, ie a concentration of 1.5 µg/ml of Haemagglutinin antigens

equivalent of each of the strains.

Once the optimal dose of the vaccine was determined, I determined the optimal incubation time by stimulating PBMCs for 16, 24 or 48 hours with 1.5 µg/ml of Haemagglutinin antigens equivalent (10µl of the vaccine solution). The optimal time of incubation was found to be 24 hours, and was used for the subsequent experiments.

The reproducibility of this protocol was confirmed by comparing fresh and frozen PBMC from the same donor. Briefly PBMCs were thawed and rested overnight at 37 ºC, 5% CO2, to minimize background cytokine production secondary to lymphocyte manipulation. PBMCs were counted and plated at 1 to 2 $x10^6$ cells/ 200 µL RPMI supplemented with 10% FCS per well of a 96-well plate (BD FalconTM, Oxford, UK). Cells were stimulated using 3 different conditions of antigen for 19 hours at 37 ºC, 5% Co2:

1- CD28/49d co-stimulatory reagent only (negative control), added at Hour (H) 0

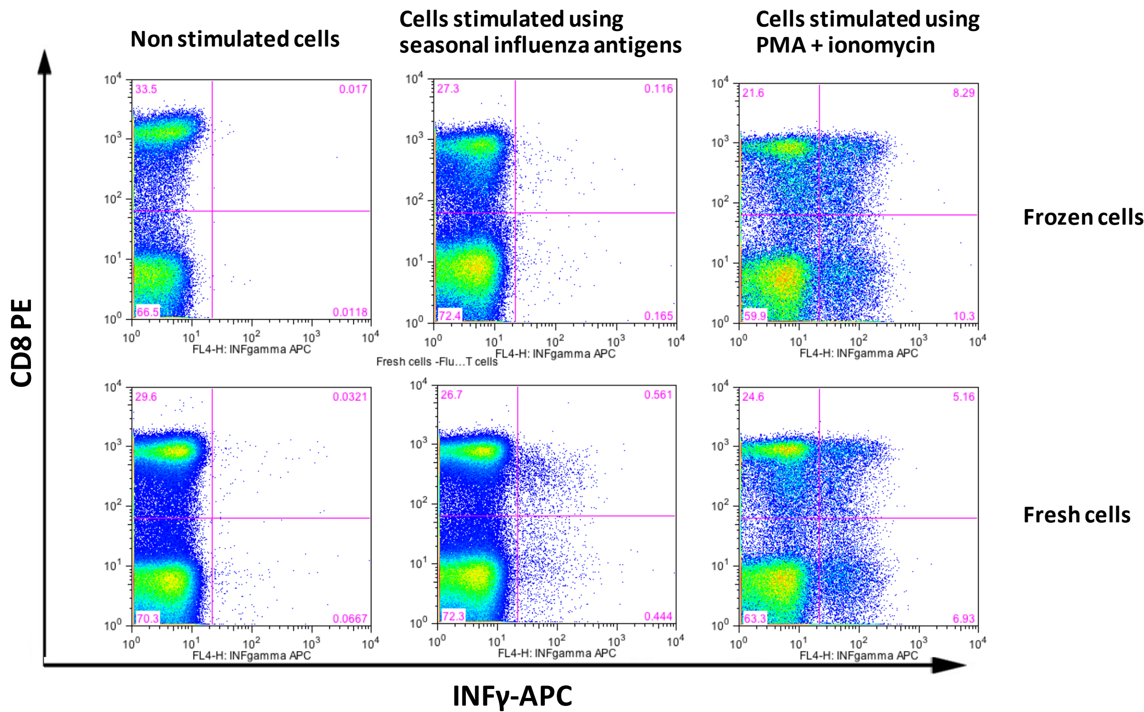
2- PMA (50ng/ml)/Ionomycin (2ug/ml) (Sigma Aldrich), (positive control) added at H+19

3- CD28/49d co-stimulatory reagent and influenza antigens added at H0

After 19 hours of stimulation, BFA or monensin + CD107a and PMA/ionomycin (where appropriate) were added for another 5 hours (see II.5.1) incubation at 37ºC, 5% Co2 followed by intracellular staining (see II.5.1). PBMC were stimulated with influenza antigens for a total of 24 hours (19 + 5).

Comparison with fresh cells was performed and the results were reproducible, as shown in figure II.4.

Figure II.4: Comparison between fresh and frozen cells (INFγ)



Stimulation using 2009 H1N1 influenza vaccine solution

The 2009 H1N1 influenza vaccine solution is a monovalent vaccine and contains one strain of A/California/07/2009(H1N1)v-like strain in various proportion. We tested two vaccines:

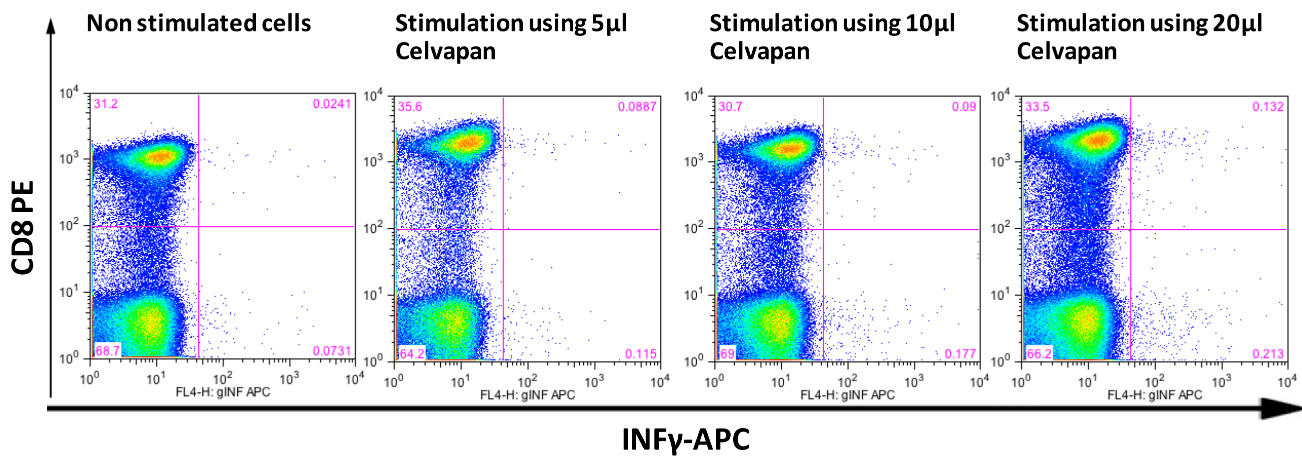
- Pandemrix (GSK, UK) vaccine containing 3.75 µg A/California/07/2009(H1N1)v-like strain Haemagglutinin antigen in 0.5 ml to be mixed with adjuvant SF03

- Celvapan (Baxter, UK) containing 7.5 µg A/California/07/2009(H1N1)v-like strain Haemagglutinin antigen in 0.5 ml without adjuvant

Protocol optimization was first performed on fresh PBMCs from adult controls at various time points from 4 weeks onward after vaccination with 2009 H1N1 vaccine. Briefly cells were incubated for 24 hours with 5µl, 10 µl, 20 µl or 50 µl of both vaccine solutions. Importantly stimulation with Pandemrix was done using only the solution containing the antigens (separated from the one containing the

adjuvant). A quantity of 20 µl Celvapan was found optimal, which correspond to a final concentration of 1.5 µg/ml of Haemagglutinin antigens, equivalent to the titration obtained with the seasonal influenza vaccine (Figure II.5). Concentration of Haemagglutinin antigens were comparable to that described by others (Vogt *et al*, 2008). Definition for a positive flu-specific cytokine immune response was comparable to that used by others (Vogt *et al*, 2008).

Figure II.5: Optimisation of Celvapan concentration to induce optimal T cell stimulation



II.5.3 Detection of H1N1-specific T cells in the study

To assess T-cell responses to H1N1, PBMCs collected before vaccination and on day 49 were thawed and stimulated for 24 hours with or without H1N1 vaccine (A/California/07/2009(H1N1)v-like strain, Baxter, United Kingdom) or seasonal influenza vaccine (A/Brisbane 59/2007(H1N1), A/Brisbane/10/2007(H3N2)- and B/Florida/30/2008-like strain, CSL Biotherapies, Germany) (used as positive control) at a final concentration of 1.5 µg/ml of haemagglutinin antigens. Assessment of the effector function of antigen-specific CD8+ and CD4+ T-cells was performed by intracellular-cytokine staining for interferon-γ (INF-γ) and tumour

necrosis factor-α (TNF-α) as described in section II.5.1. Optimisation of antigen stimulation was performed as described in section II.5.2. Allophycocyanin (APC)-conjugated antibodies to INF-γ and TNF-α were employed to detect the frequencies of INF-γ or TNF-α producing T-cells. A response was considered positive if the combined percentage of H1N1-specific TNF-α plus IFN-γ producing CD4+ or CD8+ T-cells was 2-fold or higher compared to background level (non-stimulated PBMC) and if there was a minimum of 0.05% H1N1-specific TNF-α plus IFN-γ producing CD4+ or CD8+ T-cells (after subtracting the background).

II.5.4 Detection of seasonal influenza-specific T cells

The immunological T-cell responses to seasonal influenza virus were analyzed both quantitatively and qualitatively using flow cytometry and intracellular cytokine assay for TNF-α, IFN-γ, IL-2 and the cytotoxicity marker CD107a as described in section II.5.1. PBMC collected before and 2-3 months post-vaccination were thawed and stimulated for 24 h with or without seasonal influenza vaccine at a final concentration of 1.5 µg/mL of hemagglutinin antigens or with PMA (50ng/ml) and ionomycin (2ug/ml, Sigma Aldrich) (positive control) for 19 h at 37°C. Brefeldin A (10 µg/mL) (Sigma Aldrich, Gillingham, UK) was added alone or with monensin (0.7µl/mL) (BD/Pharmingen, San Diego, CA) and the degranulation marker CD107a-FITC (BD/Pharmingen, San Diego, CA). Influenza vaccine Ph. Eur. 2008/2009 was used to stimulate PBMC collected from subjects vaccinated in 2008 and Influenza vaccine Ph. Eur. 2009/2010 in those who received the vaccine in 2009. PBMCs were washed and stained with anti-CD3 (BD Biosciences, Oxford UK) and anti-CD8 antibodies (BD Biosciences, Oxford UK), fixed/permeablized (BD Biosciences, Oxford UK) and stained with anti–IFN-γ, anti–TNF-α anti-IL-2

antibodies (all BD/Pharmingen, San Diego, CA). Optimisation of antigen stimulation was performed as described in section II.5.2.

The threshold of positivity for cytokines and CD107a was set in order to minimize non-specific staining in non-stimulated cells (negative control). The results were evaluated by subtracting the background positivity, and by comparing the fold increase in frequency of cytokine positive CD8+ and CD4+ T-cells. Following vaccination, a response was considered positive if there was a minimum of 0.10% Flu-specific TNF-α or INF-γ-producing T-cells and the percentage of antigen-specific TNF-α or INF-γ-producing T-cells was 2-fold or higher compared to pre-vaccination level.

II.5.5 CD154 staining on CD4+ T cells

CD154 expression has a fundamental role in the interactions between CD4+ T cells and B cells and antigen presenting cells. Indeed recently activated CD4+ T cells express (CD40L), which provides costimulatory signals to activate B cells while resting CD4+ T cells do not express CD154 (Brines & Klaus, 1993;Kawabe *et al*, 1994). By providing crucial costimulatory signals, the expression of CD154 by antigen-specific T cells identifies cells that may provide B-cell help. In addition stimulated CD4+ T cells expressing TNFα, INFγ or Il-2 are predominantly CD154+ cells. CD154 expression is therefore a marker that represents multiple functional responses of CD4+ T cells.

To determine the CD154 (CD40L) expression on CD4+ cells after vaccination with 2009 H1N1 vaccine or seasonal influenza vaccine, protocol optimization was based

on previously publications (Chattopadhyay *et al*, 2005;Chattopadhyay *et al*, 2006); briefly fresh PBMCs from adult controls that had been vaccinated with both 2009 H1N1 vaccine and seasonal influenza vaccines were incubated with different concentrations (1µl, 5 µl, 10 µl and 20 µl) of anti-CD154 (CD40L) PE-conjugated antibody to obtain the highest signal-to-noise ratio (see Figure II.4).

PBMCs were thawed and rested overnight at 37°C in 96-well plate in 200 µl volume; after 18 hours stimulation with and without 1.5 µg/ml of the 2009 H1N1 vaccine solution or 1.5 µg/ml of the 2009 seasonal influenza vaccine (CSL Biotherapy) cells were incubated for 6 hours with 0.7µL/mL monensin, 5 µl CD154 (CD40L) PE-conjugated antibody and 10 µl CD107a FITC-conjugated antibody (all BD/Pharmingen, San Diego, CA). Finally cells were stained with 5 µl CD3 PerCP-conjugated antibody and 10 µl CD4 APC-conjugated antibody for 20 minutes. CD154 expression was found to correlate with TNFα production and CD107a expression on CD4+ T cells as shown in Figure II.5.

Figure II.6: Antibody titration for CD154 staining: A dose of 5 µl CD154 was found to be optimal.

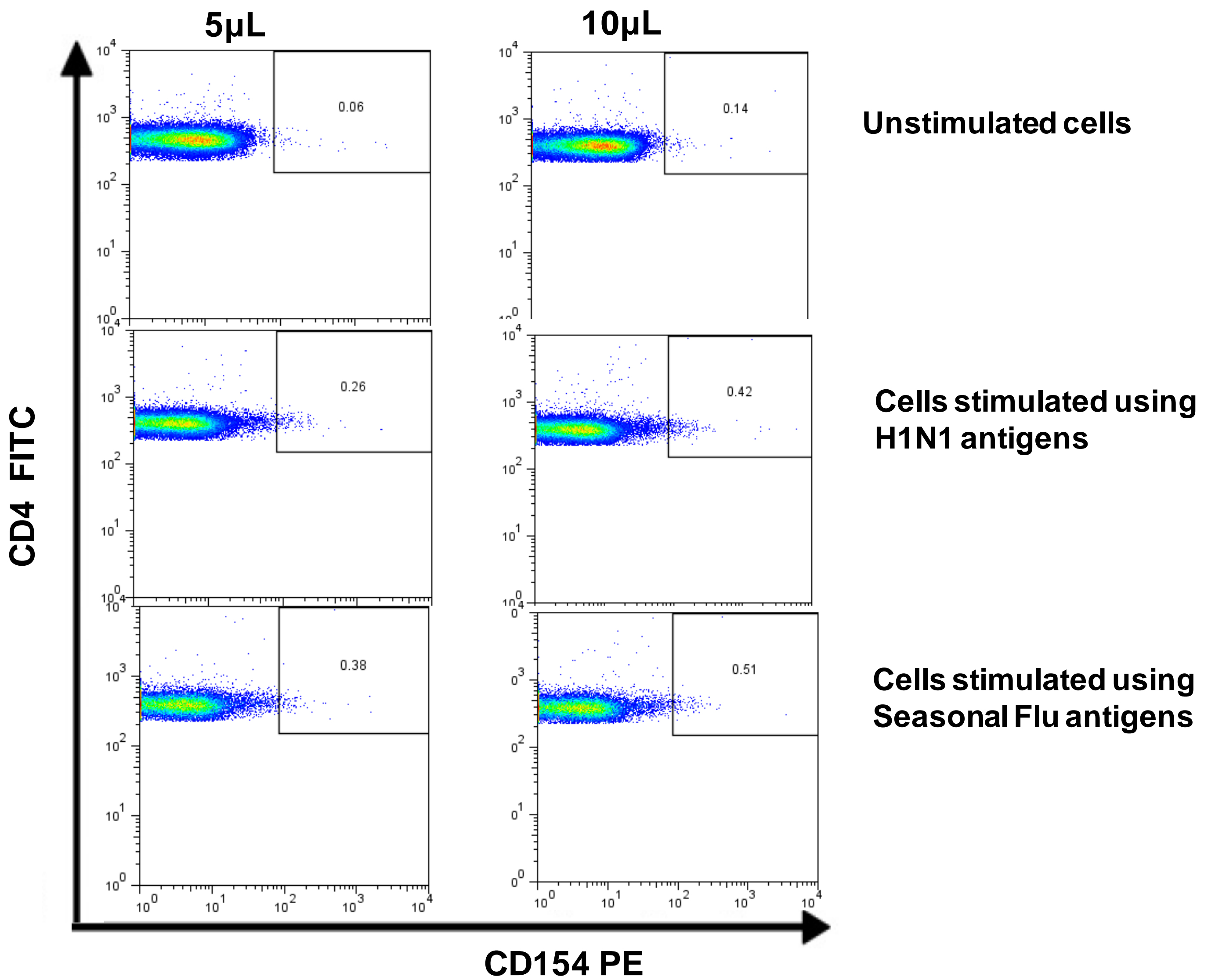
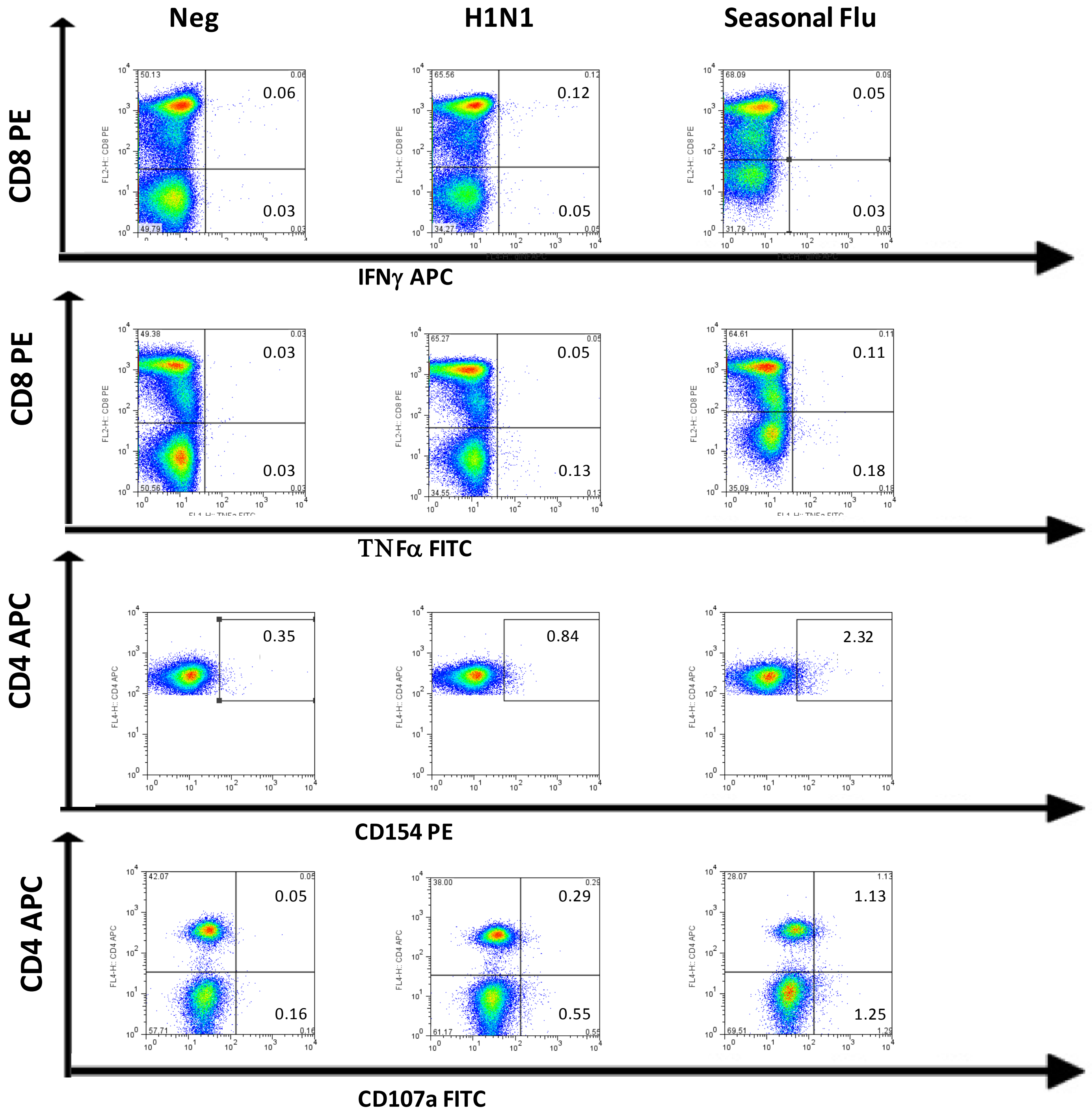


Figure II.7: Functional characterization of CD8+ and CD4+ T-cell responses to H1N1 and seasonal influenza in a multiple myeloma patient with confirmed H1N1 infection.

A response was considered positive if the percentage of antigen-specific IFN-γ, TNF-α, or CD107a expressing T-cells was 2-fold or higher compared to background (unstimulated PBMC) and if there was a minimum of 0.05% antigen-specific T-cells (after subtracting the background).



II.6 FOUR-COLOUR MEMORY B CELL PHENOTYPING

B cell phenotyping was adapted from a protocol kindly provided by Miss Melanie Hart in Dr William P Kelleher's laboratory (Chelsea and Westminster Hospital, Department of Immunology). The mAbs used to define the human B cell subsets were: PE-cyanin 7 (PC7) conjugated anti-CD19 (Coulter Immunotech High Wycombe, UK), PE-conjugated anti-human IgD (Southern Biotechnology Associates, Birmingham, USA), APC-conjugated anti-human IgM (The Jackson Laboratory, Bar Harbor USA), FITC-conjugated anti-CD27 (DakoCytomation, Glostrup Denmark).

Briefly frozen PBMCs were incubated for 30 minutes in the dark at room temperature with the antibodies shown on the panel (see Table II.2), and washed twice using PBS. A minimum of three thousand events were acquired on the B cell gate and the results are expressed as a percentage of CD19 events. FlowJo software (TreeStar, San Carlos, CA) was used for data analysis. Calibration was performed using cells alone and single labelled tubes, and acquired on a FACScalibur. Instrument settings were stored and used for subsequent experiments. Gating strategy was performed as shown in Figure II-6 and the threshold for CD27 FITC positivity was determined using the Fluorescence minus one (FMO) method (see Table II.3, tube 6).

Since IgM memory B cells express both IgD and IgM, co-expression of either IgD or IgM together with CD27 was used to define this cell subset. IgM memory B cells (defined as CD19+ CD27+ IgMhigh IgD+/lo) and switched memory B cells (defined as CD19+ CD27+ IgM- IgD-) subsets were calculated according to a modified Piqueras classification,(Piqueras *et al*, 2003) where both IgM memory B cells and

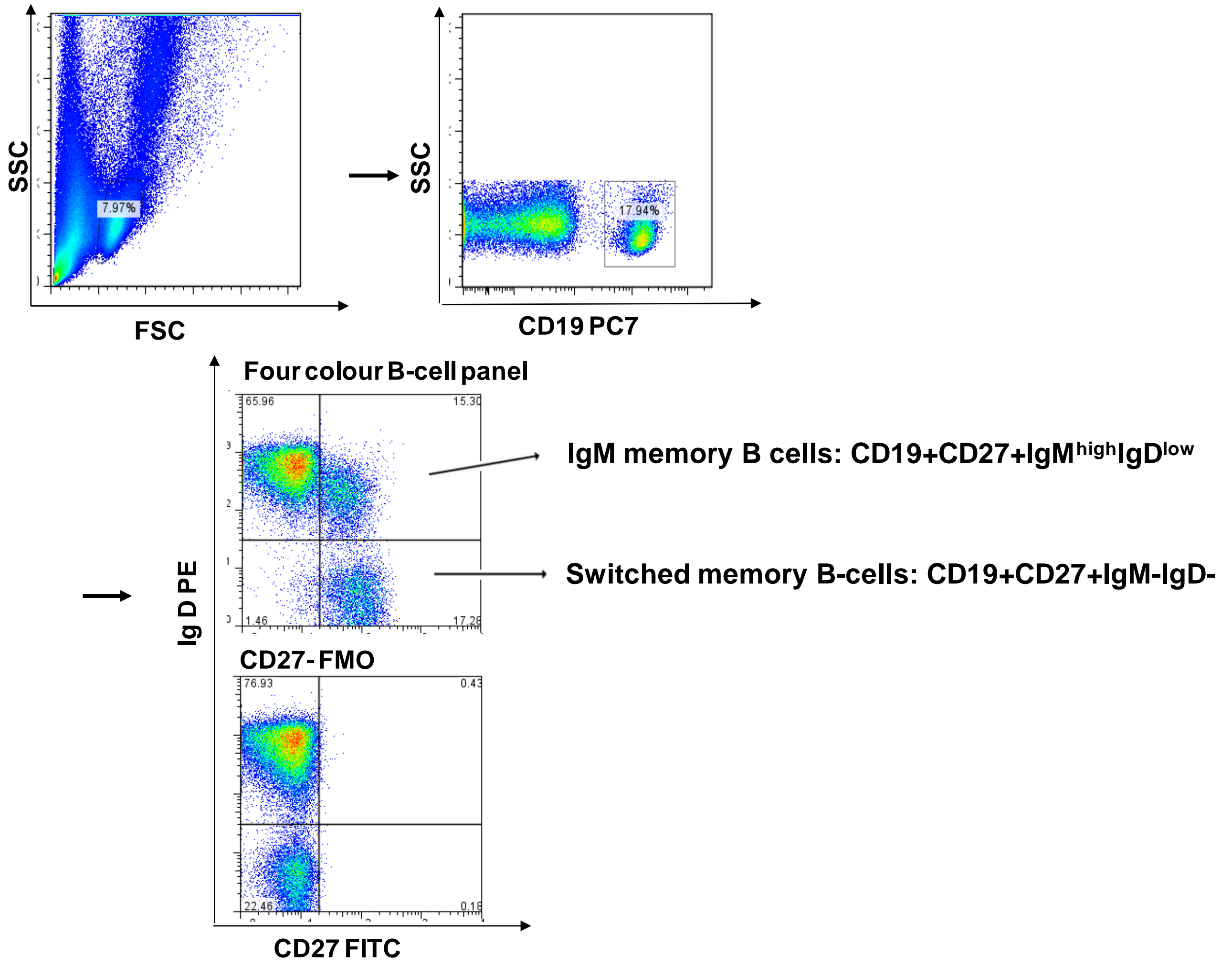
switched memory B cells are calculated as a percentage of the total B cell population.

Table II.3: Compensation tubes and FMO, B cell panel

Tube No	Tube	FL Channel	Antibody	Volume
1	Cells alone	Nil	Nil	
2	CD4 FITC	FL1	CD4 FITC (BD)	5μl
3	CD8 PE	FL2	CD8 PE (BD)	5μl
4	CD19 PC7	FL3	CD19 PC7 (Coulter)	10μl
5	CD27 APC	FL4	CD27 APC (BD)	5μl

	Tube	Antibody	Volume
6	FMO	IgD PE (Southern Biotech) CD19 PC7 (Coulter) (10UL) IgM APC (Coulter)	5μl (1:40) 10μl 5μl (1:10)
7	27,D,19,M	CD27 FITC (Dako) IgD PE (Southern Biotech) CD19 PC7 (Coulter) (10UL) IgM APC (Coulter)	5μl (1:5) 5μl (1:40) 10μl 1μl (1:10)

Figure II.8: memory B-cell gating



II.7 DETERMINATION OF ANTIPNEUMOCOCCAL SERUM TITRES for IGM AND IGG

This work was performed by Miss Melanie Hart (Chelsea and Westminster Hospital, Department of Immunology, Dr P. Kelleher, Imperial College London). Serum titres of IgM and IgG antipneumococcal were determined using ELISA technology. Briefly, 96-well plates (Maxisorp; Nunc; Fisher-Scientific) were coated with Pneumovax II 23-valent vaccine overnight at 4°C. Patient samples and reference standards were incubated with cell wall pneumococcal polysaccharide Ag (5 μg/ml; Statens Serum Institut) in PBS/0.1% Tween 20/1% BSA solution for 1 h at room temperature to remove nonspecific Abs which do not play a role in mediating protective immune responses. The 96-well plates were then washed three times with PBS/Tween 20 solution. Patient' samples (duplicates at four different dilutions) were then added and incubated at room temperature for 1.5 h. Plates were washed and then the appropriate HRP-labeled IgG or IgM conjugate (Sigma-Aldrich) was added. After a second wash step, plates were developed using o-phenylenediamine substrate, with results calculated from a standard curve. Pneumococcal IgG standard, assigned a value of 70 U (Protein Reference Unit, Sheffield, U.K.), was used together with an in-house standard for pneumococcal IgM levels to set up the standard curve.

A positive IgM Pneumovax II response was defined as a 4-fold rise in serum IgM titres or an IgM titre > 200 U/ml 4 weeks post-immunization irrespective of the pre-immunization titre. A positive IgG response was defined as a 2-fold rise in serum IgG titre or an IgG titre >200 U/ml at 1 or 3 months (Hart *et al*, 2007).

II.8 HEMAGGLUTINATION-INHIBITION ASSAY TO DETECT 2009 H1N1 ANTIBODY

This work was performed by Dr Katja Hoschler (Centre for Infections, Health Protection Agency,London, U.K). Antibody responses were detected by means of hemagglutination-inhibition assays, according to standard methods (Miller *et al*, 2010) and with the use of cell-culture X-179A H1N1 vaccine virus and egg-grown NIBRG-121 virus. Serum samples obtained from subjects were tested with the use of 1:2 serial dilutions; serum samples were tested in duplicates at an initial dilution of 1:8 and a final dilution of 1:1024. Haemagglutination-inhibition antibody titres were reported according to the criteria conventionally used to assess the immunogenicity of H1N1 influenza vaccines, i.e. geometric mean titres (GMT), geometric mean titre ratio, seroprotection rate (proportion with titres ≥1:32) and seroconversion rate (proportion with pre-vaccination titre <1:8 and a post-vaccination titre ≥1:32, or a pre-vaccination titre ≥1:8 and an increase in the titre by a factor of four or more), (The European Agency for the Evaluation ofMedicinal Products (EMEA), 1997).

Specific humoral responses to the seasonal flu vaccine were not measured as these have been extensively described previously (Kunisaki & Janoff, 2009;Ljungman & Avetisyan, 2008;Pollyea *et al*, 2010;Ring *et al*, 2002).

II.9 IDENTIFICATION OF BCR-ABL POSITIVE B-CELLS AND 8-COLOUR B CELL PANEL

II.9.1 Sample thawing and B-cell enrichment

Samples used in this experiment were apheresis-derived MNCs cryopreserved and stored by the JGCCT. Sample identification, request, storage and thawing was jointly performed with Dr Alexandra Bazeos who used the CD34 positive fraction for her PhD work while the resulting CD34 negative fraction was used for the purposes of this work.

Cell thawing was performed as described in II.2.6. CD34 positive selection was performed using a Miltenyi CD34 positive selection kit by Dr. Bazeos. The unlabelled, CD34 negative fraction was used for lymphocyte staining and selection. An aliquot of 10 x 10^6 cells was taken for staining with the extended B cell panel (see II.9.5) and the remaining cells were use for B cell enrichment and sorting.

B cell enrichment was performed prior to cell sorting on the FACS ARIA II to increase cell yield. Enrichment using microbeads and magnetic separation was performed using either CD19 positive selection for panel #1 (see II.9.6: isolation of transitional T1 B-cells, naïve B-cells, IgM memory B-cells and Switched memory B-cells) or negative B cell isolation (B cell isolation kit II', Myltenyi) for panel #2 (see II.9.6: isolation of LSCs and progenitors).

A buffer solution (containing PBS, pH 7.2, 0.5% BSA and 2 mM EDTA) was prepared by diluting MACS BSA Stock Solution (CN 130-091-376) 1:20 with autoMACS Rinsing solution (CN 130-091-222).

Cells for B cell enrichment were filtered before selection using a 30 µm lylon mesh pre-separation filters (Myltenyi CN 130-041-407) in order to remove cell clumps.

B cell enrichment using the ‘CD 19 Microbeads’ positive selection (MACS, Myltenyi Biotech, CN 130-050-301)

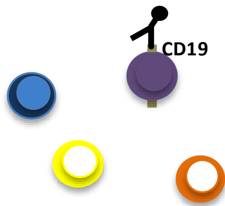
Cells were washed and resuspended in 80 µL of buffer per 10^7 total cells. Magnetic labelling was performed by adding 20 µL of CD19 MicroBeads per 10^7 total cells. After 15 minutes incubation in the refrigerator, cells were washed and resuspended at a maximum concentration of 10^9 cells per 5ml.

Magnetic separation was then performed using LS Columns on a MACS Separator. After rinsing the column with 3mL of buffer, cells were applied to the LS column and the column was washed 3 times with 3ml of buffer. The column was then removed from the separator and placed into a collection tube. 5mL of buffer was placed onto the column to flush the magnetically labelled cells out, as shown in figure II.7 (left panel). Cells were then resuspended in PBS 200 µL and used for the sorting panel #1 (see II.9.3).

Figure II.9: B cell enrichment using microbeads (adapted from Miltelnyi Biotech, Bisely, UK).

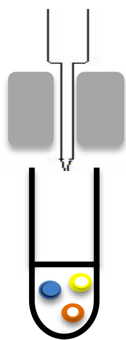
CD19 Positive selection strategy

Target cells are magnetically labeled and isolated as the magnetically retained cell fraction



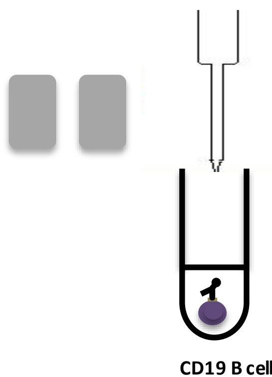
Magnetic labeling

Cells of interest are magnetically labeled with CD19 MicroBeads.



Magnetic separation

Cells are separated in a MACS Column placed in a MACS Separator. The flow-through fraction can be collected as negative fraction depleted of the labeled cells.

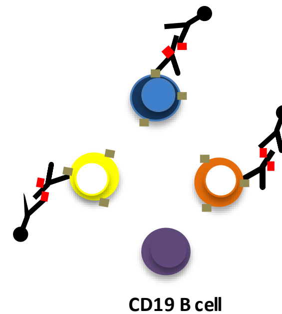


Elution of the labeled cell fraction

The column is removed from the separator. The retained cells are eluted as the enriched, positively selected CD19 cell fraction.

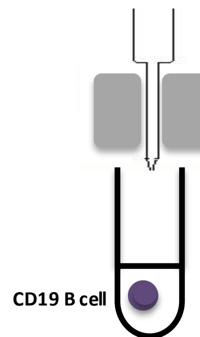
Untouched isolation

Non-target cells are magnetically labeled and eliminated from the cell mixture. The non-magnetically labeled, untouched cell fraction contains the target cells



Magnetic labeling

Non-target cells are magnetically labeled with a biotin-antibody cocktail and Anti-Biotin MicroBeads.



Magnetic separation

Undesired cells are retained in a MACS Column placed in a MACS Separator. The target cells pass though the column and are collected as the enriched, unlabeled cell fraction, depleted of non-target cells.

B cell enrichment using the ‘B cell isolation kit II’ (MACS, Myltenyi Biotech, CN 130-091-151)

Cells were washed and resuspended in 40 µL of buffer per 10^7 total cells. A labelling of the non-B cell fraction was performed by adding 10 µL of Biotin-Antibody Cocktail per 10^7 total cells. After 10 minutes incubation in the refrigerator cells were resuspended in 30 µL of buffer per 10^7 total cells and 20 µL of Anti-Biotin MicroBeads per 10^7 total cells was added. After an additional 15 minutes incubation in the refrigerator, cells were washed and resuspended at a maximum concentration of 10^8 cells per 5ml.

Magnetic separation was then performed using LS Columns on a MACS Separator. After rinsing the column with 3mL of buffer, cells were applied to the LS column and the column was washed 3 times with 3ml of buffer. The unlabelled cells that passed through were collected and resuspended in PBS 200 µL as shown in Figure II.7. Cells were then used for the sorting panel #2 (see II.9.3).

II.9.2 Panels for lymphocyte subset isolation

Cells were stained before transfer to the Flow Cytometry Department of Respiratory Medicine (St. Mary's Medical School, Imperial College London) for cell separation using a BD FACS Aria2 cell sorter.

Panel #1: isolation of transitional T1 B-cells, naïve B-cells, IgM memory B-cells and Switched memory B-cells

This panel was derived from the 4-colour B-cell panel that was used in the previous experiments (see II.6). Antibody titration for this panel was performed in order to optimise cell sorting on the BD FACS Aria2.

The objective was to sort the following B-cell subsets:

- Transitional T1 B-cells: CD27- CD10+ IgM+
- Naïve + transitional T2 and T3 B-cells: CD27- CD10- IgM+
- IgM memory B-cells: CD27+ CD10- IgM+
- Switched memory B-cells: CD27+ CD10- IgM-

The antibodies used were:

- CD27 FITC: DakoCytomation (clone M-T271, Catalog No. F7178)
- CD19-PC7: BC/Immunotech (Catalog No.3628)
- PerCP cy5.5 antihuman IgM (BD Cat # 561285)
- CD10 APC (BD, catalog No. 332777)

A Live/dead marker was included to optimise cell sorting. Propidium iodide (PI) 1.0 mg⁄mL (invitrogen, P3566) was chosen as this dye can be added after staining and right before cell sorting at the St Mary’s facility with no need for further wash out; therefore the cells that would be apoptosing or dying during the transfer from the TIL (tumour immunology lab-Hammersmith Hospital campus) to the St. Mary's

campus would be stained and excluded through gating

This dye was also compatible with the 4 colour panel as shown below:

Live/Dead Discriminators
(unfixed cells)

Dye	Excitation	Emission	Preference
Hoechst dyes Hoechst 33342 Hoechst 33258 DAPI	UV (355 nm) Exλ$_{max}$ 350 nm Exλ$_{max}$ 352 nm UV (355 nm) Exλ$_{max}$ 358 nm	Emλ$_{max}$ 461 nm Emλ$_{max}$ 461 nm Emλ$_{max}$ 461 nm	AT-base pair AT-base pair
PI 7AAD	488 nm Exλ$_{max}$ 535 nm 488 nm	Emλ$_{max}$ 617 nm Emλ$_{max}$ 647 nm	None G-C base pair
To-Pro-3 DRAQ 7	633/635/640 nm Exλ$_{max}$ 642 nm 633/635/640 nm Exλ$_{max}$ 599/644 nm	Emλ$_{max}$ 661 nm Emλ$_{max}$ 678 nm / 694 nm	None (?) AT-base pair

The panel below was used after titration for diagnostic CML samples and adapted to the cell count:

Antibody	**R/ µl**
CD27 FITC	2µl
CD19-PC7	10µl
IgM PerCP cy 5.5	3µl
CD10 APC	10µl

Cells were incubated for 20 to 30 min at RT in the dark, washed and pelleted. PI 1µl was added before cell sorting.

Panel #2: isolation of leukaemia stem cell and progenitors

The objective of this panel was to isolate CD34+CD38-cells (Leukaemia stem cell and multipotent progenitor), CD34+CD38+CD19- (common myeloid progenitor and

granulocyte-macrophage progenitor) and CD34+CD38+CD19+ (common lymphoid progenitors) from CD34 non depleted samples.

The panel below was used after titration for diagnostic CML samples and adapted to the cell count:

Antibody	**R/ µl**
CD34 FITC	5µl
CD19-PE	10µl
CD38 PE cy7	3µl

Cells were incubated for 20 to 30 min at RT in the dark, washed and pelleted.

Panel #3: isolation of T cells, NK cells and CD3+CD56+ cells

The objective was to sort the following lymphocyte subsets:

- T-cells: CD3+ cells
- NK cells: CD3-CD56+
- CD3+CD56+ cells

The panel below was used:

Antibody	**R/ µl**
CD56 FITC	10µl
CD3-PE	5µl

Cells were incubated for 20 to 30 min at RT in the dark, washed and pelleted.

II.9.3 Lymphocytes isolation using BD FACS ARIA2

Apheresis-derived MNCs materials were thawed as described in section II.2.6 (‘Apheresis-derived MNCs: thawing’). In order to determine B cells subsets, an 8 colour mAb panel was employed as detailed in section II.9.5 ‘B-cell extended panel’. The remainder of the sample underwent CD34+ positive selection (this cell fraction was used for a different project) and the negative fraction was used for positive B cell enrichment using the ‘CD 19 Microbeads’ positive selection (MACS, Myltenyi Biotech, CN 130-050-301), as detailed in section II.9.2 ‘Sample thawing and B-cell enrichment’. Stained cells were incubated for 20 to 30 min at RT and processed on a BD FACS Aria2 cell sorter. The sorted populations were collected in MACS buffer and stored on ice.

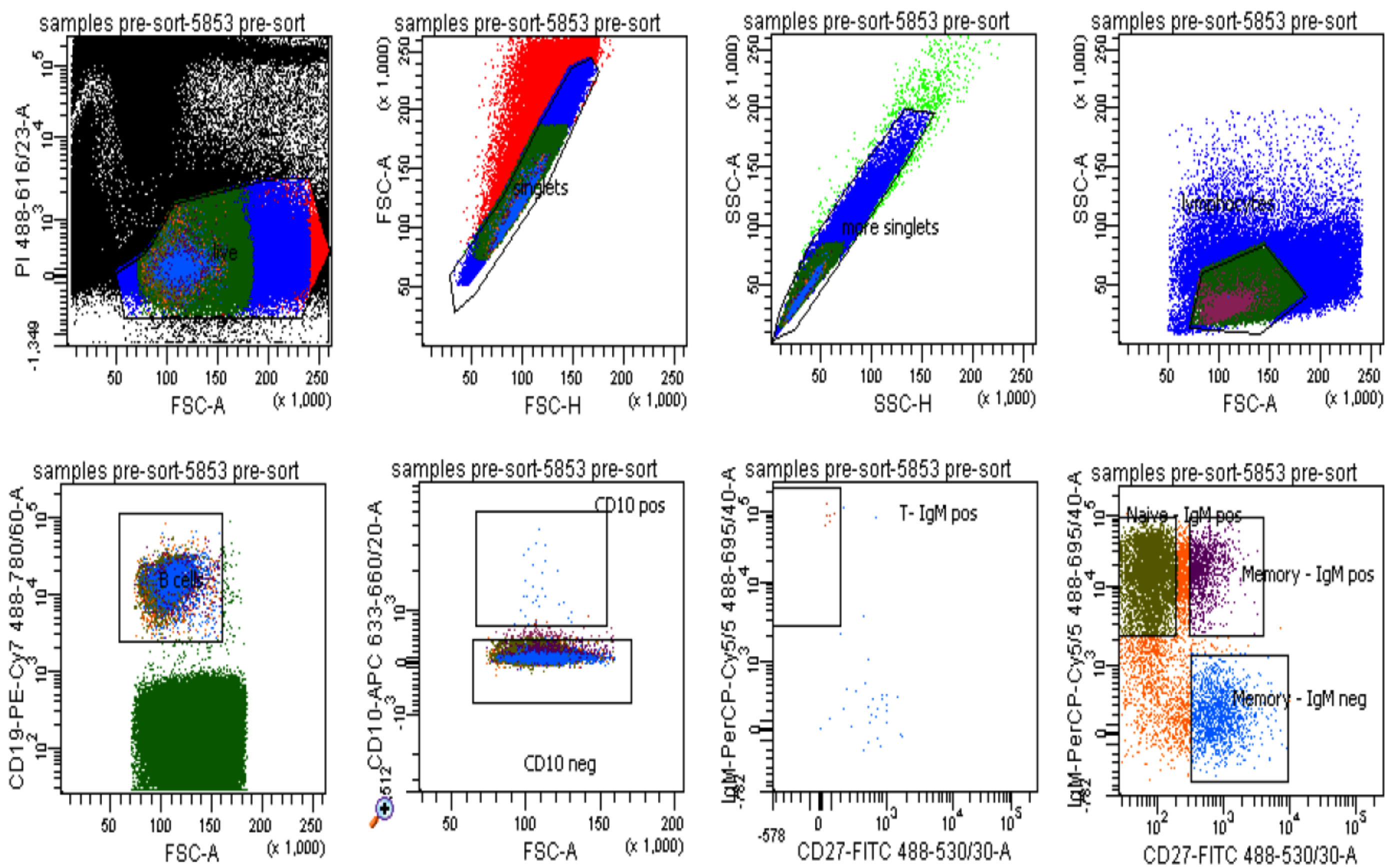
The handling and management of the FACS Aria2 cell sorter was performed by Mr Robert Sampson, senior technician at the Flow Cytometry Department of Respiratory Medicine.

Gating strategy for B-cell subsets (panel 1):

The purpose of this panel was to select transitional T1 B-cells (CD27- CD10+ IgM+), naïve B-cells + transitional T2 and T3 B-cells (CD27- CD10- IgM+), IgM memory B-cells (CD27+ CD10- IgM+) and switched memory B-cells (CD27+ CD10- IgM-).

The B cells subsets were obtained by applying a gating strategy as shown below:

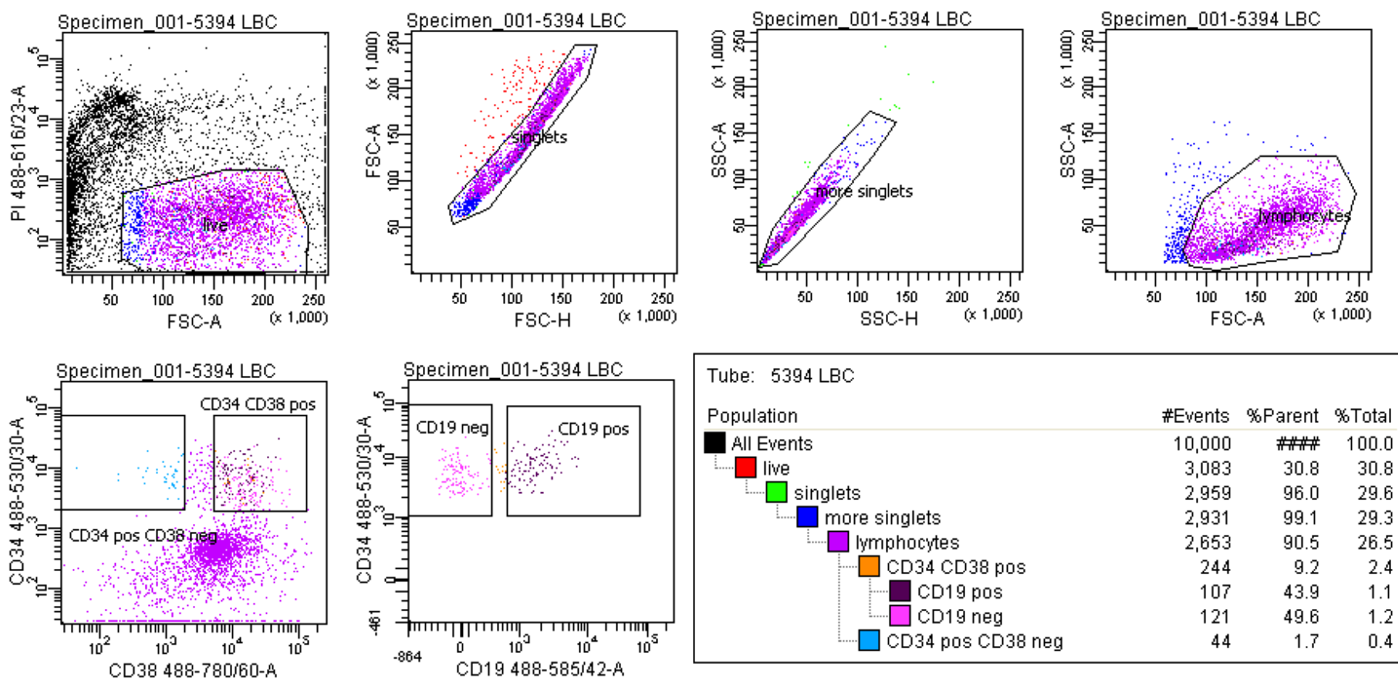
FACSDiva Version 6.1.2



Gating strategy for leukaemia stem cell and progenitors (panel 2):

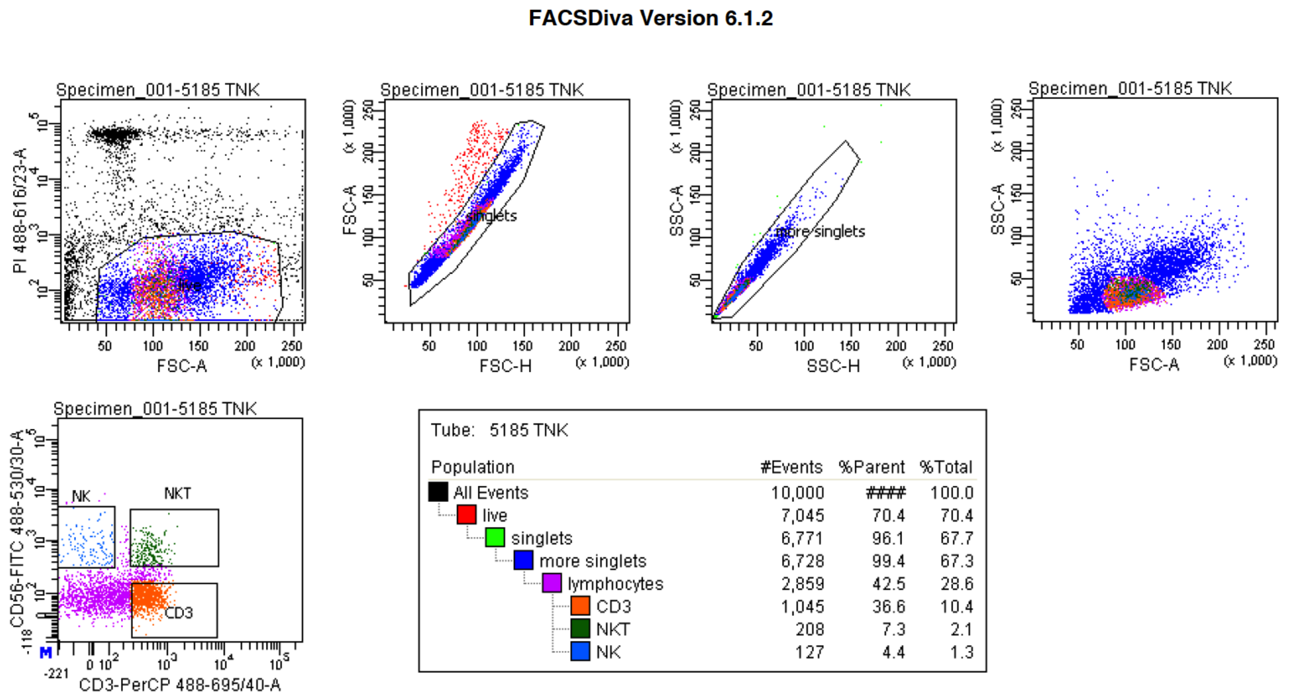
This second panel was standardised for isolation of CD34+CD38-cells (Leukaemia stem cell and progenitor), CD34+CD38+CD19- (common myeloid progenitor and granulocyte-macrophage progenitor) and CD34+CD38+CD19+ (common lymphoid progenitors) from CD34 non depleted samples.

Only strongly positive cells for marker of interest were selected for cell sorting. Using this strategy the specificity of target cell sorted was very high although sensitivity for cells of interest was reduced.



Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
live	3,083	30.8	30.8
singlets	2,959	96.0	29.6
more singlets	2,931	99.1	29.3
lymphocytes	2,653	90.5	26.5
CD34 CD38 pos	244	9.2	2.4
CD19 pos	107	43.9	1.1
CD19 neg	121	49.6	1.2
CD34 pos CD38 neg	44	1.7	0.4

Gating strategy for lymphocytes subsets (panel 3):



Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
live	7,045	70.4	70.4
singlets	6,771	96.1	67.7
more singlets	6,728	99.4	67.3
lymphocytes	2,859	42.5	28.6
CD3	1,045	36.6	10.4
NKT	208	7.3	2.1
NK	127	4.4	1.3

A purity check was performed after cell sorting for each of the cell subset and on each occasion of cell sorting.

The number of cell subsets obtained after each sorting was recorded. An example is listed below:

Sample #5185 (CML leukapheresis sample at diagnosis):

- Transitional: 73494
- Naive: 446546
- Switched: 23540
- IgM Memory: 40596
- NKT: 18528
- T cells: 101851
- NK cells: 10158

II.9.4 BCR-ABL detection using FISH and DNA/RNA extraction.

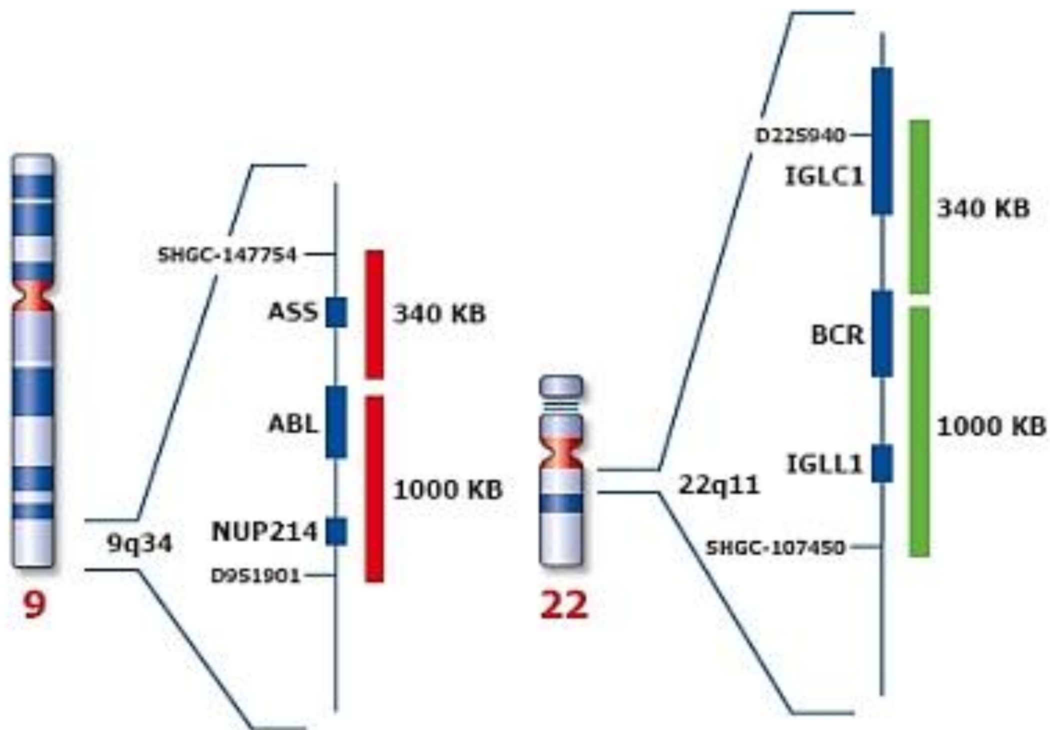
Stained cells were transferred in MACS buffer on ice shielded from light to the cytogenetic laboratory at the Hammersmith Hospital for Fluorescence In Situ Hybridization (FISH).

Depending on the cell number for each subset, cells were also stored in RLT plus buffer for future DNA/RNA extraction.

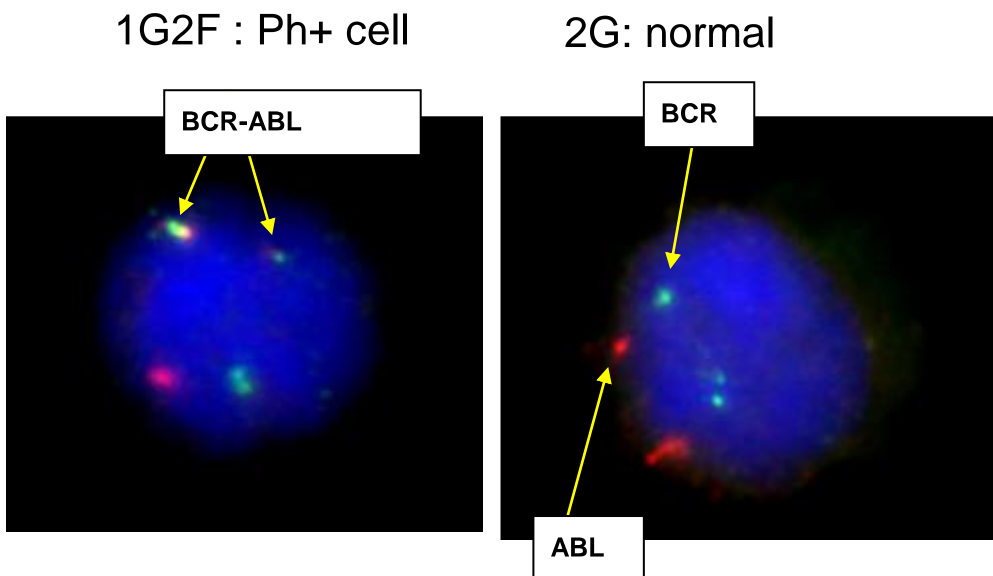
BCR-ABL1 detection using fluorescence In Situ Hybridization (FISH)

Samples were fixed using a Methanol-Acetone solution, transferred into 1.5ml eppendorf tubes and stored at -20* for future FISH and analysis.

FISH for *BCR-ABL1* detection and its analysis was performed by Mrs Philippa May (Department of cytogenetics, Hammersmith Hospital) *BCR/ABL1* t(9;22) Dual-Color, Dual-Fusion Probe (Kreatech Diagnostics, KBI-10005) were used, were *BCR* is labelled in green and *ABL1* is labelled in red (as shown below).



The ‘standard’ abnormal signal pattern is 1R1G2F, but other signal patterns are possible if the patient has deletions of 3’ BCR or 5’ ABL1. A classical example of a *BCR/ABL1* positive signal is shown below (left hand side panel) together with a negative example (right hand side).



DNA/RNA lysis for future extraction

Part of the cells of each subset were lysed in 350 µl RLT plus buffer (Quiagen) and stored for future DNA/RNA extraction using AllPrep kit (Quiagen).

II.9.5 B-cell extended panel (8 colours).

The following staining panel was used for extended B cell phenotyping:

Reagents:

- invitrogenTM LIVE/DEAD Fixable Aqua Dead cell stain Kit (Cat # L34957)
- BD PharmingenTM FITC mouse anti human CD24 (Cat # 555427)
- BD PharmingenTM PE Mouse Anti-Human CD27 (Cat # 555441)
- eBioscience PE Cy7 mouse anti human CD38 (Cat # 25-0389-42)
- BD PharmingenTM PerCP cy5.5 antihuman IgM (Cat # 561285
- eBioscience APC mouse anti human CD21 (Cat # 17-0219 -42)
- BD PharmingenTM APC H7 mouse anti human CD19 (Cat # 560177)
- Invitrogen Qdot® 605 mouse anti-human CD10 (Cat # Q10153)
- BD Horizon™ V450 Mouse Anti-Human IgD (Cat # 561309)

Briefly, approximately 1×10^6 PBMCs were washed and stained with the viability marker; invitrogenTM LIVE/DEAD Fixable Aqua Dead cell stain Kit cat# L34957 according the manufacturer’s protocol. Cells were then washed and incubated with the panel shown below in PBS in the dark for 30 min at room temperature. Cells were then washed once before analysis on BD FACSLSR2.

Antibody	**Full panel R/ µl**
CD24 FITC	5
CD27 PE	5
CD38 PE cy7	3
IgM PerCP cy 5.5	3
CD21 APC	3
CD19 APC H7	5
CD10 QDOT	2
IgD PB	5
Live/ dead	Boil cells for 60 sec mix with live cells

Transitional B cells were defined as CD24high CD38high and naïve B cells as CD24int CD38int cells.

II.10 DETECTON OF BTK AND PLCgamma2 PHOSPHORYLATION

The purpose of this experiment was to detect the phosphorylated conformation of the kinase Btk and the phosphorylation of its downstream kinase PLCγ2 using the BD Phosflow antibody for flow cytometry. Ramos cell line was used as a positive control for this experiment.

II.10.1 Ramos cell line subculture

The Burkitt's lymphoma-derived cell line Ramos (RA #1, ATCC, CN CRL-1596)

was thawed and cultured in 10%FCS RPMI complete media. It was used as a positive control to set up this experiment.

II.10.2 PhosphoBtk and PhosphoPLCγ2 using BD Phosflow antibodies: detection in Ramos cell line

10^7 ramos cells were stimulated with using 10 ml 50 mM H2O2 PBS and incubated for 15 minutes. Cells were then fixed with 5 ml of pre-warmed cytofix buffer (cat 554655) and incubate for 10 min at 37°. After a wash cells were pelleted and permeabilized using 1 ml of BD Phosflow Perm buffer II (cat 558050) and incubate on ice for 30 min. After two washes 5 µl PE mouse anti-Btk(pY551) or 5 µl PE mouse anti-PLC- γ2 (pY759) (both from BD Biosciences, San Jose, CA) were added and cells were incubated at room temperature for 30 min in the dark. Cells were washed with BD Stain buffer and processed on a FACS Calibur.

II.10.3 PhosphoBtk and PhosphoPLCγ2 staining with or without co-incubation with tyrosine kinase in human B cells

This work was performed by Miss Anushruti Sarvaria.

To assess the impact of TKI on normal B cells, PBMC from healthy controls were isolated and cultured in the presence or absence of increasing concentrations of TKIs namely, 1-50 µM of imatinib (LC Laboratories, Woburn, MA), 1-25 µM of nilotinib (LC Laboratories, Woburn, MA) and 1-100 nM of dasatinib (LC Laboratories, Woburn, MA) for 72 hours.

Following incubation, PBMC were stimulated with 5mL of 50 mM of H2O2 for 20 minutes at 37˚C. The stimulation was terminated by the addition of 5 mL pre-

warmed Cytofix Buffer (BD Biosciences, San Jose, CA) at 37°C for 12 minutes. Cells were then fixed and permabilized in Perm buffer II (BD Biosciences, San Jose, CA) and resuspended in 200 µL of staining buffer (BD Pharmingen, Oxford UK). Cells were stained with PE-conjugated anti-phosphorylated Btk (pBtk-PE) or PE-conjugated anti-phosphorylated PLC-γ2 (pPLC-γ2-PE), PerCP-conjugated anti-human IgM (BD Biosciences, Oxford UK), APC-conjugated anti-CD19 (BD Biosciences, San Jose, CA), and FITC-conjugated anti-CD27 (DakoCytomation, Glostrup Denmark). Data acquisition was performed on the FACSCaliburTM (BD Biosciences, Oxford UK). TKIs inhibited phosphorylation of Btk and PLC-γ2 in B cells in a dose and time-dependent manner.

To assess the impact of physiological doses of imatinib, dasatinb and nilotinib on Btk phosphorylation in B cells, cyopreserved PBMCs from CML patients on TKI (imatinib, n=3, nilotinib n=4 and dasatinib n=3) were thawed, washed and co-cultured with autologous plasma or RPMI/10%FCS overnight. PBMCs were then stimulated with 5mL of 50 mM of H2O2 for 15 minutes at 37˚C. The stimulation was terminated by the addition of 5 mL pre-warmed Cytofix Buffer (BD Biosciences, San Jose, CA) at 37°C for 12 minutes. Cells were fixed and permabilized in Perm buffer II (BD Biosciences, San Jose, CA) and resuspended in 200 µL of staining buffer (BD Pharmingen, Oxford UK). Cells were stained with PE-conjugated anti-phosphorylated Btk (pBtk-PE) (BD Biosciences, San Jose, CA) and APC-conjugated anti-CD19 (BD Biosciences, San Jose, CA). Data acquisition was performed on the FACSCaliburTM (BD Biosciences, Oxford UK) and FlowJo software (TreeStar, San Carlos, CA) was used for analysis.

II.11 STATISTICAL ANALYSIS

II.11.1 Study 'Immune response following vaccination against influenza A (H1N1) 2009 virus'

Groups were compared using Fisher's exact test for categorical data and the Mann-Whitney test for continuous variables. To evaluate the effect of a second vaccine dose, paired sample analysis was performed using a Mc-Nemar test. GMT values, with 95% confidence intervals (CIs), were calculated by use of the mean, and lower and upper limits of the 95% CIs of log-transformed titres. The influence of variables on the rates of seroprotection or seroconversion was studied using a logistic regression model. All reported p values are two-sided and without adjustment for multiple testing. Analyses were done for the full-analysis set using the software package SPSS (version 17).

II.11.2 Study 'T cell and B cell responses after vaccination against seasonal influenza and pneumococcus'

Fisher's exact test was used to compare proportions. Continuous variables were compared using the Mann-Whitney test or the Kruskal Wallis test. Paired samples were compared using the Wilcoxon signed rank. Multivariate analysis was performed using a logistic regression model. All reported p values are two-sided. Samples were correlated using Spearman rank correlation test. Analyses were performed using the software package SPSS version 17

CHAPTER 3 COMPARISON OF HUMORAL AND CELLULAR IMMUNE RESPONSES TO 2009 H1N1 VACCINE IN CML PATIENTS, PATIENTS WITH OTHER HAEMATOLOGICAL MALIGNANCIES AND HEALTHY CONTROLS

III-1 INTRODUCTION

In 2009 the spread of influenza A (H1N1) satisfied the World Health Organization (WHO) criteria of a global-pandemic and led to the initiation of a vaccination campaign to ensure protection for the most vulnerable patients, including those with haematological malignancies. However, the immunogenicity of the 2009 H1N1 vaccine in immunocompromised patients has not been specifically tested. Furthermore, the number of doses of vaccine required for effective immunization against novel influenza A (H1N1) has not been established. Whereas the European Medicines Agency (EMEA) (The European Agency for the Evaluation ofMedicinal Products (EMEA),) and the UK Department of Health (DoH) (Department of Health, 2009) recommend the injection of two doses of inactivated H1N1 vaccine with a minimum of three weeks between doses for immunocompromised individuals, the Centers for Disease Control and Prevention (CDC) recommend immunization with one dose of inactivated H1N1 vaccine for patients with cancer receiving chemotherapy, followed by a booster vaccine 3 months after completion of treatment if the pandemic continues (Pollyea *et al*, 2010).

The vaccination against a novel H1N1 influenza virus strain has created a unique opportunity to study the immune response in CML patients compared to other subgroups of patients with haematological malignancies. In order to gain further

understanding on the capability of such patients to develop an immune response to novel leukaemia associated antigen vaccine, I decided to compare the immune response to the novel pandemic 2009 H1N1 influenza vaccine between CML patients and adult controls as well as patients with other haematological malignancies; the assumption being that most patients would not have been previously exposed to the pandemic H1N1 virus. To that end I conducted a prospective study to determine the safety and immunogenicity of the vaccination program against the 2009 pandemic H1N1 in chronic phase CML patients on tyrosine kinase inhibitor (TKI) as well as in patients with other haematological malignancies and healthy controls; I also characterized the different components of the immune response to H1N1.

III-2 AIM

The aims of this study were

- to determine the humoral and cellular immune responses to 2009 H1N1 vaccine in CML patients on TKI, and the role of a booster injection
- to compare the humoral and cellular immune responses to the vaccine in CML patients and healthy controls
- to evaluate the humoral and cellular immune responses to the vaccine in CML patients in CCyR on TKI compared to other groups of patients with haematological malignancies such as non-Hodgkin lymphoma (NHL), chronic lymphocytic leukaemia (CLL) or allogeneic stem cell transplant (allo-SCT) recipients
- to provide a more complete picture of the host response to 2009 H1N1 vaccination, and facilitate the development of improved vaccination strategies for immunosuppressed individuals

III-3 RESULTS

III.3.1 Patients characteristics

Clinical characteristics of patients and healthy controls are summarized in Table III.1. Of the 97 patients, 89 received the recommended booster at a median of 27 days (range, 18-57) after the first vaccine dose. Eight patients failed to receive a booster dose, either due to patient refusal (n=3) or limited access to their primary health care physician (n=5). Twenty five healthy controls received one dose of the vaccine only as per UK DoH guidelines.

Table III.1: Patient and healthy control characteristics.

Abbreviations: M: Male; F: Female; Allo-SCT: allogeneic stem cell transplantation; CML: chronic myeloid leukaemia; CML CP: CML in chronic phase; RIC: reduced intensity conditioning; MAC: myeloablative conditioning; SIB: identical sibling donor; MUD: matched unrelated donor; GVHD: graft-versus-host disease; CLL: chronic lymphocytic leukaemia; SLL: small lymphocytic lymphoma; DLBCL: diffuse large B-cell lymphoma; CCyR: complete cytogenetic response; Y: yes; N: no.

Characteristics	**Patients N=97**	**Healthy controls N=25**	**P value**
Age, median (range)	57.0 (22.8-88.1)	37.9 (25.6-61.8)	0.001
Allo-SCT	38.6 (22.8-63.4)		
B-cell malignancies	66.0 (29.9-82.1)		
CML	53.9 (25.0-88.1)		
Gender (M/F)	58/39	10/15	
Disease			
Allo-SCT	26		
Underlying disease:			
CML CP	11		
Other myeloid malignancies	5		
Lymphoid malignancies	5		
Other	5		
Month from allo-SCT, median (range)	39 (6-127)		
RIC/MAC	11/15		
SIB/MUD	11/15		
Acute GVHD (Y/N)	10/16		
Chronic GVHD (Y/N)	7/19		
B-cell malignancies	39		
CLL/SLL	19		
Follicular lymphoma	7		
DLBCL	4		
Hodgkin’s lymphoma	2		
Others	7		
Chronic phase CML in CCyR on	32		
Imatinib	23		
Dasatinib	9		
Seasonal influenza vaccination in 2008 (Y/N)	57/35	10/15	

III.3.2 Toxicity profile following vaccination with 2009 H1N1 and seasonal influenza vaccines

In general the vaccines were well tolerated. Table III.2 shows the adverse events during the first 7 days after the first dose. Overall 86/95 evaluable patients (90.5%) reported adverse reactions after the first vaccine dose, including local reactions in 84/95 (88.4%) and systemic adverse events in 41/95 (43.2%), of which 2.1% and 3.2% respectively were reported as severe adverse events. We solicited side effects after the second vaccine dose in 72 patients: 9/72 (12.5%) reported worsening side effects, including exacerbation of local reactions (pain or tenderness) in 6 and systemic adverse events (fever, nausea or malaise) in 3 patients. No patient required hospital admission as a consequence of vaccine-related adverse events.

In comparison 22/25 healthy controls (88%) reported adverse events, of whom 22/25 had local reactions (88%) and 10/25 (40%) had systemic adverse events. There were no obvious difference in the side effect profiles or frequencies of adverse events between patients and controls (data not shown).

Table III.2: Injection-site and systemic adverse effects within 7 days after the first dose of vaccine among patients.

Adverse event	Mild	Moderate	Severe	All grades
		percent (95% confidence interval)		
local event				
Any	64.2 (54.6-73.9)	22.1 (13.8-30.4)	2.1 (0-5.0)	88.4 (82.0-94.9)
Pain	54.7 (44.7-64.7)	18.9 (11.1-26.8)	1.0 (0-3.1)	74.7 (66.0-83.5)
Tenderness	57.9 (48.0-67.8)	20.0 (12.0-28.0)	2.1 (0-5.0)	80.0 (72.0-88.0)
Redness	13.7 (6.8-20.6)	4.2 (0.2-8.2)	0	17.9 (10.2-25.6)
Induration	15.8 (8.5-23.1)	3.2 (0.4-6.7)	0	18.9 (11.1-26.8)
Ecchymosis	4.2 (0.2-8.2)	0	0	4.2 (0.2-8.2)
systemic event				
Any	28.4 (19.4-37.5)	11.6 (5.1-18.0)	3.2 (0-6.7)	43.2 (33.2-53.1)
Fever	5.3 (0.8-9.8)	3.2 (0-6.7)	1.1 (0-3.1)	9.5 (3.6-15.4)
Headache	14.7 (7.6-21.9)	3.2 (0-6.7)	0	17.9 (10.2-25.6)
Malaise	17.9 (10.2-25.6)	10.5 (4.4-16.7)	1.1 (0-3.1)	29.5 (20.3-38.6)
Myalgia	11.6 (5.1-18.0)	6.3 (1.4-11.2)	0	17.9 (10.2-25.6)
Chills	6.3 (1.4-11.2)	3.2 (0-6.7)	0	9.5 (3.6-15.4)
Nausea	7.4 (2.1-12.6)	1.1 (0-3.1)	1.1 (0-3.1)	9.5 (3.6-15.4)

III.3.3 Clinical efficacy of vaccination

Five patients reported influenza-like illness by the end of the influenza season on 31st March 2010, of whom one required admission to hospital. None of these 5 patients had a RT-PCR-confirmed H1N1 influenza illness or received antiviral therapy, and all had achieved seroconversion after vaccination. Similarly, H1N1 infection was not diagnosed in any of the vaccinated controls during follow-up.

III.3.4 Seroprotection rates to 2009 H1N1 in controls and patients

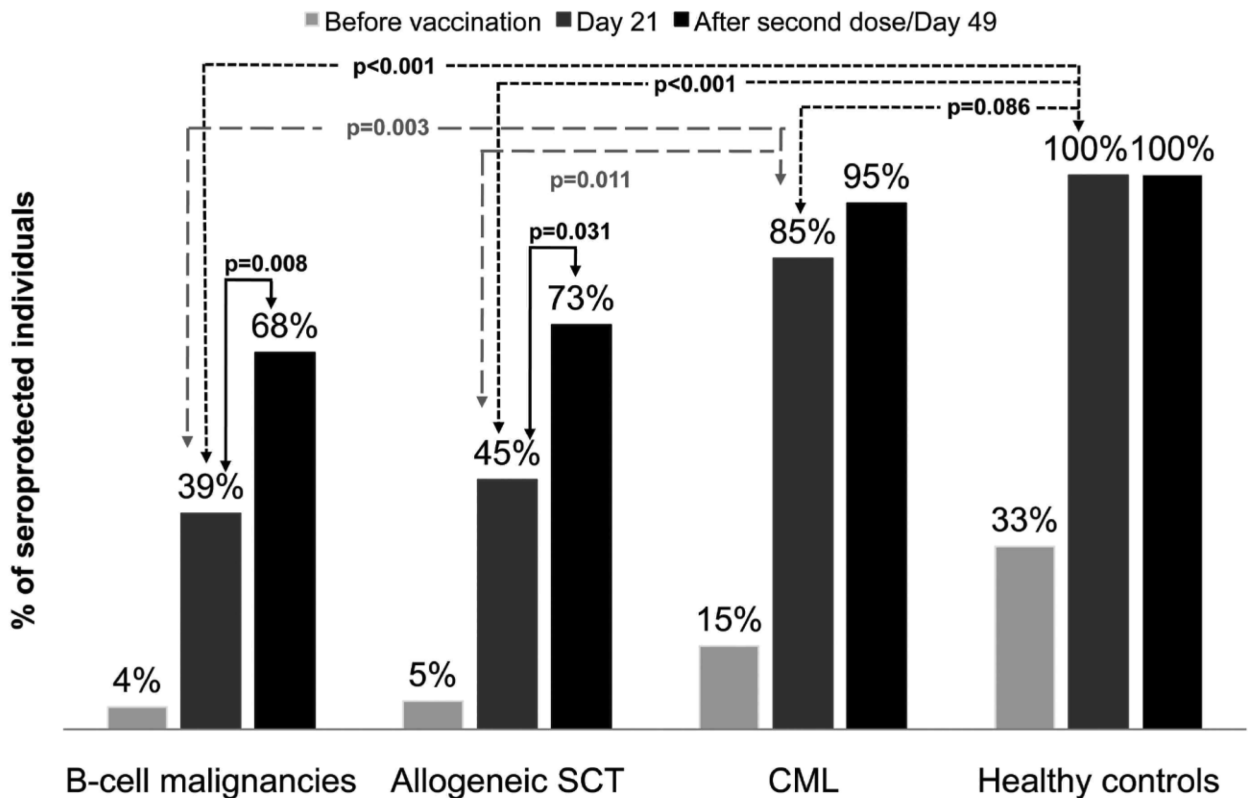
We evaluated the humoral response in 70 patients and 24 healthy controls in whom antibody titres were available at all study time points (before vaccination and at days 21 and 49); patients who failed to receive a second dose were also excluded from this analysis. Before vaccination protective antibody titres of 1:32 or more were seen in 8/24 (33.3%) controls compared to 1/28 (3.6%) patients with B-cell malignancies (p=0.008), 1/22 (4.5%) allo-SCT recipients (p = 0.023) and 3/20 (15.0%) CML patients (p=0.29) as shown in Table III.3 and Figure III.1.

On day 21 after vaccination, protective antibody titres of 1:32 or more were seen in 24/24 (100%) controls compared to only 11/28 (39.3%) patients with B-cell malignancies (p<0.001), 10/22 (45.5%) of allo-SCT recipients (p<0.001) and 17/20 (85.0%) CML patients (p=0.086), Table III.3 and Figure III.1. The GMT was also significantly higher in healthy controls compared to all patients groups, namely 362 vs. 18 (p=0.001) in patients with B-cell malignancies, 362 vs. 42 (p=0.001) in allo-SCT recipients and 362 vs. 100 (p=0.004) in CML patients (Table III.3). The seroprotection rates achieved in CML patients were significantly higher than those in patients with B-cell malignancies (p=0.003) and recipients of allo-SCT (p=0.011). Similar results were obtained when looking at the rate of seroconversion after the first injection (Table III.3).

Table III.3: Antibody response to the first (day 21) and second dose (day 49, patients only) of vaccine as measured with the haemagglutination-inhibition assay, according to patient group. Geometric mean ratios are calculated by comparing the ratios between the geometric mean titre (GMT) after vaccination to the GMT before vaccination. Seroprotection was defined as an antibody titre of 1:32 or more. Seroconversion was defined as a pre-vaccination antibody titre of 1:8 or less and a post-vaccination titre of 1:32 or more, or a pre-vaccination titre greater than 1:8 and an increase in the antibody titre by a factor of four or more. P values correspond to the comparison between controls and each patient subgroup.

Value	B-cell malignancies (n=28)	Allo-SCT (n=22)	CML (n=20) Imatinib, n=15 Dasatinib n=5	Controls (n=24)
Pre- H1N1 vaccination				
Geometric mean titre (95% CI)	5.5 (3.8-7.8) p=0.010	5.1 (3.8-6.9) p=0.016	6.6 (4.3-10.3) p=0.11	10.7 (6.5-17.4)
Seroprotection -% (95% CI)	3.6 (0-10.4) p=0.008	4.5 (0-13.2) p=0.023	15.0 (0-30.6) p=0.29	33.3 (14.5-52.2)
Day 21				
Geometric mean titre (95% CI)	17.7 (8.7-35.7) p<0.001	41.8 (14.5-120.3) p=0.001	100.4 (54.2-186.0) p=0.004	362.0 (216.4-605.5)
Geometric mean ratio (95% CI)	3.2 (1.7-6.0)	8.1 (3.0-22.2)	15.2 (8.2-28.0)	33.9 (21.4-53.6)
Seroconversion -% (95% CI)	35.7 (18.0-53.5) p<0.001	45.5 (24.6-66.3) p<0.001	80.0 (62.5-97.5) p=0.036	100
Seroprotection -% (95% CI)	39.3 (21.2-57.4) p<0.001	45.5 (24.6-66.3) p<0.001	85.0 (69.4-100) p=0.086	100
Day 49				
Geometric mean titre (95% CI)	57.2 (24.2-135.2) p=0.012	130.0 (43.8-386.4) p=0.67	130.2 (67.6-251.0) p=0.17	248.7 (144.1-429.2)
Geometric mean ratio (95% CI)	10.5 (4.6-24.2)	25.3 (8.3-76.8)	19.7 (9.9-39.0)	23.3 (14.3-37.7)
Seroconversion -% (95% CI)	64.3 (46.5-82.0) p=0.001	72.7 (54.1-91.3) p=0.008	90.0 (76.9-100) p=0.20	100
Seroprotection -% (95% CI)	67.9 (50.6-85.2) p=0.002	72.7 (54.1-91.3) p=0.008	95.0 (85.4-100) p=0.46	100

Figure III.1: Frequency of seroprotected individuals after one dose (patients and controls) and two doses (patients only) of vaccine.



III.3.5 Humoral response to the second dose of vaccine

When I analyzed the antibody response to H1N1 at day 49 post-vaccination the seroprotection rates were significantly lower in patients with B-cell malignancies (p=0.002) and in allo-SCT recipients (p=0.008) than in healthy controls (Table III.3). The seroprotection rates achieved in CML patients at day 49 were significantly higher than those achieved in patients with B-cell malignancies (19/20 vs. 19/28 respectively; p=0.031) but not significantly different to recipients of allo-SCT controls (19/20 vs. 16/22; p=0.096) or healthy controls (p=0.46).

In order to assess the effect of the second booster dose, I performed a paired sample analysis using a Mc-Nemar test. The second vaccine dose induced a

significant increase in the seroprotection rates from 39% to 68% (11/28 vs. 19/28; p=0.008) in patients with B-cell malignancies and from 45% to 73% (10/22 vs. 16/22; p=0.031) in allo-SCT recipients. However, after the second booster dose, the seroprotection rate for CML patients did not change significantly (17/20 after the 1st dose and 19/20 after 2nd dose; p=0.5). The seroconversion rates followed the same pattern (data not shown).

III.3.6 Impact of age on the level of seroprotection and seroconversion

The median age of controls was 37.9 years (range, 25.6-61.8) compared to 66.0 years (range, 29.9-82.1) in patients with B-cell malignancies, 38.6 years (range, 22.8-63.4) in allo-SCT recipients and 53.9 years (range, 25.0-88.1) in CML patients. We studied the relationship between age and the rate of seroconversion or seroprotection by constructing a logistic regression model for each outcome in which we entered the baseline disease (B-cell malignancies, CML, allograft or control) and the age of the patient or control. Age, either as continous variable or as a categorical variable (quartiles) did not influence the seroconversion or seroprotection rates as measured on day 21 or day 49 (data not shown).

III.3.7 Effect of chemotherapy and rituximab on the humoral response to vaccination

Among the 28 evaluable patients with B-cell malignancies, 9 patients had not received chemotherapy. Of the 19 treated patients 12 patients had received rituximab-based treatment or were on maintenance rituximab (Table III.4). The period of time between chemotherapy and vaccination was significantly longer in patients who were seroprotected at day 49 compared to those who were not (4.7

vs. 17.5 months, p=0.001). Of the 19 patients who had received prior chemotherapy, 8/8 (100%) patients vaccinated more than 12 months after chemotherapy achieved seroprotection after two doses of the vaccine, compared to 3/6 (50%) vaccinated between 6-12 months and 0/5 (0%) vaccinated within 6 months of chemotherapy (p=0.001, Chi-squared trend test). There were no significant differences in the seroprotection rates of the 8 patients who were vaccinated more than 12 months following chemotherapy compared to the 9 patients who had not been previously treated (100% versus 89%, p = 0.99), Table 4. Importantly, when restricting the analysis to the 17 patients with B-cell malignancies who were vaccinated more than 12 months after receiving chemotherapy (n=8) or those who had never been treated with chemotherapy (n=9), 16/17 (94%) achieved a level compatible with seroprotection after a second dose, which was not statistically significantly different from the healthy controls (p=0.415). However, it appears that 2 doses of vaccine are still necessary to induce a significant antibody titre in these patients as only 10 of the 17 seroconverted after the 1st dose (p = 0.031).

Table III.4: Comparison of antibody response to 2009 H1N1 vaccination in patients with B-cell malignancies according to time from chemotherapy

Only patients in whom antibody titres were available at all time points and who received two vaccine doses were included in this table.
Abbreviation: HI: haemagglutination-inhibition; DLBCL: diffuse large B-cell lymphoma; SLL: small lymphocytic lymphoma; FL: follicular lymphoma; CLL: chronic lymphocytic leukaemia; HL: Hodgkin's lymphoma; MCL: mantle cell lymphoma; NHL: non-Hodgkin's lymphoma; LPL: lymphoplasmacytoid lymphoma; R: rituximab; Chl: chloraminophen; Fluda: fludarabine; Cycl: cyclophosphamide; CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; R-CHOP: rituximab-CHOP; R-CVP: rituximab, cyclophosphamide, vincristine, prednisone; ABVD: doxorubicin, bleomycin, vinblastine, dacarbazine; DHAP: dexamethasone, cytarabine, cisplatin; Codox M: cyclophosphamide, vincristine, doxorubicin, methotrexate; IVAC: ifosfamide, etoposide, cytarabine; NA: not available.

Patient #	Disease type	Chemotherapy received	Maintenance Rituximab	Time from chemotherapy or maintenance rituximab(months)	HI assay		
					Day 0	Day 21	Day 49
1	DLBCL	3*R-CVP, 4*R-CHOP	N	1.7	<1:8	<1:8	<1:8
2	SLL	6*R-Chl	Y	1.8	<1:8	<1:8	<1:8
3	FL	6*R-CVP	Y	2.4	<1:8	<1:8	<1:8
4	FL	3*R-CVP, 3 * R-CHOP	Y	3.6	<1:8	<1:8	<1:8
5	CLL	3*Fluda-Cycl, 6*R-Cycl	N	5.9	<1:8	<1:8	<1:8
6	HL	6*AVBD	N	6.2	<1:8	<1:8	1:32
7	FL	6*R-CHOP	Y	6.4	<1:8	<1:8	<1:8
8	CLL	5*Fluda-Cycl	N	6.6	<1:8	<1:8	1:8
9	MCL	5*R-CHOP, 1*R-CVP	N	6.8	<1:8	1:23	1:64
10	FL	6*Chl, 2 * DHAP, 2*R-CVP	Y	6.9	<1:8	<1:8	<1:8
11	DLBCL	6* R-CVP	N	10.5	16	1:256	1:256
12	CLL	1*Fluda-Cycl	N	16.7	<1:8	<1:8	1:32

Patient #	Disease type	Chemotherapy received	Maintenance Rituximab	Time from chemotherapy or maintenance rituximab(months)	HI assay	Patient #	Disease type
13	CLL	5*Chl, 4*Fluda, 6*Fluda-Cycl, Campath	N	16.9	<1:8	<1:8	1:362
14	CLL	Campath	N	17.6	<1:8	1:256	1:4096
15	DLBCL	6 * R-CHOP	N	31.9	<1:8	1:32	1:512
16	Hairy cell leukaemia	Cladribine	N	58.7	<1:8	1:64	1:5792
17	CLL	6*Fluda-Cycl, Campath	N	58.9	1:16	1:512	1:512
18	FL	6*R-CVP	N	66.7	<1:8	1:64	1:32
19	Splenic NHL	8*R-CHOP	N	82.9	<1:8	1:16	1:256
20	LPL	None			<1:8	1:512	1:512
21	CLL	None (Binet stage A)			<1:8	<1:8	1:64
22	CLL	None (Binet stage A)			1:8	1:64	1:128
23	CLL	None (Binet stage A)			1:362	1:128	1:362
24	CLL	None (Binet stage A)			1:8	1:362	1:256
25	CLL	None (Binet stage A)			<1:8	<1:8	1:256
26	CLL	None (Binet stage A)			<1:8	1:32	1:32
27	CLL	None (Binet stage A)			<1:8	<1:8	1:33
28	CLL	None (Binet stage B)			<1:8	<1:8	<1:8

III.3.8 Impact of time from transplant on humoral response to vaccination

In the allo-SCT recipient group, we studied the impact of a number of factors including conditioning regimen (myeloablative versus reduced-intensity), donor type (sibling or matched unrelated donor- MUD), time from transplant, previous history of acute GVHD or chronic GVHD, and underlying disease on seroconversion and seroprotection rates following H1N1 vaccination (Table III.1). The time from transplantation was the only significant predictive variable: patients who achieved seroprotection had a significantly longer transplant-to-vaccination interval compared to patients who failed to achieve seroprotection (6.5 vs. 48 months; p=0.015). Of note only 2 patients were on low dose immunosuppressive therapy with cyclosporin A, neither of whom developed a seroprotective humoral response to vaccination.

III.3.9 H1N1-specific T-cell response to vaccination

The induction of virus-specific T-cell responses by H1N1 vaccination was assessed directly ex-vivo by flow cytometric enumeration of antigen-specific CD8+ and CD4+ T lymphocytes using an intracellular cytokine assay for IFN-γ and TNF-α (Th1 effector cytokines) production. PBMC were available for analysis at baseline and at day 49 in 23 controls and 81 patients. Prior to H1N1 vaccination, pre-existing T-cell responses against 2009 H1N1 influenza could be detected in 10/23 (43%) controls compared to 2/25 (8%) allo-SCT recipients (p=0.007), 2/28 (7%) patients with B-cell malignancies (p=0.003) and 6/28 (21%) of CML patients (p=0.131).

Following vaccination, H1N1-specific T-cells were induced in a significant proportion of allo-SCT recipient, (2/25 pre-vaccine vs. 10/25 post-vaccine; p=0.008, Mc-Nemar test) and patients with B-cell malignancies (2/28 pre-vaccine vs. 10/28

post-vaccine; p=0.008). There appeared to be no effect of prior chemotherapy or time from transplant on the induction of H1N1-specific T-cells after two doses of vaccine. In contrast, there was no significant increase in the proportion of individuals with H1N1 specific T-cell response following H1N1 vaccination in CML patients (6/28 pre-vaccine vs.9/28 post-vaccine; p=0.51) and healthy controls (10/23 pre-vaccine vs. 11/23 post-vaccine; p = 0.51), table III.5. Figure III.2 depicts the Fluorescent Activated Cell Sorting (FACS) plots from 3 representative patients and a control with robust T-cell responses to H1N1 vaccines.

Furthermore, I did not find an association between vaccine-induced T and B-cell responses following H1N1 vaccination in the 81 patients for whom both day 49 PBMC and sera were available; 19/81 patients mounted both cellular and humoral responses to H1N1 vaccination, 10/81 patients had only T-cell responses, and 41/81 patients had only antibody responses. (p=0.32).

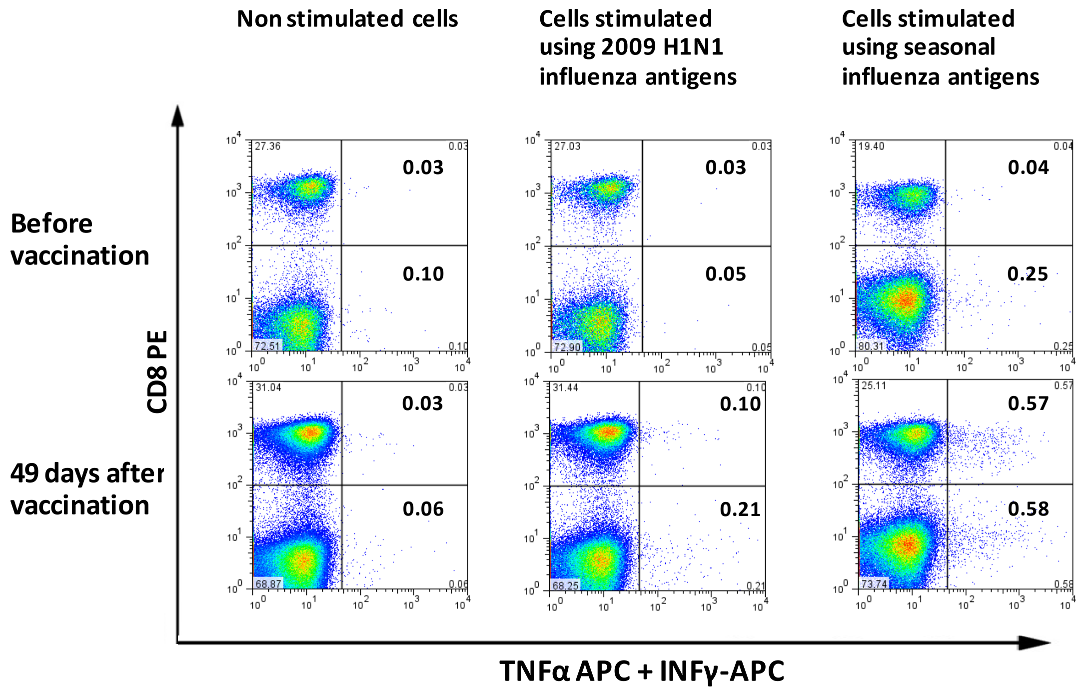
Table III.5: T-cell responses against 2009 influenza A H1N1.

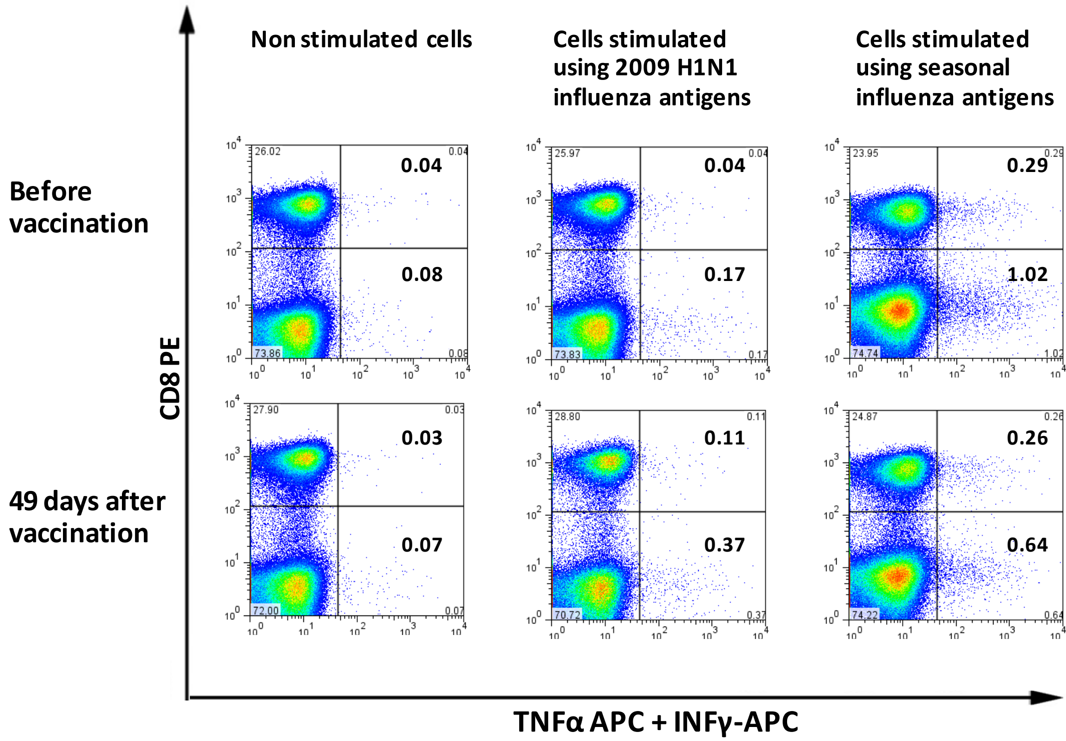
Frequencies of CD4+ and CD8+ T-cells expressing either TNF-α or IFN-γ in PBMC stimulated with H1N1 vaccine are presented. Values are shown with the background (unstimulated cells- negative control) subtracted. A response was considered positive if the percentage of antigen-specific IFN-γ or TNF-α expressing T-cells was 2-fold or higher compared to background (unstimulated PBMC) and if there was a minimum of 0.05% antigen-specific T-cells (after subtracting the background).
*Median and range are calculated on samples with a positive T-cell response.

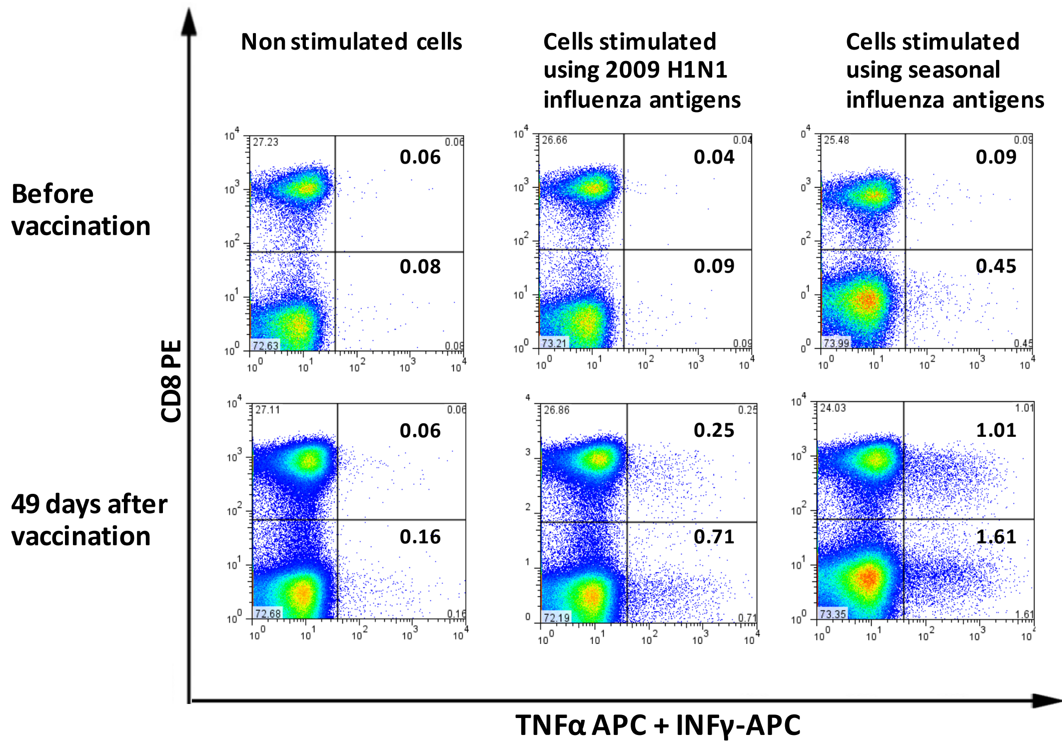
Value	B-cell malignancies (n=28)	Allo-SCT (n=25)	CML (n=28)	Controls (n=23)
Pre- H1N1 vaccination				
Number of patients with T- cell responses against H1N1 influenza - (%)	2/28 (7.1)	2/25 (8.0)	6/28 (21.4)	10/23 (43.5)
Median CD8+ T-cells against H1N1 influenza, % (range)*	0.07 (0.03-0.12)	0.03 (0.03-0.03)	0 (0-0.09)	0.05 (0-0.08)
Median CD4+ T-cells against H1N1 influenza. % (range)*	0.09 (0.08-0.11)	0.11 (0.07-0.16)	0.08 (0.06-0.27)	0.08 (0-0.15)
Day 49				
Number of patients with T- cell responses against H1N1 influenza - (%)	10/28 (35.7)	10/25 (40.0)	9/28 (32.1)	11/23 (47.8)
Median CD8+ T-cells against H1N1 influenza, % (range)*	0.02 (0-0.14)	0.04 (0.01-0.19)	0.05 (0-0.08)	0.05 (0-0.12)
Median CD4+ T-cells against H1N1 influenza, % (range)*	0.10 (0.05-0.34)	0.09 (0.06-0.55)	0.08 (0-0.30)	0.10 (0.06-0.27)

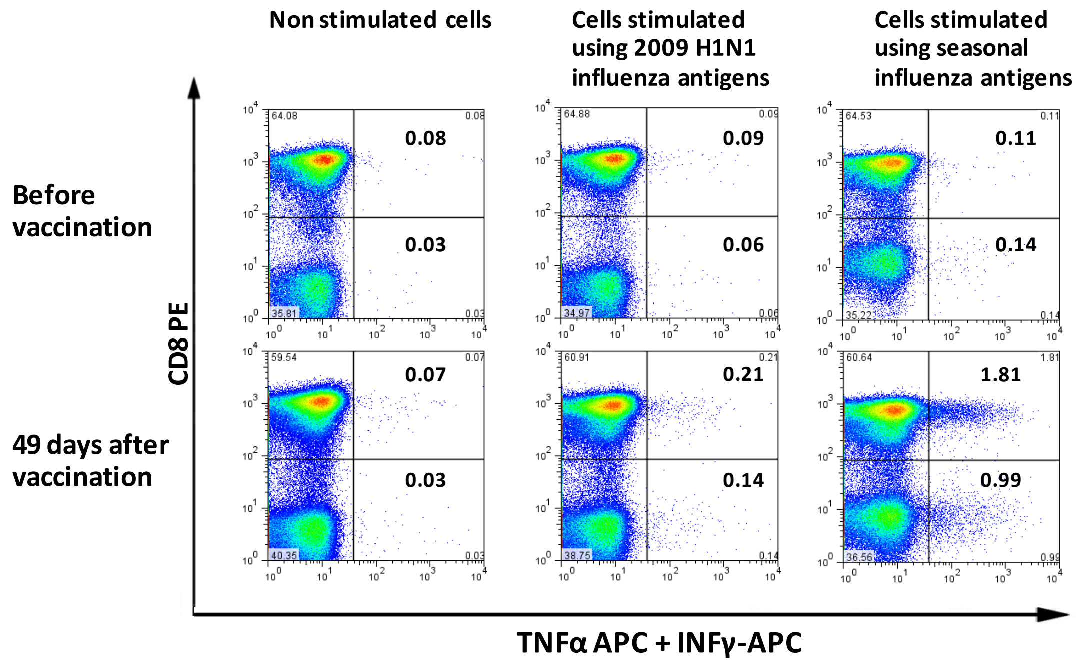
Figure III.2: Fluorescent Activated Cell Sorting (FACS) plots from 3 patients (one from each group- Allo-SCT, B-cell malignancy and CML) and a control with robust T-cell responses to H1N1 vaccine.

Cells were gated on CD3 positive lymphocytes. The Y axis represents CD8 expression and X axis TNFα + INFγ production. The allo-SCT patient (upper left panel), CLL patient (lower left panel) and the healthy control (lower right panel) received both the 2009 H1N1 vaccine and the 2009/2010 seasonal influenza vaccine, while the CML patient (upper right panel) was only vaccinated with the 2009 H1N1 vaccine.


Control
Non stimulated cells
Cells stimulated using 2009 H1N1 influenza antigens
Cells stimulated using seasonal influenza antigens
Before vaccination
49 days after vaccination
CD8 PE
TNFα APC + INFγ-APC
0.03
0.10
0.03
0.05
0.04
0.25
0.03
0.06
0.10
0.21
0.57
0.58


CML patient
Non stimulated cells
Cells stimulated using 2009 H1N1 influenza antigens
Cells stimulated using seasonal influenza antigens
Before vaccination
49 days after vaccination
CD8 PE
TNFα APC + INFγ-APC
0.04
0.08
0.04
0.17
0.29
1.02
0.03
0.07
0.11
0.37
0.26
0.64


Allo-SCT patient
Non stimulated cells
Cells stimulated using 2009 H1N1 influenza antigens
Cells stimulated using seasonal influenza antigens
Before vaccination
49 days after vaccination
CD8 PE
TNFα APC + INFγ-APC
0.06
0.08
0.04
0.09
0.09
0.45
0.06
0.16
0.25
0.71
1.01
1.61


CLL patient
Non stimulated cells
Cells stimulated using 2009 H1N1 influenza antigens
Cells stimulated using seasonal influenza antigens
Before vaccination
49 days after vaccination
CD8 PE
TNFα APC + INFγ-APC
0.08
0.03
0.09
0.06
0.11
0.14
0.07
0.03
0.21
0.14
1.81
0.99

III.4 DISCUSSION

A number of publications have studied the efficacy of vaccination with seasonal influenza in protecting against influenza like illnesses (Kunisaki & Janoff, 2009;Pollyea *et al*, 2010). Although the incidence of proven influenza infection in the vaccinated population is the preferred clinical endpoint, the low incidence of influenza like illnesses (ILI) in these studies makes the seroprotection rate an acceptable surrogate endpoint in normal controls (Clark *et al*, 2009;Greenberg *et al*, 2009;Liang *et al*, 2010;Plennevaux *et al*, 2010) and in the immunocompromised population (Kunisaki & Janoff, 2009;Ljungman *et al*, 2005;Ljungman & Avetisyan, 2008;Pollyea *et al*, 2010). In contrast T-cell protection against influenza remains poorly understood. In a study performed in elderly patients, the antibody response to influenza was reported not to be reliable at predicting risk for laboratory diagnosed influenza (LDI) while the T cell response –as assessed by cytokine and granzyme B production- was predictive for LDI (McElhaney *et al*, 2006). The same group recently suggested a link between cell-mediated immunity and influenza A/H3N2 illness severity in vaccinated older adults (Shahid *et al*, 2010).

The benefit of a seasonal influenza booster vaccine in patients with haematological malignancies remains controversial despite a number of well designed studies (Smithson *et al*, 1978;Ljungman *et al*, 2005;Lo *et al*, 1993). Even less is known about the safety, immunogenicity and optimal dosing regimen of 2009 H1N1 vaccine in this group of patients, although several investigators have reported efficacy of single dosing in healthy adults and children (Clark *et al*, 2009;Greenberg *et al*, 2009;Liang *et al*, 2010;Plennevaux *et al*, 2010).

Our data demonstrate that vaccination against 2009 pandemic H1N1 is associated with an acceptable safety profile in patients with treated and untreated haematological malignancies. As previously reported (Clark *et al*, 2009;Greenberg *et al*, 2009;Liang *et al*, 2010;Plennevaux *et al*, 2010), 100% of healthy controls in our study seroconverted after one vaccine dose. In contrast the level of humoral immunity induced by the vaccine in patients appears to be influenced by both the underlying malignancy and the time from last chemotherapy or transplantation. The seroprotection rates were significantly higher in patients with CML, patients with B-cell malignancies who had never received chemotherapy or who were vaccinated more than 12 months after chemo-immunotherapy and in intermediate to long-term survivors of transplant, compared to all other groups.

The recovery of peripheral blood B-lymphocytes following rituximab-induced B-cell depletion begins 6 months after treatment(Maloney *et al*, 1997;van der Kolk *et al*, 2002) and does not return to pre-treatment levels for up to 1 year (McLaughlin *et al*, 1998). Although the effect of rituximab on the immunogenicity of seasonal influenza vaccination remains unclear (Ljungman *et al*, 2005;Takata *et al*, 2009), it has been suggested that rituximab negatively impacts the ability to respond to novel influenza antigens (Takata *et al*, 2009). Indeed, we recently showed that patients treated with rituximab with confirmed H1N1 infection fail to mount an antibody response to H1N1 (Garland *et al*, 2011). In our current study none of the patients treated with rituximab within 6 months of vaccination achieved detectable antibody titres to H1N1. However our results show that the immune responses in untreated patients with B-cell malignancies, those who are more than 6 months from treatment and

allo-SCT recipients can be substantially improved by a second dose of vaccine confirming the need for a booster in these patient groups. In view of the limited efficacy, the advisability of vaccination in recently treated patients remains unclear and must be balanced against the high degree of mortality associated with H1N1 infection in the immunocompromised. Reassuringly the incidence and severity of side effects were no greater in this group than in any other cohort.

I evaluated the immunogenicity of H1N1 vaccine in CML-CP patients stably treated with the tyrosine kinase inhibitors (TKI), imatinib and dasatinib and compared the humoral and cellular immune responses to the vaccine in CML patients to other groups of patients with haematological malignancies and to healthy controls. Indeed some of the tyrosine kinase targets of these drugs play a role in immune responses such that there are theoretical reasons to postulate altered immune reactivity. A number of reports have documented seemingly contradictory immunomodulatory effects of TKI, ranging from impaired T-cell responses (Fraser *et al*, 2009b;Mumprecht *et al*, 2006;Seggewiss *et al*, 2005) to enhanced responses to vaccination (Larmonier *et al*, 2008). Also, little is known on the potential effect of TKI on humoral responses and few studies have examined the impact of TKIs on B cells (Kawamata *et al*, 2012;Paniagua *et al*, 2006).

Our results show that patients with CML treated on TKI can mount effective humoral immune responses to H1N1 vaccination that is not significantly different from that achieved in healthy controls. Moreover the humoral response to H1N1 is significantly better in patients with CML compared to patients with B-cell malignancies and recipients of allo-SCT. Those data would suggest that TKI do not impair humoral responses in CML patients with a good remission status and are

seemingly in contradiction with the study by Paniuga and colleagues which shows that imatinib inhibits murine B cell proliferation and immunoglobulin production by naive B cells. However the method used in our study to evaluate humoral response, namely the haemagglutination-inhibition antibody titres, cannot distinguish an IgM from an IgG response. A haemagglutination-inhibition assay detects the presence of an antibody to a virus (in this case antibodies to H1N1 virus) that will interfere with viral attachment to red blood cells. The presence of antibody will therefore inhibit hemagglutination and red cell lysis, irrespectively of the isotype of antibody. A haemaglutination-assay cannot distinguish selective impairments in IgM or IgG responses and more detailed analysis is therefore needed to study the impact of TKI on B cell responses to vaccination.

Of note this study was not designed to look at differences in vaccine-induced immune responses between imatinib and dasatinib treated patients and we cannot determine whether dasatinib may be more immunosuppressive than imatinib as suggested in a number of in vitro studies (Schade *et al*, 2008;Weichsel *et al*, 2008).

Seasonal influenza vaccine fails to produce cross-reactive antibodies to pandemic H1N1(Hancock *et al*, 2009) because H1N1 virus and conventional influenza strains differ in their haemagglutinin and neuraminidase sequences, the two surface proteins that are the primary targets of neutralizing antibodies (Greenbaum *et al*, 2009). In contrast, recent in vitro data show up to 69% cross-reactivity in CD8+ T-cell epitopes derived from pandemic H1N1 and other seasonal influenza strains (Greenbaum *et al*, 2009). Prior to vaccination, pre-existing T-cell responses to H1N1 could be detected in a significant proportion of healthy controls and CML

patients, possibly related to previous exposure to 2009 H1N1 virus but more likely due to the presence of cross-reactive seasonal and pandemic H1N1 specific T-cells (Greenbaum *et al*, 2009). This possibility is supported further by a recent study demonstrating the existence of cross-reactive seasonal and 2009 H1N1 specific T-cells of similar avidity with a memory phenotype in healthy controls (Ge *et al*, 2010). Following vaccination, H1N1-specific T-cells were induced in a significantly greater proportion of allo-SCT recipient and patients with B-cell malignancies than in CML patients or healthy controls. The limited ability of vaccination to significantly increase pre-existing influenza-specific T-cells has been previously reported although the mechanism for this phenomenon has not yet been fully elucidated (He *et al*, 2008;Keynan *et al*, 2010). A potential mechanism could be the exhaustion of influenza-specific T-cells upon repeated stimulation with the same influenza antigens (McElhaney *et al*, 2005).

Combining cellular and humoral measures of vaccine efficacy may increase the ability to predict the risk of influenza illness. Indeed cellular immune responses to influenza have been shown to correlate with protection against influenza in the absence of strong serum antibody responses among the elderly (McElhaney *et al*, 2006). Moreover studies in patients vaccinated against other viruses such as Hepatitis B have demonstrated persistence of HBsAg-specific memory T cells in the circulation for a long time after vaccination, even when serum anti-HBs antibodies were no longer detectable (Wang *et al*, 2004). This phenomenon may also apply to rituximab treated patients in whom no antibody responses were detected, yet cellular responses were present. It is possible that the effector cytotoxic T cells seen in this group can provide protection against H1N1 infection,

supporting vaccination for this subgroup of patients. I found no significant correlation between the H1N1 vaccine-induced humoral and cellular immune responses. Furthermore, none of the vaccinated patients in our study contracted H1N1 infection; therefore we are unable to evaluate the relationship between the development of influenza illness, serum antibody titres and ex vivo cellular immune responses to 2009 H1N1.

In summary, these data suggest that following vaccination with 2009 H1N1 vaccine, the humoral response to H1N1 in CML patients and healthy controls is significantly better than that achieved in patients with B-cell malignancies and recipients of allo-SCT. However, due to the inherent limitations of the haemagglutination-inhibition assay, we cannot confidently surmise that the B cell response in CML patients is not affected by TKI. Following vaccination, healthy controls and CML responded in a similar manner to H1N1 vaccination, probably due to the presence of pre-existing T-cell immunity which most likely had been lost in the all-SCT and B-cell malingnancies patient groups. I could not find a statistically significant difference in T cell responses in CML compared to healthy controls. It is possible that with a larger study, and greater power, small differences in T cell response to vaccination can be detected between CML patients on TKI and healthy controls. Finally our results unequivocally support the EMEA and the UK DoH guidelines for the administration of 2 vaccine doses in patients with B-cell malignancies and stem cell transplant recipients to induce protective immune response against 2009 H1N1 influenza and may contribute towards the development of evidence-based guidelines for influenza vaccination in patients with haematological malignancies or other immunocompromised hosts.

CHAPTER 4 TYROSINE KINASE INHIBITORS IMPAIR B-CELL IMMUNE RESPONSES IN CML THROUGH OFF-TARGET INHIBITION OF KINASES IMPORTANT FOR B-CELL SIGNALLING

IV-1 INTRODUCTION

The tyrosine kinase inhibitors (TKIs) imatinib, nilotinib and dasatinib are remarkably effective as single-agent therapy for chronic myeloid leukaemia (CML) in chronic phase (CP) (de Lavallade H. *et al*, 2008;Kantarjian *et al*, 2010;Saglio *et al*, 2010). To date very few in vivo human studies have addressed the long-term impact of these molecular-targeted drugs on the immune function. Data from in vitro and animal studies have documented seemingly contradictory effects of imatinib on the immune response, ranging from impaired antigen-specific T-cell responses(Cwynarski *et al*, 2004;Mumprecht *et al*, 2006;Seggewiss *et al*, 2005) to reversal of T cell tolerance (Wang *et al*, 2005) and potentiation of anti-tumor immune responses (Larmonier *et al*, 2008;Balachandran *et al*, 2011). The limited in vitro data available with second-generation TKIs nilotinib (Tasigna; Novartis) and dasatinib (Sprycel; Bristol-Myers Squibb) all report impaired antigen-specific T-cell responses (Blake *et al*, 2008a;Blake *et al*, 2009;Chen *et al*, 2008;Fei *et al*, 2008;Fraser *et al*, 2009a;Weichsel *et al*, 2008). Few studies have examined the impact of TKIs on B cells (Paniagua *et al*, 2006), although hypogammaglobulinaemia has been reported in CML patients treated with imatinib (Steegmann *et al*, 2003). A recent murine study reported that imatinib may directly impair class switch recombination following B cell activation through downregulation of activation-induced cytidine deaminase (AID) (Kawamata *et al*, 2012).

In our previous study we have shown that patients with CML treated on TKI can mount effective humoral immune responses to H1N1 vaccination. However due to the limitation of the haemagglutination-inhibition assay, we could not confidently exclude possible impairments in IgM or IgG response to vaccination in patients with CML on TKI, as previously suggested by murine studies (Paniagua *et al*, 2006). It is however important to note that clinically significant antibody deficiency requiring IgG therapy is not a recognised complication of imatinib.

In our previous study on immune responses to H1N1 vaccination, we did not find a significant difference in the proportion of individuals with H1N1 specific T-cell response in CML patients and healthy controls. However, I did not compare the quality of the T cell response between CML patients and healthy controls. Assessment of the polyfunctionality of the T cell response to vaccination would represent a better model to assess any possible impairment in cellular immune response in CML patients on TKI.

ABL- and SRC-dependent intracellular signalling molecules are also involved in normal T- and B-cell activation (Blake *et al*, 2009;Lee *et al*, 2010;Rix *et al*, 2007), as well as other kinases such as Btk or Syk (Atwell *et al*, 2004;Hantschel *et al*, 2007). I hypothesise that TKI may interfere with vaccine-induced cellular and humoral immune responses in patients with CML on TKI through their off-target multi-kinase inhibitory effects. To investigate this, I characterized T and B cell responses to vaccination against seasonal influenza and pneumococcus in CML-CP patients receiving imatinib, dasatinib and nilotinib and healthy controls. I found that the B cell response to pneumococcal vaccine is significantly impaired in CML

patients, associated with loss of memory B cell subsets. Furthermore, I showed that all 3 TKIs suppress an important kinase in B cell receptor signalling and survival, namely Bruton's tyrosine kinase (Btk) and its downstream substrate phospholipase C (PLC)-γ2 in a dose-dependent manner. Our findings suggest that TKIs may interfere with B-cell activation and induction of humoral immune responses in vivo through their off-target multi-kinase inhibitory effects.

IV-2 AIM

The aims of this study were

- to evaluate the cellular immune response both qualitatively and qualitatively, including analysis of the cytokine profile and effector function of vaccine-induced T cell responses
- to determine if TKI have a differential effect on IgM and IgG humoral responses to antigen
- to understand the potential mechanisms responsible for the immunomodulatory effect of TKIs on B cells

IV-3 RESULTS: T CELL RESPONSES TO INFLUENZA

Patient characteristics are shown in table IV.1.

Table IV.1: Patient characteristics: The characteristics of 51 CML-CP patients on TKI and 24 healthy controls in this study are presented

Characteristics	Patients N=51	Healthy controls N=24
Age, median (range)	52 (27-75)	41 (29-70)
Gender (M/F)	33/18	13/11
Tyrosine kinase inhibitor		N/A
Imatinib	26	
Dasatinib	13	
Nilotinib	12	

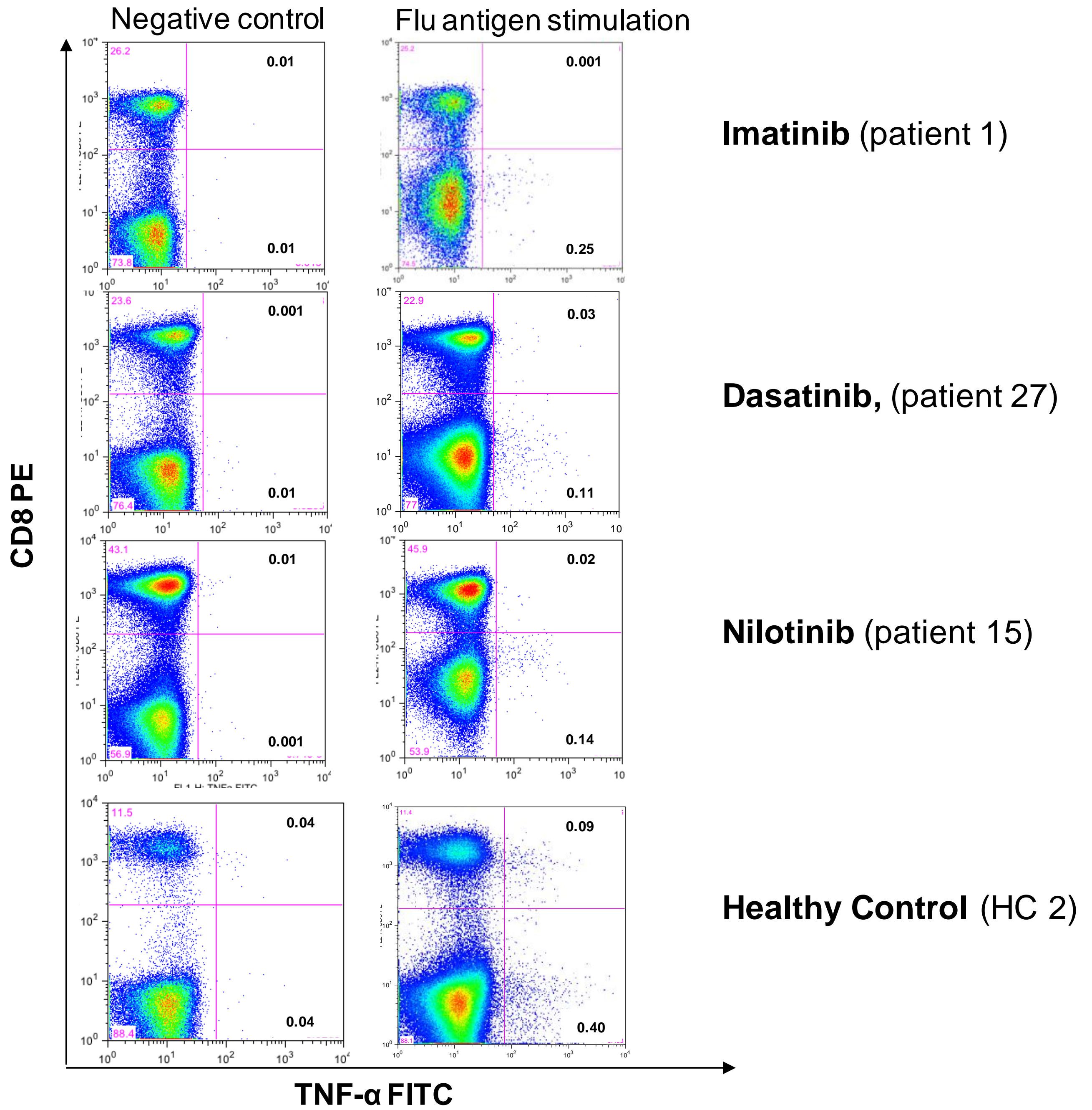
The induction of virus-specific T-cell responses by Flu vaccination was assessed directly ex vivo by flow cytometric enumeration of antigen-specific CD8+ and CD4+ T lymphocytes using an intracellular cytokine assay for IFN-γ and TNF-α. Specific T-cell responses to Flu were based on the detection of at least one intracellular cytokine above levels seen in unstimulated cells (as defined in the materials and Methods section).

IV.3.1 T cell responses to Influenza A are detectable in patients with CML on TKI prior to vaccination

The induction of virus-specific T-cell responses to Flu vaccination was assessed directly ex vivo by flow cytometric enumeration of antigen-specific CD8+ and CD4+ T lymphocytes using an intracellular cytokine assay for IFN-γ and TNF-α. A T cell response was defined to be Flu-specific if at least one cytokine was detected following in vitro antigen-stimulation as defined in Materials and Methods. Prior to vaccination, T cell responses against Flu could be detected in 21/51 (41.2%)

patients on TKI and 12/24 (50%) healthy controls ($p=0.15$), indicating the presence of pre-existing memory T cell responses to Flu in patients with CML on TKI and in healthy controls (Figure IV.1).

Figure IV.1: Examples of pre-existing CD8+ and CD4+ T cell responses to Influenza before vaccination in patients on TKI and one healthy control



IV.3.2 Vaccination with influenza A induces CD8+ and CD4+ T cell responses in patients on TKI and healthy controls

A response was considered positive if there was a minimum of 0.10% Flu-specific TNF-α or INF-γ-producing T-cells and the percentage of antigen-specific TNF-α or INF-γ-producing T-cells was 2-fold or higher compared to pre-vaccination level. Following vaccination, flu-specific T-cells were induced in 24/51 (47.0%) patients on TKI (median 0.15% TNF-α+ CD3+ T cells, range 0.05–0.64%) and 15/24 (62.5%) healthy controls (median 0.40% TNF-α+ CD3+ T cells, range 0.12–2.0%), $p=0.16$ (Table IV.2). FACS plots showing examples of T cell response to influenza vaccine in patients on TKI are shown in Figure IV.2.

Table IV.2. T cell responses in 51 CML patients and 24 healthy controls.

ND: not detected; NA: not available

Patient		Time point	pre-vaccine memory T-cells	Response	TNFa		gammaINF	
					CD4+	CD8+	CD4+	CD8+
1	**CML, imatinib**	pre-vaccine	Yes	No	0.24	ND	NA	NA
		4 weeks			0.13	ND	NA	NA
2	**CML, imatinib**	pre-vaccine	Yes	Yes	0.05	0.06	0.19	0.22
		4 weeks			0.05	0.06	ND	ND
		3 months			**0.42**	ND	ND	ND
3	**CML, imatinib**	pre-vaccine	No	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
4	**CML, imatinib**	pre-vaccine	Yes	Yes	0.1	ND	ND	ND
		4 weeks			0.1	ND	ND	ND
		3 months			**0.2**	0.05	ND	ND
5	**CML, imatinib**	pre-vaccine	Yes	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			**0.1**	ND	ND	ND
6	**CML, imatinib**	pre-vaccine	Yes	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
7	**CML, imatinib**	pre-vaccine	Yes	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND

Patient		Time point	pre-vaccine memory T-cells	Response	TNFa		gammaINF	
					CD4+	CD8+	CD4+	CD8+
8	**CML, imatinib**	pre-vaccine	Yes	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
9	**CML, imatinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			**0.15**	ND	ND	ND
10	**CML, imatinib**	pre-vaccine	No	No	ND	ND	ND	ND
		3 months			ND	ND	ND	ND
11	**CML, imatinib**	pre-vaccine	No	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
12	**CML, imatinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			**0.15**	ND	ND	ND
		3 months			NA	NA	NA	NA
13	**CML, imatinib**	pre-vaccine	Yes	Yes	0.14	ND	ND	ND
		4 weeks			0.05	ND	ND	ND
		3 months			**0.36**	ND	ND	ND
14	**CML, imatinib**	pre-vaccine	Yes	Yes	**0.14**	ND	ND	ND
		4 weeks			NA	NA	ND	ND
		3 months			**0.36**	ND	ND	ND
15	**CML, nilotinib**	pre-vaccine	Yes	No	0.14	ND	ND	ND
		4 weeks			0.05	ND	ND	ND
		3 months			0.06	ND	ND	ND
16	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	0.1	ND

Patient		Time point	pre-vaccine memory T-cells	Response	TNFa		gammaINF	
					CD4+	CD8+	CD4+	CD8+
		3 months			**0.1**	ND	ND	ND
17	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	0.13	ND
		4 weeks			ND	ND	ND	ND
		3 months			**0.1**	ND	ND	ND
18	**CML, nilotinib**	pre-vaccine	No	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
19	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	**0.1**	ND	ND
20	**CML, nilotinib**	pre-vaccine	Yes	No	0.36	ND	ND	ND
		3 months			0.08	ND	ND	ND
21	**CML, nilotinib**	pre-vaccine	No	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
22	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		3 months			**0.49**	ND	ND	ND
23	**CML, nilotinib**	pre-vaccine	Yes	No	0.07	ND	0.09	0.09
		3 months			ND	ND	ND	ND
24	**CML, nilotinib**	pre-vaccine	No	No	ND	ND	ND	ND
		3 months			ND	ND	ND	ND
25	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			**0.1**	ND	ND	ND
		3 months			0.05	ND	ND	ND

Patient		Time point	pre-vaccine memory T-cells	Response	TNFa		gammaINF	
					CD4+	**CD8+**	**CD4+**	**CD8+**
26	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	0.14	ND
27	**CML, dasatinib**	pre-vaccine	Yes	No	0.1	ND	0.08	ND
		4 weeks			0.09	ND	ND	ND
		3 months			ND	ND	ND	ND
28	**CML, dasatinib**	pre-vaccine	No	No	ND	ND	ND	ND
		3 months			ND	ND	ND	ND
29	**CML, dasatinib**	pre-vaccine	No	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			0.05	ND	ND	ND
30	**CML, dasatinib**	pre-vaccine	Yes	Yes	ND	ND	0.22	ND
		4 weeks			**0.07**	ND	0.16	ND
		3 months			NA	NA	NA	NA
31	**CML, dasatinib**	pre-vaccine	No	Yes	0.05	NA	NA	NA
		4 weeks			0.13	NA	NA	NA
		3 months			**0.64**	NA	NA	NA
32	**CML, dasatinib**	pre-vaccine	No	No	ND	ND	0.07	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
33	**CML, dasatinib**	pre-vaccine	Yes	No	0.36	ND	ND	ND
		4 weeks			0.1	ND	ND	ND
		3 months			0.3	ND	ND	ND
34	**CML, dasatinib**	pre-vaccine	No	No	ND	ND	ND	ND

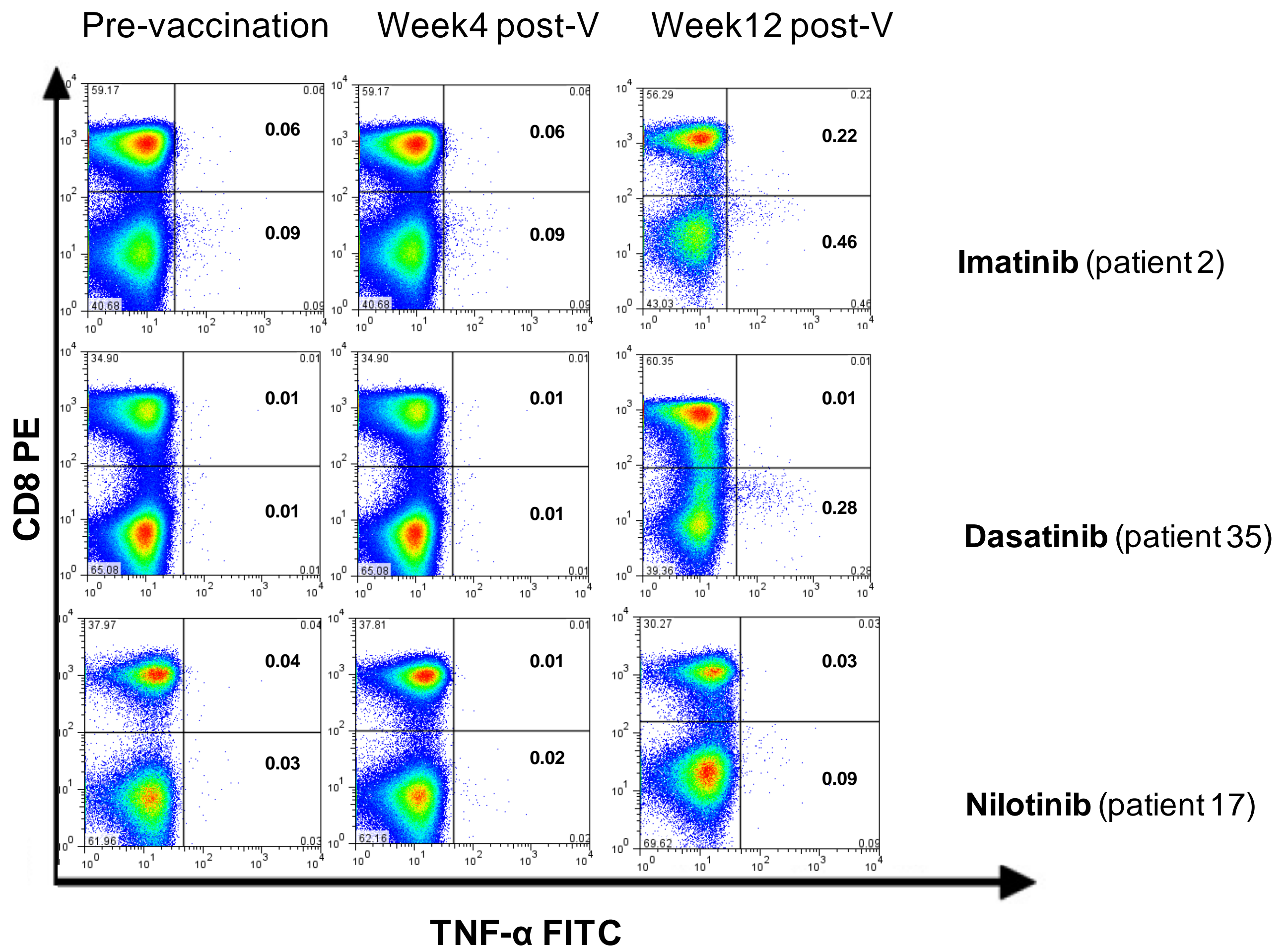
Patient		Time point	pre-vaccine memory T-cells	Response	TNFa CD4+	TNFa CD8+	gammaINF CD4+	gammaINF CD8+
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
35	**CML, dasatinib**	pre-vaccine	No	Yes	ND	ND	ND	ND
		3 months			**0.28**	ND	ND	ND
36	**CML, dasatinib**	pre-vaccine	Yes	No	0.07	0.11	NA	NA
		3 months			0.1	0.13	NA	NA
37	**CML, imatinib**	pre-vaccine	No	No	0.08	0	NA	NA
		3 months			0.12	0	NA	NA
38	**CML, dasatinib**	pre-vaccine	No	No	0.07	0.05	NA	NA
		3 months			ND	0.07	NA	NA
39	**CML, dasatinib**	pre-vaccine	Yes	Yes	0.16	0	NA	NA
		3 months			**0.33**	0.06	NA	NA
40	**CML, imatinib**	pre-vaccine	No	Yes	0	0	NA	NA
		3 months			**0.18**	0.09	NA	NA
41	**CML, imatinib**	pre-vaccine	No		0.08	0	NA	NA
		3 months			0.1	0.05	NA	NA
42	**CML, imatinib**	pre-vaccine	No	Yes	0.08	0	NA	NA
		3 months			**0.17**	0.07	NA	NA
43	**CML, imatinib**	pre-vaccine	No	Yes	0.07	0	NA	NA
		3 months			**0.15**	0	NA	NA
44	**CML, imatinib**	pre-vaccine	No	Yes	0	0	NA	NA
		3 months			**0.1**	0	NA	NA
45	**CML, dasatinib**	pre-vaccine	Yes	No	0.12	0	NA	NA
		3 months			0	0.05	NA	NA

Patient		Time point	pre-vaccine memory T-cells	Response	TNFa		gammaINF	
					CD4+	CD8+	CD4+	CD8+
46	**CML, imatinib**	pre-vaccine	Yes	No	0.18	0	NA	NA
		3 months			0	0	NA	NA
47	**CML, imatinib**	pre-vaccine	Yes	No	0.16	0	NA	NA
		3 months			0.06	0	NA	NA
48	**CML, imatinib**	pre-vaccine	No	Yes	0.08	0	NA	NA
		3 months			**0.17**	0.07	NA	NA
49	**CML, imatinib**	pre-vaccine	Yes	No	0.12	0	NA	NA
		3 months			0.2	0.08	NA	NA
50	**CML, imatinib**	pre-vaccine	No	Yes	0.06	0	NA	NA
		3 months			**0.12**	0	NA	NA
51	**CML, imatinib**	pre-vaccine	No	No	0	0	NA	NA
		3 months			0.08	0.06	NA	NA
1	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			**0.18**	0.09	ND	ND
2	**Healthy control**	pre-vaccine	Yes	No	0.36	0.1	0.25	0.08
		4 weeks			0.4	ND	ND	ND
		3 months			0.39	ND	0.3	0.16
3	**Healthy control**	pre-vaccine	No	No	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	0.14	0.11
4	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			**0.21**	ND	ND	ND
		3 months			**0.22**	ND	ND	ND

Patient		Time point	pre-vaccine memory T-cells	Response	TNFa		gammaINF	
					CD4+	CD8+	CD4+	CD8+
5	**Healthy control**	pre-vaccine	No	Yes	ND	ND	0.1	ND
		4 weeks			ND	ND	ND	ND
		3 months			**0.5**	ND	ND	ND
6	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			**0.17**	ND	ND	ND
7	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			**0.49**	ND	ND	ND
8	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			**0.16**	ND	ND	ND
		3 months			ND	ND	ND	ND
9	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			**0.1**	0.01	ND	ND
		3 months			**0.4**	0.05	ND	ND
10	**Healthy control**	pre-vaccine	Yes	No	0.16	0.1	0.12	0.08
		4 weeks			ND	ND	ND	ND
		3 months			ND	ND	ND	ND
11	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND
		3 months			**0.15**	ND	ND	ND
12	**Healthy control**	pre-vaccine	Yes	No	0.4	0.11		
		3 months			0.28	0.2		
13	**Healthy control**	pre-vaccine	Yes	No	0.1	0.07		

Patient		Time point	pre-vaccine memory T-cells	Response	TNFa		gammaINF	
					CD4+	CD8+	CD4+	CD8+
		3 months			0.12	0.05		
14	**Healthy control**	pre-vaccine	No	Yes	ND	ND		
		3 months			**0.42**	0.24		
15	**Healthy control**	pre-vaccine	Yes	Yes	0.18	0		
		3 months			**0.7**	**1.3**		
16	**Healthy control**	pre-vaccine	No	Yes	ND	ND		
		3 months			**0.22**	0.09		
17	**Healthy control**	pre-vaccine	Yes	Yes	0.14	0.06		
		3 months			0.21	**0.47**		
18	**Healthy control**	pre-vaccine	Yes	Yes	0.15	0		
		3 months			**0.52**	**0.54**		
19	**Healthy control**	pre-vaccine	Yes	No	0.76	0		
		3 months			0.27	0.08		
20	**Healthy control**	pre-vaccine	Yes	No	0.13	0		
		3 months			0.11	0.02		
21	**Healthy control**	pre-vaccine	No	No	0	0		
		3 months			0.09	0.07		
22	**Healthy control**	pre-vaccine	Yes	Yes	0.16	0.07		
		3 months			**0.22**	0.2		
23	**Healthy control**	pre-vaccine	Yes	Yes	0.28	0.11		
		3 months			**0.45**	0.24		
24	**Healthy control**	pre-vaccine	Yes	No	0.11	0.06		
		3 months			0.1	0.11		

Figure IV.2: Examples of T cell responses to influenza A vaccination in patients on TKI using intracellular cytokine assay.



IV.3.3 Detection of Influenza-specific CD8+ T cells using HLA-A2 restricted GILGFVFTL (FluMP) pentamers.

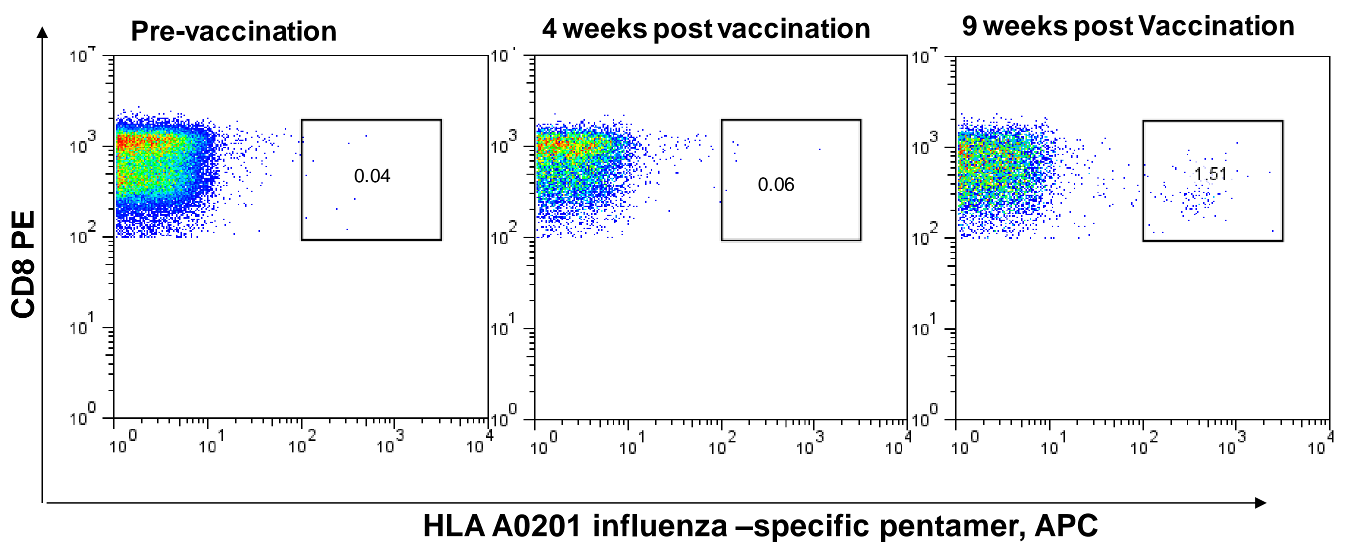
In 12 HLA-A*0201+ patients with CML (including 3 imatinib, 6 dasatinib and 3 nilotinib treated patients), and 4 HLA-A*0201+ healthy donors I also confirmed the presence of circulating Flu-specific memory CD8+ T cells using the HLA-A2 restricted GILGFVFTL (FluMP) pentamer. An increase of at least 2 fold in the frequencies of Flu-specific CD8+ T cells was detected in 2/4 controls and 5/12 patients (median 0.44% of total CD8+ T cells, range 0.1 to 1.51%) as shown in

Table IV.3. All patients and donors with detectable Flu-specific CD8+ T-cell responses by pentamer analysis also had detectable responses by intracellular cytokine assay, indicating that HLA-A2/FluMP CD8+ T cells are functional (data not shown). An example of a patient on dasatinib with a robust CD8+ T cell response (Patient 33) to influenza vaccination is presented in Figure IV.3.

Table IV.3: influenza-specfic CD8+ T-cells responses in 4 patients and two healthy controls using an HLA-A2 restricted GILGFVFTL (FluMP) pentamer.

Patient		Pre-vaccination (%)	4weeks post-vaccination (%)	12 weeks post vaccination (%)
27	CML, dasatinib	0.07	0.15	0.14
28	CML, dasatinib	0.05	0.1	NA
31	CML, dasatinib	0.03	0.44	0.09
33	CML, dasatinib	0.04	0.06	1.51
39	CML, dasatinib	0.16	0.46	NA
7	Healthy control	0.07	0.08	0.22
11	Healthy control	0.11	0.26	0.3

Figure IV.3: Detection of influenza-specific CD8+ T-cells using an HLA-A2 restricted GILGFVFTL (FluMP) pentamer: the FACS plot from a CML patient on dasatinib showing a robust CD8+ T cell response to influenza vaccination is presented.



IV.3.4 Vaccination with influenza A induces polyfunctional CD8 and CD4 T cell responses in patients on TKI.

I next evaluated the functional quality of the influenza vaccine-induced T-cell response by flow-cytometric analysis of markers related to T-helper cell-1 (TNF-α, IFN-γ and IL-2) and degranulation/cytotoxicity (CD107a) in individuals with a positive vaccine-induced T cell response (responders) and in whom sufficient numbers of cells were available for the analysis, i.e. 9 of 24 'responders' in the CML and 7 of 15 'responders' in the healthy control groups.

A polyfunctional response was defined by the production of 2 or more cytokines or one cytokine and the cytotoxic marker CD107a. Such polyfunctional responses were detected at baseline in 2/9 CML patients (two functions, n=1; three functions, n=1) and 2/7 controls (two functions, n=2). Following vaccination, polyfunctional responses were induced in 6/9 evaluable patients (2 functions, n=3, three functions, n=3) and 3/7 normal controls (two function, n=2; three function, n=1).

I found no significant differences in the quality of the T cell response to influenza vaccine in the two groups as summarized in Table IV.4.

Table IV.4: Polyfunctional T cell responses in 9 evaluable CML patients and 7 evaluable healthy controls

ND: not detected; NA: not available

Patient		Time point	pre-vaccine memory T cells	Response	TNFa		gammaINF		CD107a			IL2	
					CD4+	**CD8+**	**CD4+**	**CD8+**	**CD4+**	**CD8+**	**CD3+**	**CD4+**	**CD8+**
2	**CML, imatinib**	pre-vaccine	Yes	Yes	0.05	0.06	0.19	0.22	0.2	0.25	0.68	ND	ND
		4 weeks			0.05	0.06	ND	ND	0.21	0.23	0.81	ND	ND
		3 months			**0.42**	ND	ND	ND	0.17	0.17	0.77	ND	ND
4	**CML, imatinib**	pre-vaccine	Yes	Yes	0.1	ND	ND	ND	NA	NA	NA	ND	ND
		4 weeks			0.1	ND	ND	ND	NA	NA	NA	ND	ND
		3 months			**0.2**	0.05	ND	ND	NA	NA	NA	NA	NA
5	**CML, imatinib**	pre-vaccine	Yes	Yes	ND	ND	ND	ND	0.2	0.08	0.44	ND	ND
		4 weeks			ND	ND	ND	ND	0.3	0.15	0.62	ND	ND
		3 months			**0.1**	ND	ND	ND	0.53	0.17	0.57	ND	ND
7	**CML, imatinib**	pre-vaccine	Yes	Yes	ND	ND	ND	ND	0.2	0.09	0.38	ND	ND
		4 weeks			ND	ND	ND	ND	**0.42**	**0.18**	0.48	ND	ND
		3 months			ND	ND	ND	ND	0.38	0.14	0.2	ND	ND
13	**CML, imatinib**	pre-vaccine	Yes	Yes	0.14	ND	ND	ND	NA	0.72	0.49	ND	ND
		4 weeks			0.05	ND	ND	ND	NA	0.29	0.28	0.08	ND
		3 months			**0.36**	ND	ND	ND	NA	NA	0.67	0.08	ND

Patient		Time point	pre-vaccine memory T cells	Response	TNFa		gammaINF		CD107a			IL2	
					CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD3+	CD4+	CD8+
14	**CML, imatinib**	pre-vaccine	Yes	Yes	**0.14**	ND	ND	ND	NA	ND	ND	ND	ND
		4 weeks			NA	NA	ND	ND	NA	ND	ND	ND	ND
		3 months			**0.36**	ND	ND	ND	NA	NA	NA	NA	NA
16	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	ND	ND	0.26	0.27	0.67	ND	ND
		4 weeks			ND	ND	0.1	ND	NA	NA	NA	0.06	0.06
		3 months			**0.1**	ND	ND	ND	0.53	0.21	0.73	NA	NA
26	**CML, nilotinib**	pre-vaccine	No	Yes	ND	ND	ND	ND	ND	ND	0.32	ND	ND
		4 weeks			ND	ND	ND	ND	0.31	0.13	0.21	ND	ND
		3 months			ND	ND	0.14	ND	0.99	0.37	0.59	ND	ND
30	**CML, dasatinib**	pre-vaccine	Yes	Yes	ND	ND	0.22	ND	ND	ND	ND	NA	NA
		4 weeks			**0.07**	ND	0.16	ND	0.58	0.11	0.78	ND	ND
		3 months			NA	NA	NA	NA	NA	NA	0.58	NA	NA
1	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND	ND	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND	ND	ND	ND	ND	ND
		3 months			**0.18**	0.09	ND	ND	ND	ND	ND	ND	ND
4	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND	ND	ND	ND	ND	ND
		4 weeks			**0.21**	ND	ND	ND	NA	NA	0.88	ND	ND
		3 months			**0.22**	ND	ND	ND	NA	NA	1.37	ND	ND

Patient		Time point	pre-vaccine memory T cells	Response	TNFa		gammaINF		CD107a			IL2	
					CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD3+	CD4+	CD8+
5	**Healthy control**	pre-vaccine	No	Yes	ND	ND	0.1	ND	NA	NA	NA	0.11	ND
		4 weeks			ND	ND	ND	ND	NA	NA	NA	ND	ND
		3 months			**0.5**	ND	ND	ND	NA	NA	NA	NA	NA
6	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND	ND	ND	ND	ND	ND
		4 weeks			ND	ND	ND	ND	ND	ND	ND	ND	ND
		3 months			**0.17**	ND	ND	ND	NA	NA	NA	NA	NA
7	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND	NA	NA	NA	ND	ND
		4 weeks			ND	ND	ND	ND	NA	NA	NA	ND	ND
		3 months			**0.49**	ND	ND	ND	NA	NA	NA	NA	NA
8	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND	NA	NA	ND	ND	ND
		4 weeks			**0.16**	ND	ND	ND	NA	NA	0.1	NA	NA
		3 months			ND	ND	ND	ND	NA	NA	ND	ND	ND
9	**Healthy control**	pre-vaccine	No	Yes	ND	ND	ND	ND	0.19	0.11	0.27	0.05	0.05
		4 weeks			**0.1**	0.01	ND	ND	0.38	0.17	0.32	ND	ND
		3 months			**0.4**	0.05	ND	ND	NA	NA	NA	NA	NA

IV-4 RESULTS: B CELL RESPONSES TO PPS VACCINE

IV.4.1 CML patients on TKI have lower pneumococcal IgM titres after vaccination

Forty five patients with CML on TKI and 12 healthy controls were vaccinated against the PPS vaccine (Pneumovax II) and all could be evaluated for response. Prior to vaccination, the median pneumococcal IgG levels were 123 U/ml in CML patients compared to 71.5 U/ml in controls (p=0.3); 16/45 patients and 3/12 controls had a pre-vaccination pneumococcal IgG levels >200 U/ml (p=0.7). In contrast, the pre-vaccine pneumococcal IgM levels were significantly lower in CML patients on TKI (median 15, range 3 to 72 U/ml) compared to healthy controls (median 38, range 13 to 78 U/ml), p=0.002.

We assessed the humoral response to PPS vaccine by measuring pneumococcal IgM levels 4 weeks following vaccination. Eleven of 12 (92%) of controls had a positive IgM humoral response (defined as a 4-fold rise in serum IgM titre or IgM > 200 U/ml post-vaccination) compared to only 18 of 45 (40%) of CML patients on TKI (p=0.002). Moreover, pneumococcal IgM titres achieved at 4 weeks were significantly lower in patients with CML on TKI compared to healthy controls (median, 79 U/ml, range 5-200 vs. 200 U/ml, range 58-200, p=0.0006; Figures IV.4 and IV.5), supporting the notion that CML patients on TKI have an impaired IgM response to vaccination. Of note we only found a weak correlation between the pre- and post-vaccination IgM levels in CML patients on TKI (R2=0.17).

Figure IV.4: Pneumococcal IgM response following vaccination: pneumococcal IgM titres are presented before and 4 weeks following vaccination in healthy controls and CML patients on TKI. A positive IgM pneumococcal response was defined as a 4-fold rise in serum IgM titres or an IgM titre > 200 U/ml 4 weeks post-immunization irrespective of the pre-immunization titre.

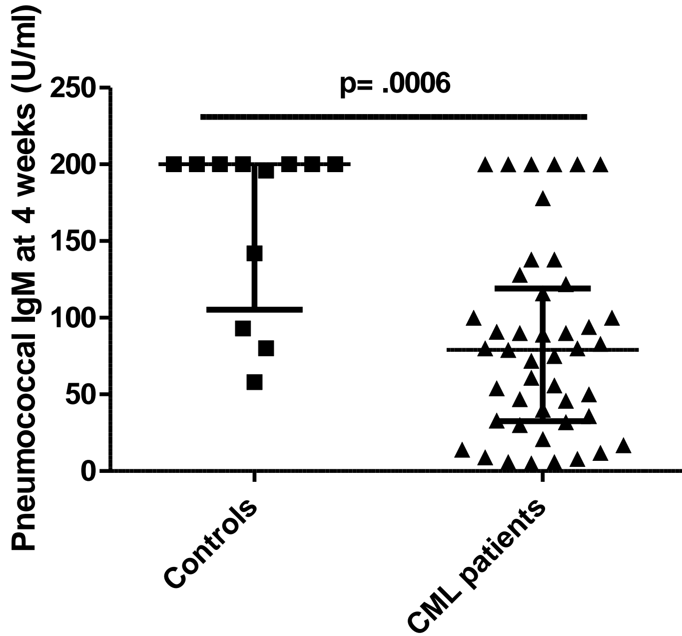
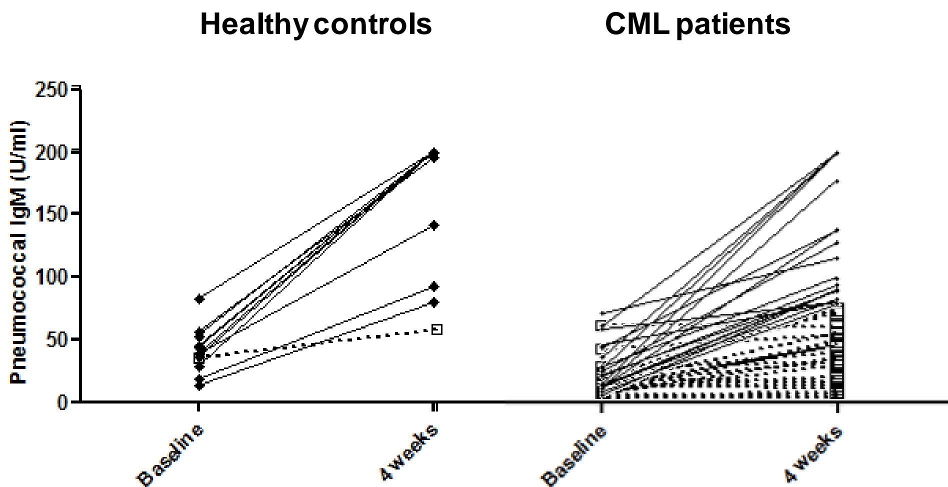


Figure IV-5: Pneumococcal IgM response following vaccination: The pneumococcal IgM response is presented before and 4 weeks after vaccination in responders (black lines) and non-responders (dashed lines) for healthy controls and CML patients on TKI



We also assessed humoral responses to the vaccine in patients and controls without prior evidence of pneumococcal infection or immunization (defined as IgG <200 U/ml prior to vaccination). Of 24/45 evaluable patients, 6/24 (25%) failed to mount both an IgM and IgG response compared to 0/9 healthy controls (p=0.15), suggesting the presence of global B cell memory impairment in a proportion of CML patient on TKI. The characteristics of the 6 CML patients who failed to mount an IgM and IgG response to PPS vaccine is presented in Table IV.5. We found no significant differences in the underlying characteristics (including age, Sokal risk score and spleen size) between the 6/24 patients who failed to mount a humoral response compared to the 18/24 responders.

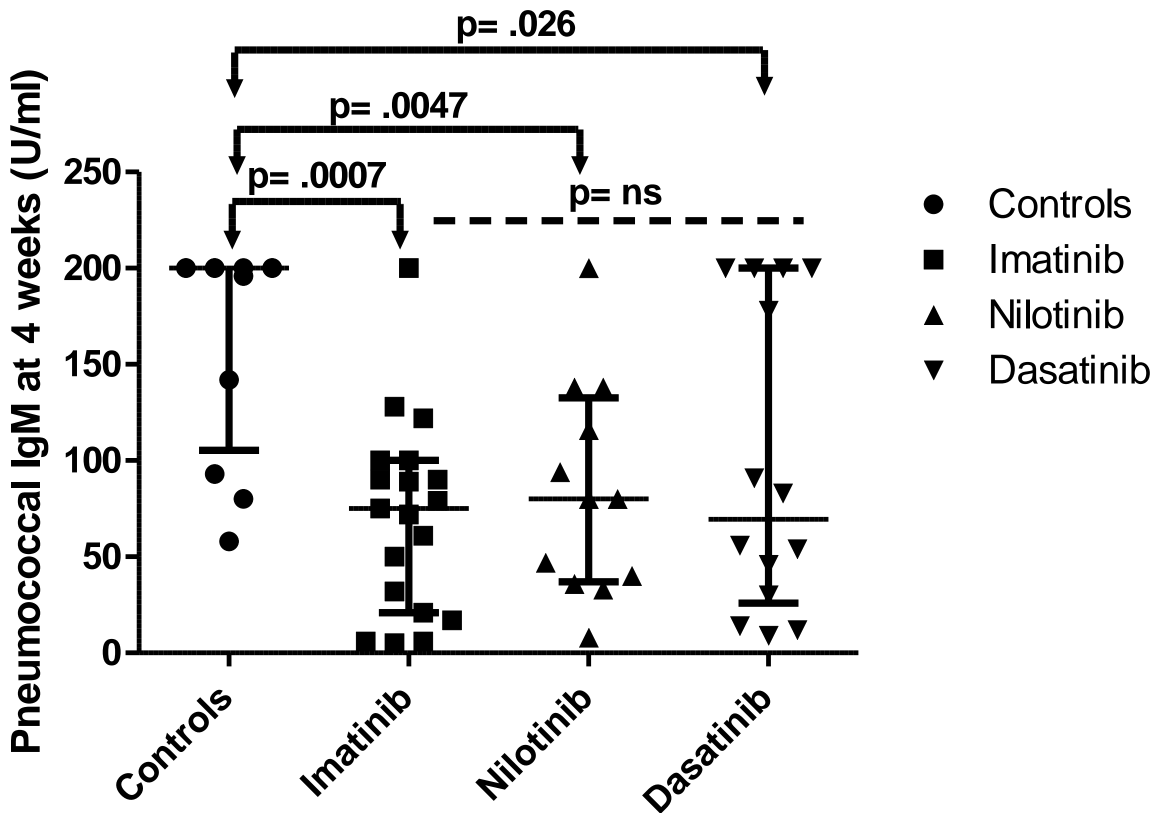
Table IV.5: Patient characteristics: The characteristics of the 6 patients who failed to mount an appropriate IgM and IgG response to pneumococcal vaccination are presented.

Patient	Age/ gender	TKI	IgM titres U/ml		IgG titres U/ml		IgM memory B cell frequencies [%]	Class switched memory B cell frequencies [%]
			Pre	Post	Pre	Post		
5	M/68	Imatinib	4	5	23	37	4.7	9.0
7	F/38	Imatinib	23	72	198	270	8.1	3.2
8	F/65	Imatinib	19	17	176	172	4.1	3.5
24	F/42	Nilotinib	8	30	49	171	4.0	11.6
28	F/60	Dasatinib	14	12	106	110	4.5	3.8
29	F/43	Dasatinib	14	46	32	170	14.9	8.8

Although this study was not specifically designed to look at differences in the vaccine response in patients treated with different TKIs, I did not find significant differences in the post-vaccine humoral response rates (7/19, 4/12 and 7/14,

p=0.87) or pneumococcal IgM serum titres (median, 75, 80 and 69 U/ml, p=0.7) in patients treated with imatinib, nilotinib or dasatinib, respectively (Figure IV.6).

Figure IV.6: Pneumococcal IgM response following vaccination: the post-immunization pneumococcal IgM titres are presented for CML patients on imatinib, nilotinib and dasatinib. Bars represent medians with interquartile range.



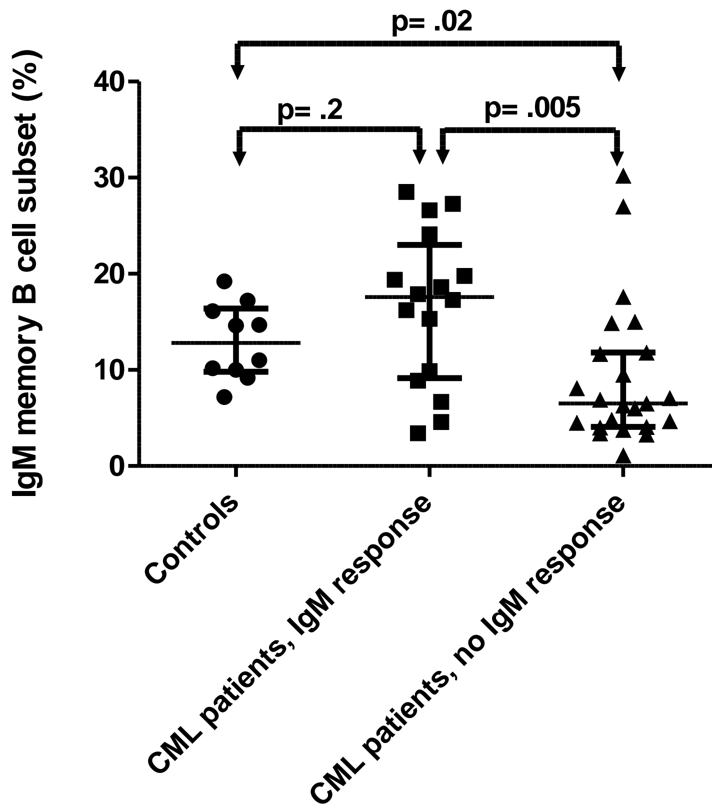
IV.4.2 IgM memory B cell subsets are markedly reduced in CML patients who do not mount an anti-pneumococcal IgM response after vaccination

To further elucidate the mechanisms underlying the impaired humoral immune response to Pneumovax II in patients with CML on TKI, I determined the

percentages of IgM memory B cells (CD19+ CD27+ IgMhigh IgD+/lo) and switched memory B cells (CD19+ CD27+ IgM- IgD-) before and 4 weeks following immunization and correlated these with pneumococcal IgM and IgG levels respectively. We had sufficient samples to perform this analysis in 39 patients and 10 healthy controls. Of note the percentage of IgM memory and switched memory B cells did not differ significantly before and after immunization.

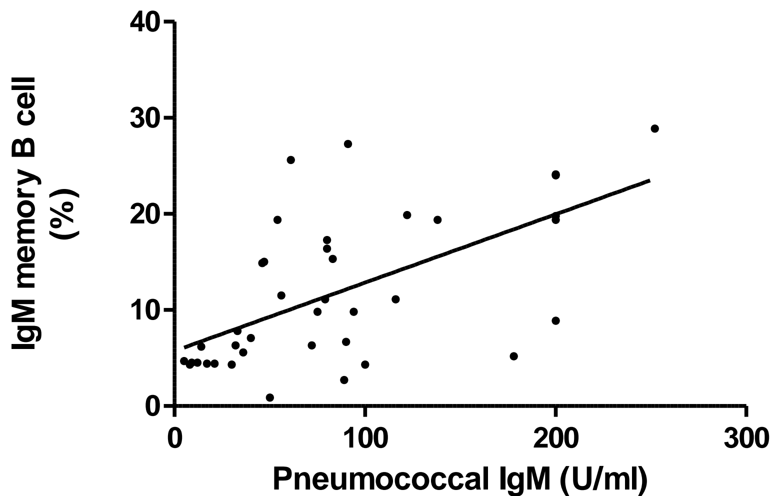
CML patients were stratified based on their 4 week pneumococcal IgM response into 2 groups of 'vaccine responders' and 'vaccine non-responders' (Figure IV.5). A positive IgM Pneumovax II response was defined as a 4-fold rise in serum IgM titres or an IgM titre > 200 U/ml 4 weeks post-immunization irrespective of the pre-immunization titre (Hart et al, 2007). CML patients who failed to mount a pneumococcal IgM response had significantly lower IgM memory B cell frequencies at vaccination compared to patients who mounted a positive pneumococcal IgM response (median, 6.5% vs 17.6%, p=0.005) and compared to healthy controls (median, 6.5% vs 12.8%, p=0.02) (Figure IV.7).

Figure IV.7: Relationship between memory B-cell subsets and pneumococcal humoral response. Patients who fail to mount a pneumococcal IgM response have significantly lower frequencies of IgM memory B cells compared to responders and healthy controls



Furthermore, in patients with CML, I found a significant correlation between IgM memory B cell frequencies at vaccination and the post-vaccine pneumococcal IgM titre (R2=0.36, p<0.0001), Figure IV.8. Interestingly, the IgM memory B cell frequency for the one healthy donor who failed to mount a positive IgM pneumococcal vaccine response was within the normal range (14.6%).

Figure IV.8: Relationship between memory B-cell subsets and pneumococcal humoral response. Scatter plot evaluating the association between pneumococcal IgM titres and IgM memory B cell frequencies in CML patients.

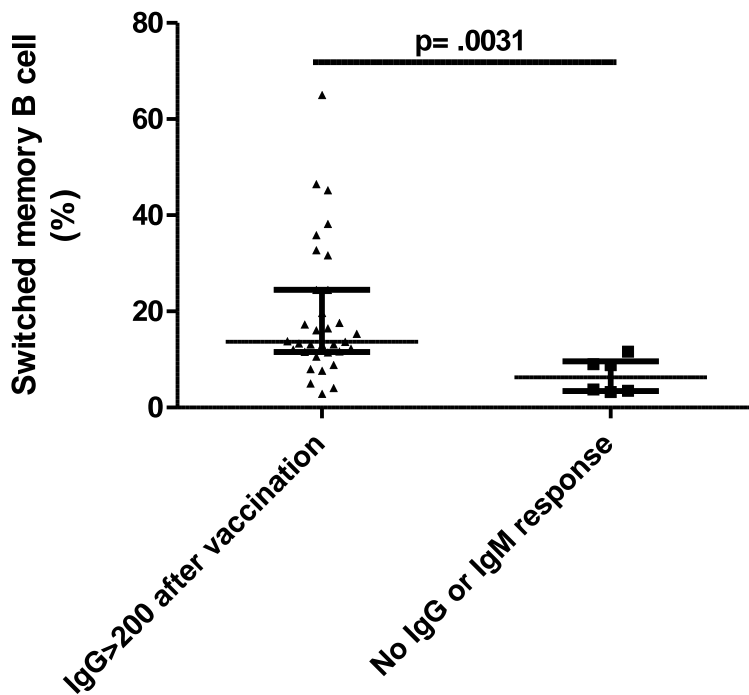


Impaired IgM responses to vaccination with PPS vaccines have been reported in the elderly population (Shi *et al*, 2005).To exclude an impact of age on the pneumococcal humoral response in our slightly older CML patient population compared to controls, we performed univariate and multivariate analyses including age, Sokal score, spleen size and IgM memory B cell frequencies. On univariate and multivariate analyses the IgM memory B cell frequency at vaccination was the only independent predictor for a positive IgM humoral response (p=0.006).

The gating strategy employed for the analysis of the B cell phenotype in vaccine responders and non-responders is shown in sectionII.6. Of note we found no significant difference in the CD19+ B frequencies between responders and non-responders (p= 0.92).

In line with the normal pre-vaccine pneumococcal IgG titres in CML, I found no significant differences in the frequencies of switched memory B cells between CML patients on TKI and controls before vaccination (median, 13.2% vs 8.9%, p=0.30 data not shown); however, the frequencies of switched memory B cells were significantly lower in the 6 patients who failed to mount an appropriate IgM and IgG response compared to the 33 patients who had an appropriate IgM or IgG response to the pneumococcal vaccine (median, 6.3% vs 13.7%, p=0.0031, Figure IV.9 and Table IV.5).

Figure IV.9: Relationship between memory B-cell subsets and pneumococcal humoral response. Frequencies of class switched memory B-cells in the 33 patients who achieved a post-immunization IgG > 200 U/ml compared to the 6 patients who failed to mount a positive pneumococcal IgM and IgG response



IV.4.3 Treatment with imatinib is associated with a significant decrease in the frequencies of IgM memory and class switched memory B cells

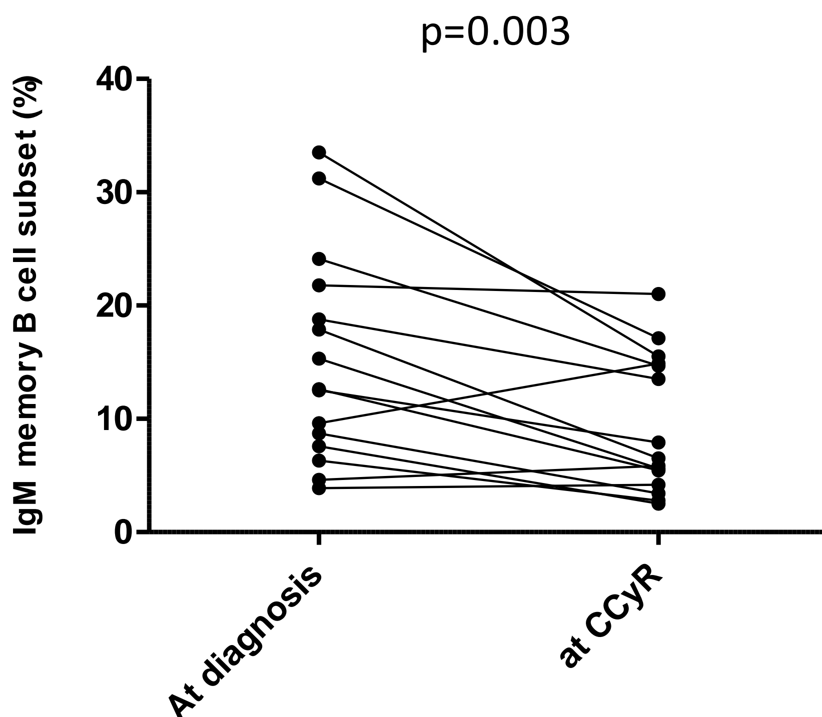
To investigate whether the loss of IgM memory B cell subset in CML patients is related to CML itself or to treatment with TKIs, I studied B cell subsets in paired samples collected from 15 CML-CP patients at diagnosis (i.e. prior to initiating imatinib therapy) and once CCyR was achieved on imatinib. The patient characteristics are summarized in Table IV.6. Only patients on imatinib were studied, as paired samples from diagnosis and following therapy were not available for patients on dasatinib and nilotinib.

Table IV.6: Characteristics of the 15 CML-CP patients whose B cell subsets were analyzed at diagnosis (i.e. prior to initiating imatinib therapy) and once CCyR was achieved on imatinib are presented. *Duration of treatment with imatinib when remission sample was collected.

Patient	Age	gender	Duration of imatinib therapy* (months)	BCR-ABL/ABL (%)*
A	50.4	F	34	0.017
B	54.8	F	42	0.014
C	37.1	M	23	0.104
D	53.6	M	32	0.338
E	44.2	M	20	0.115
F	38.0	F	18	0.619
G	35.1	M	41	0.08
H	41.5	F	54	0.003
I	46.7	F	25	0.365
J	55.9	M	21	0.003
K	59.2	M	21	0.231
L	58.1	F	30	0.017
M	56.5	F	35	0.578
N	49.6	F	33	0.005
O	27.9	M	19	0.004

No significant differences were found in the frequencies of IgM memory and switched memory B cells in CML patients at diagnosis (i.e. prior to initiation of imatinib) (n=15) compared to healthy controls (n=10) (median 12.6% vs 12.8%, p=0.85, and 14.1% vs 8.9%, p= 0.21 respectively). However, we found a significant reduction in the frequencies of IgM memory B cells in CML-CP patients following treatment with imatinib compared to diagnosis (median 6.5%, range 2.5-21.0% at CCyR, vs. 12.6%, range 3.9-33.5% at diagnosis, p=0.003), Figure IV.10.

Figure IV.10: IgM memory B-cell frequencies at diagnosis and following CCyR



Similarly, there was a significant reduction in the frequencies of class switched memory B cells following treatment with imatinib compared to diagnosis (median

7.48%, range 2.3- 22.3% at CCyR, vs. 14.1% range 6.1-28.0% at diagnosis, p= 0.001), Figure IV.11, indicating that TKI are responsible for the lower frequencies of memory B cells in CML. FACS plots from two representative patients are presented in Figure IV.12.

Figure IV.11: Class-switched memory B-cell frequencies at diagnosis and following CCyR

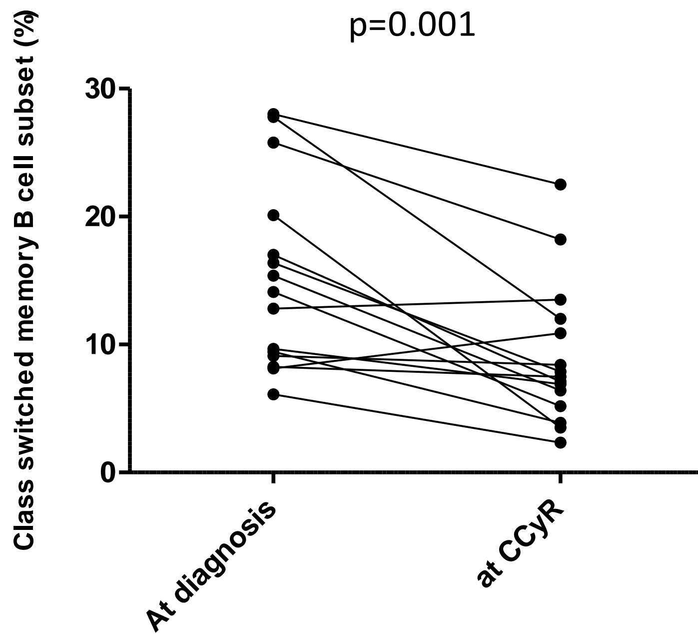
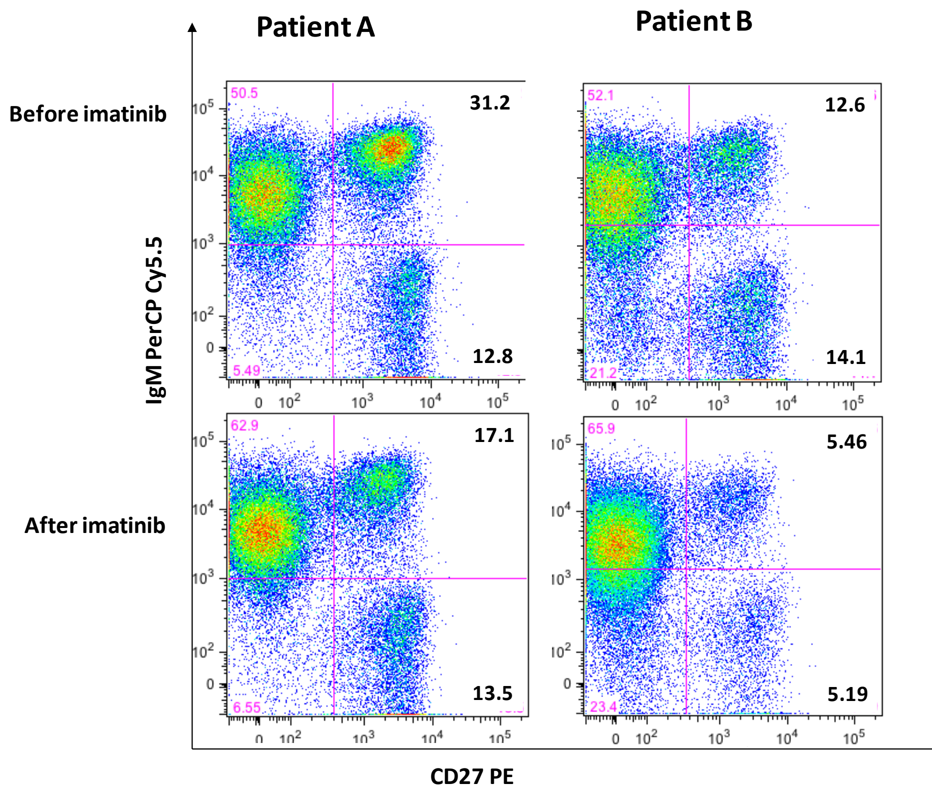


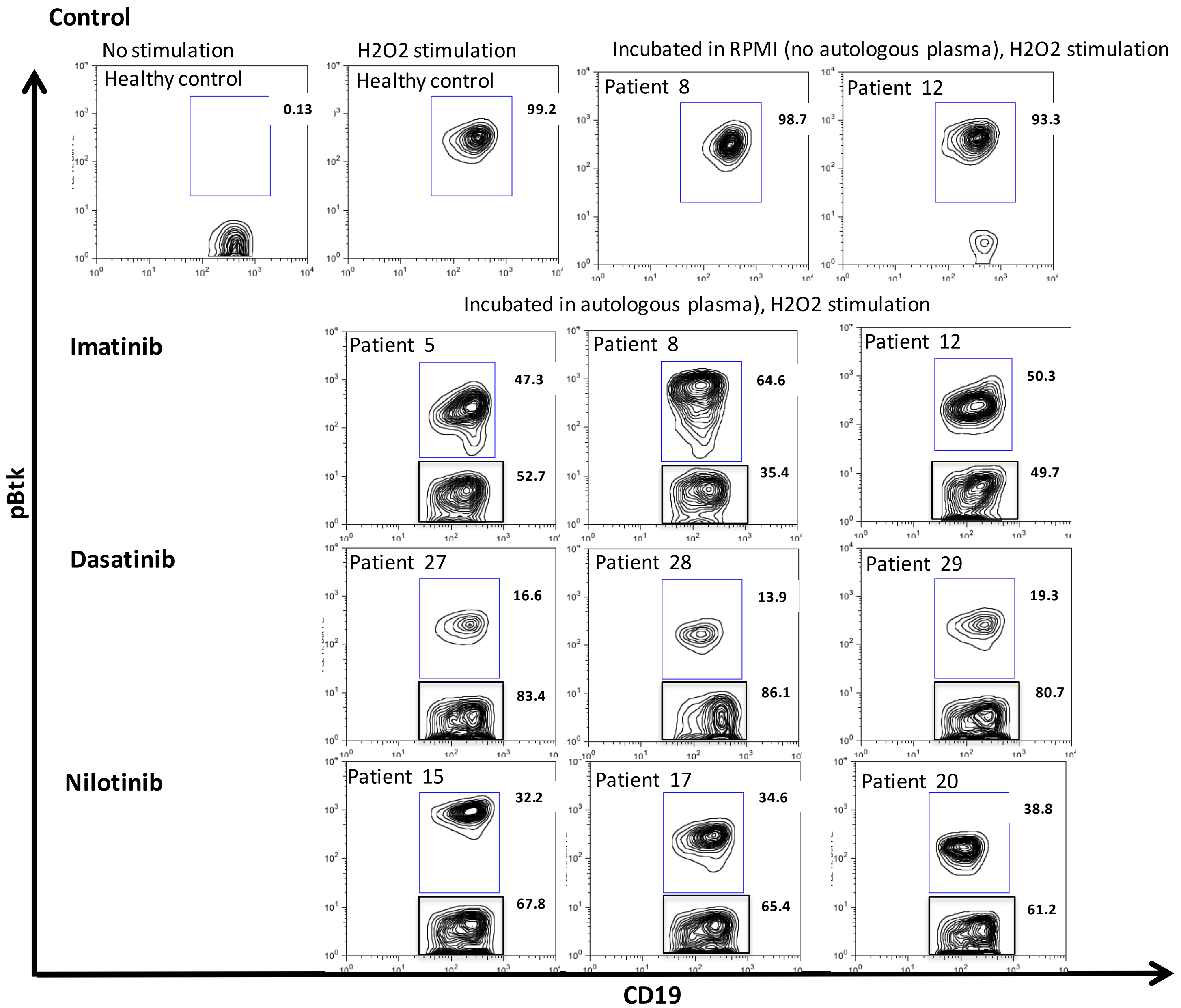
Figure IV.12: B-cell phenotype of a CML patient who developed a positive pneumococcal IgM response (Patient A) compared to a non-responder (Patient B).



IV.4.4 Plasma from CML patients on TKI co-incubated with autologous B cells inhibits Btk phosphorylation

To understand the molecular basis through which TKIs inhibit B cell activation, we co-incubated plasma from 3 vaccinated CML patients on imatinib (Patients 5, 8 and 12), 4 on nilotinib (Patients 15, 17 and 20) and 3 on dasatinib (Patients 27, 28 and 29) with autologous B cells overnight, and assessed their impact on Btk phosphorylation by phosphoflow analysis on gated CD19+ B cells. We noted significant inhibition in Btk phosphorylation in B cells of patients treated with imatinib (median inhibition 50%, range 35- 53%), nilotinib (median inhibition 65%, range 61-68%) and dasatinib (median inhibition 83%, range 81-86%), (Figure IV.13).

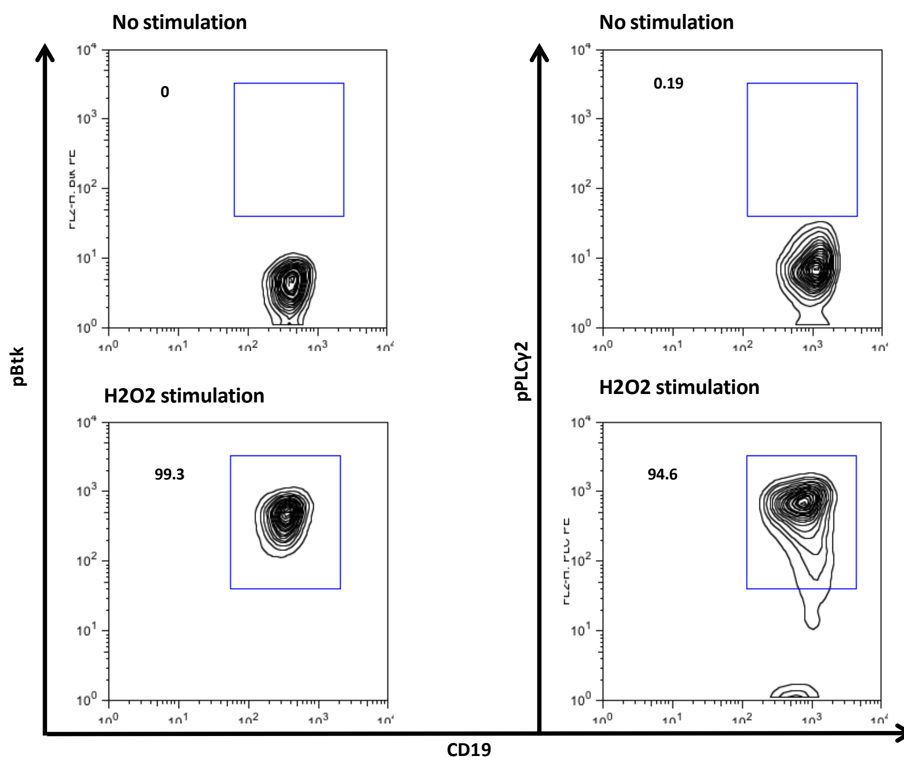
Figure IV.13: Inhibition of Btk phosphorylation in CD19+ B cells from CML patients on TKI co-incubated with autologous plasma. PBMC from CML patients were co-incubated with autologous CML plasma and stimulated with 50mM of H2O2 for 15 minutes. Phosphorylation of Btk was assessed in gated CD19+B cells. Negative controls include PBMC from healthy donors and CML patients co-incubated overnight with RPMI/10% FCS.



IV.4.5 Imatinib, dasatinib and nilotinib inhibit Btk and PLC-γ2 phosphorylation in a dose-dependent manner

We also assessed the impact of increasing doses of imatinib, dasatinib and nilotinib on the phosphorylation of Btk, and its downstream signalling molecule PLC-γ2, in healthy donor B cells. Untreated normal B cells showed little evidence of phosphorylated PLC-γ2 (0.51%, range 0.01-2.07%) and phosphorylated Btk (0.33%, range 0.00-1.15%) (Figure IV.14, top panel). Upon stimulation with H2O2, CD19+ B cells responded by expressing increased levels of p-Btk (97.36%, range 93.10-99.83%) and p-PLC-γ2 (96.73%, range 90.83-100.00%) (Figure IV.14, bottom panel).

Figure IV.14: Btk and PLC-γ2 phosphorylation inhibition by imatinib, dasatinib and nilotinib: Cells were gated on lymphocytes: the panels on the top depict the unstimulated negative control and on the bottom H202-induced phosphorylation of Btk (left) and PLC-Y2 (right).

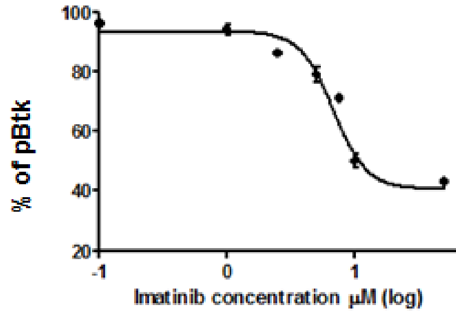


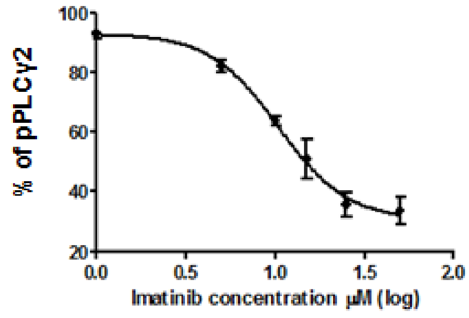
We then investigated the impact of imatinib on Btk and PLC-γ2 phosphorylation in gated CD19+ B cells derived from healthy donors. Imatinib inhibited phosphorylation of Btk (IC50 = 6.81μM) and PLC-γ2 (IC50 = 10.46 μM) in gated CD19+ B cells in a dose-dependent manner (Figure IV.15). We next determined the impact of nilotinib and dasatinib on the kinase activity of Btk and PLC-γ2. Similarly, we found that both nilotinib and dasatinib dose-dependently inhibit p-Btk (IC50 = 4.63 μM and IC50 = 27.25 nM respectively) and pPLC-γ2 (IC50 = 3.59 μM and IC50 = 25.95 nM respectively) (Figure IV.15). Each experiment was performed a minimum of 3 times.

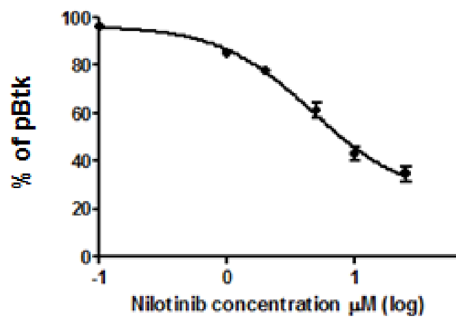
Collectively, our data provide clear evidence that all 3 TKIs can suppress B cell activation through their off-target kinase inhibition.

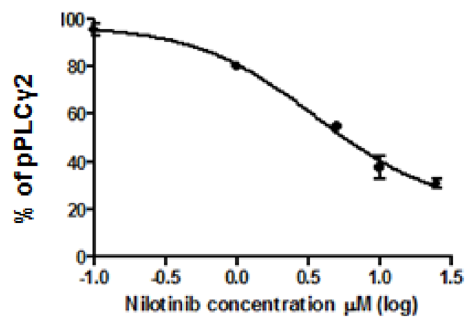
Figure IV.15: Curve fit (nonlinear regression) of log transformed TKI doses plotted against the percentage of Btk phosphorylation inhibition induced by each of the three TKI; imatinib, nilotinib and dasatinib

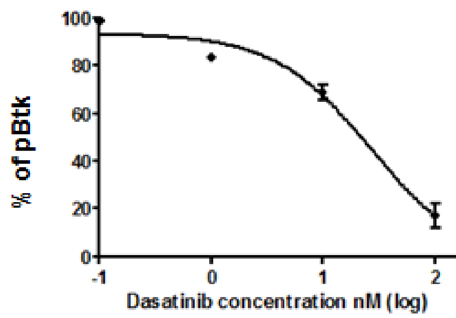
Each experiment was performed a minimum of 3 times; IC50 was calculated using nonlinear regression. The Y bar represents the percentage of gated population in which phosphorylated Btk or PLC-γ2 are detected.

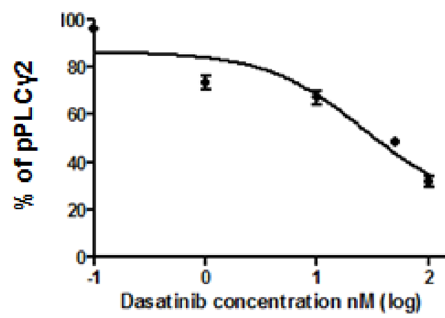












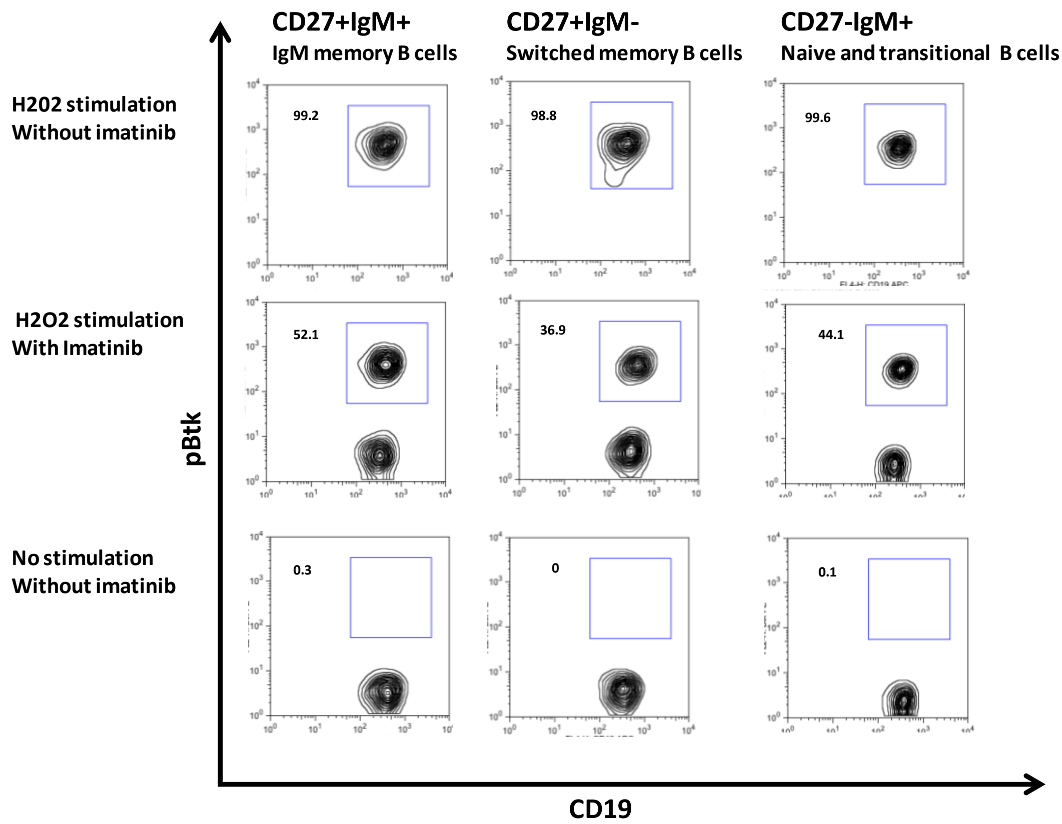
	Btk IC50 (CI 95%)	Plc γ2 IC50 (CI 95%)
Imatinib (µM)	6.81 (5.5-8.4)	10.46 (8.04-13.62)
Nilotinib (µM)	4.63 (2.86-7.44)	3.59 (1.99-6.45)
Dasatinib (nM)	27.25 (12.8-58.03)	25.95 (6.7-99.33)

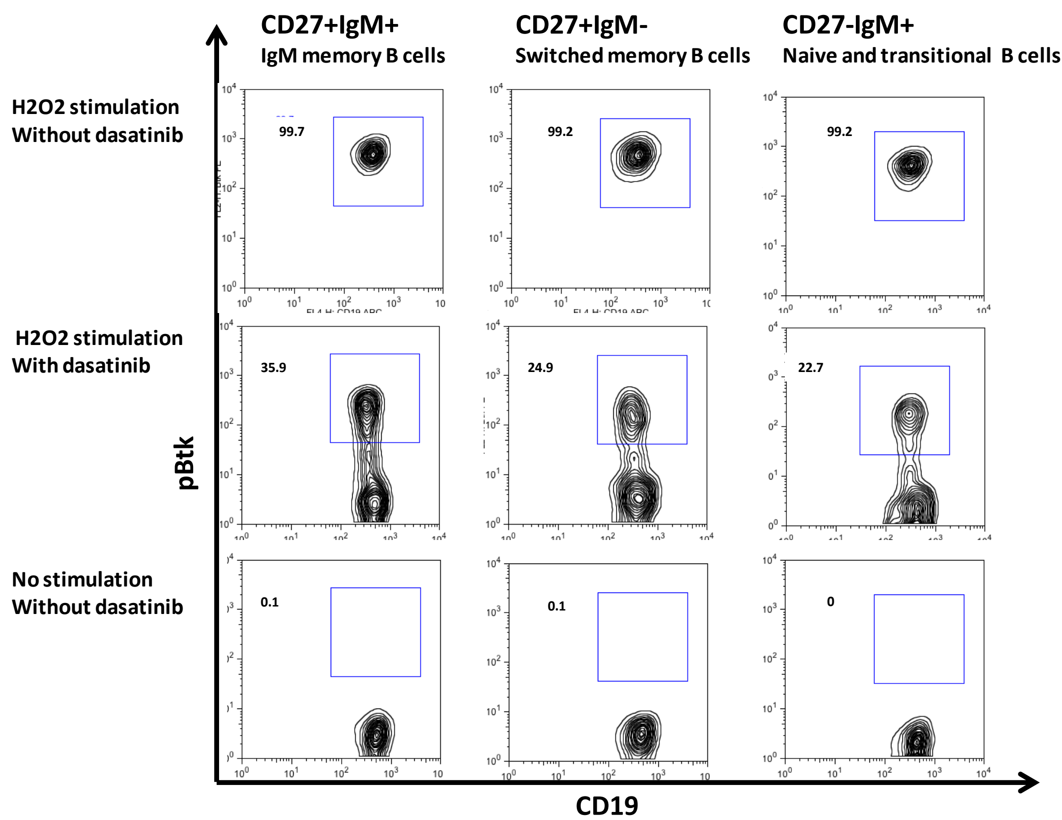
IV.4.6 Imatinib, dasatinib and nilotinib inhibit Btk and PLC-γ2 within the memory B cell subset

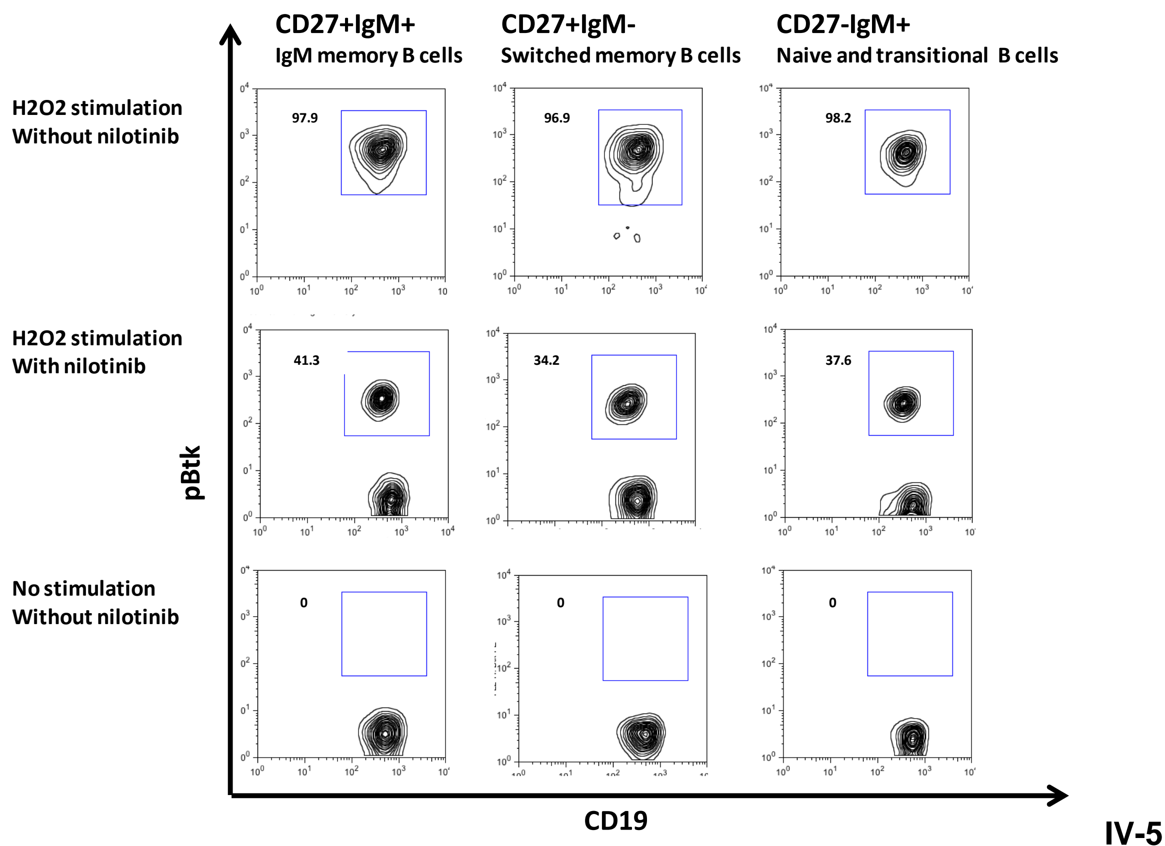
To assess if TKIs can inhibit B cell activation in memory B cell subsets, we examined the impact of these drugs on phosphorylation of Btk and PLC-γ2 by phosphoflow analysis on gated naïve B cells (CD19+CD27-), IgM memory B cells (CD19+ CD27+ IgMhigh IgD+/lo) and switched memory B cells (CD19+ CD27+ IgM- IgD-); (each experiment was performed a minimum of 3 times). With all 3 TKIs tested, inhibition of phosphorylation could be demonstrated in the memory B cell subsets as well as in naive and transitional B cells as shown in Figure IV.16.

Figure IV.16: Btk and PLC-γ2 phosphorylation inhibition in B-cell subsets

Btk phosphorylation in B cell subsets cultured in the presence or absence of Imatinib, Dasatinib or Nilotinib and stimulated with 50mM of H2O2 for 15 minutes. Effect of the TKI on pBtk inhibition is shown in gated IgM Memory B cell, Switched Memory B cell and Naive B cell subsets.


CD27+IgM+
IgM memory B cells
CD27+IgM-
Switched memory B cells
CD27-IgM+
Naive and transitional B cells
H202 stimulation
Without imatinib
H2O2 stimulation
With Imatinib
No stimulation
Without imatinib
pBtk
CD19
99.2
98.8
99.6
52.1
36.9
44.1
0.3
0
0.1


CD27+IgM+
IgM memory B cells
CD27+IgM-
Switched memory B cells
CD27-IgM+
Naive and transitional B cells
H2O2 stimulation
Without dasatinib
H2O2 stimulation
With dasatinib
No stimulation
Without dasatinib
pBtk
CD19
99.7
99.2
99.2
35.9
24.9
22.7
0.1
0.1
0



IV-5

DISCUSSION

A number of in vitro human as well as experimental models studies have investigated the potential immunomodulatory effects of TKIs on the phenotype and function of APCs and T cells. Whereas some in vitro data suggested a possible immunosuppressive effect of TKI on T cell responses (Cwynarski *et al*, 2004;Mumprecht *et al*, 2006;Seggewiss *et al*, 2005), in vivo animal models suggest a positive immunomodulatory effect of imatinib on T and APC function (Balachandran *et al*, 2011;Larmonier *et al*, 2008;Wang *et al*, 2005). Furthermore little data are available on the impact of TKIs on B cell function (Paniagua *et al*, 2006). In this study I show that CML-CP patients treated with imatinib, dasatinib or nilotinib have significant impairment in their B cell response to pneumococcal

polysaccharide vaccine. The impaired humoral response to the vaccine was associated with loss of memory B cell subsets following treatment with TKIs. Furthermore, imatinib, dasatinib and nilotinib are capable of dose-dependently suppressing one or more kinases important in BCR signalling, survival and memory formation, as demonstrated by reduced phosphorylation of Btk and indirectly its substrate PLC-γ2, providing a possible mechanism for TKI-induced B cell impairment.

In contrast, I did not find a significant difference in the T cell response to influenza vaccine in patients with CML on TKI and healthy controls. Furthermore, vaccine-induced T cells in patients with CML-CP on TKIs were functional and capable of producing effector cytokines as well as cytotoxicity, as assessed by CD107a degranulation when stimulated with the relevant antigen in vitro. Further evidence supporting the notion that prolonged TKI use may not interfere significantly with the successful induction of T cell responses in vivo has been provided by a number of clinical trials of tumor vaccination in CML patients on imatinib. Patients with CML on prolonged treatment with imatinib who were vaccinated with BCR-ABL peptides or engineered tumor cells could mount successful T cell responses against the vaccine, associated with improved cytogenetic and molecular responses (Bocchia *et al*, 2005;Cathcart *et al*, 2004;Pinilla-Ibarz *et al*, 2000;Rojas *et al*, 2007;Smith *et al*, 2010;Li *et al*, 2005). Our study is however limited by the small sample size and it is possible that with a larger cohort of patients and healthy controls, small differences in the T cell response to vaccination could be detected.

I found a strong correlation between pneumococcal IgM vaccine response and IgM

memory B cell frequencies; 60% of patients with CML-CP on TKI (27 of 45) had significantly impaired pneumococcal IgM antibody responses to vaccination, associated in almost all cases with a significant loss of IgM memory B cells. In comparison effective pneumococcal IgM responses following vaccination were seen in nearly all healthy controls (11/12) and in 18 of 45 CML patients on TKI, associated with normal IgM memory B cell frequencies. Impaired IgM responses to vaccination in association with significant reduction in IgM memory B cells has been reported in a number of conditions including common variable immunodeficiency (CVID), HIV, congenital asplenia, as well as in the elderly and children under the age of 2 years. The importance of IgM memory B cell subset in host protection against pneumococcal infection has been studied most extensively in CVID patients; in these patients a strong correlation was shown between IgM memory B cell frequencies and the incidence of encapsulated bacterial infection (Carsetti *et al*, 2005;Kruetzmann *et al*, 2003). Similarly, a number of studies analyzed the peripheral blood B lymphocyte population in HIV-positive individuals; loss of memory B cells correlated with a decrease in the pneumococcal IgM response (Hart *et al*, 2007). It is however not clear whether CML patients on TKI have more pneumococcal infection compared to the normal population. Whereas earlier studies suggested an increased infection rate in dasatinib treated CML patients,(Garcia-Munoz *et al*, 2007;Sillaber *et al*, 2009) larger prospective studies have failed to confirm these results (Kantarjian *et al*, 2010). Patients with CML-CP with low IgM memory B cell frequencies may derive some protection against infections from prior immune memory or cross-protection from other immune subsets. It is possible that patients with CML-CP on TKI who do not mount adequate responses to polysaccharide pneumococcal vaccine may respond to

vaccination with the conjugated pneumococcal vaccine, which obviously would need testing in similar settings (French *et al*, 2010). In our study we also investigated the impact of other factors including age, gender, spleen size and Sokal score on the pneumococcal humoral response. On univariate and multivariate analyses, IgM memory B cell frequency remained the only significant predictive factor for a vaccine-induced pneumococcal IgM response.

IgM memory B cells are believed to recognize T-independent (TI) antigens such as pneumococcal polysaccharide by virtue of a prediversified surface IgM and can respond immediately to antigen without T cell help. (Shi *et al*, 2003) These antibodies are of key importance in the initial phase of infection because they opsonize the pathogen and favor its phagocytosis by macrophages (Vos *et al*, 2000). Although an IgM response is believed to be the hallmark for PPS vaccine, there is evidence that switched memory B lymphocytes are also involved in the anti-PPS Ab response. Recent studies performed in SCID mice transplanted with human B lymphocytes elegantly showed that both IgM memory and switched memory B lymphocytes are involved in the anti-polysaccharide immune response (Moens *et al*, 2008). In our study a small number of CML patients (6/24 evaluable, 25%) failed to mount both an IgM and IgG response to the vaccine; the poor humoral response was associated with significantly lower switched and IgM memory B cell frequencies (Table IV.5) supporting a role for switched memory B cells in the anti-PPS humoral response. These data suggest that TKIs may affect both T-dependent and T-independent B cell activation signals in vivo, resulting in global B cell dysfunction in these patients.

The impaired humoral response and loss of B cell subsets seen in our study could be a consequence of CML itself or could be a direct effect of treatment with TKI. B cell progenitors are part of the leukaemic clone in a subset of CML patients (Takahashi *et al*, 1998) and it is therefore possible that TKIs may block BCR-ABL in Ph+ B cell lymphoid cells, thereby inducing B cell immune deficiency. Conversely, it is conceivable that Ph+ B cells may be hypo- or dysfunctional and as a result fail to mount an effective humoral response to a pathogenic antigen. However, our data do not support these scenarios as we observed a significant decrease in the frequencies of memory B cell subsets at CCyR, when a state of minimal residual disease was achieved, compared to diagnosis. Instead, our results favor a direct quantitative and qualitative effect of TKI on B cells. I found a significant reduction in IgM memory and switched memory B cell subsets following treatment with imatinib, suggesting that TKIs might interfere with the production and maintenance of B cell memory. These data may provide a possible explanation for recent reports of disease response to imatinib, despite a lack of correlation with PDGF receptor phosphorylation (Chen *et al*, 2011) and further support the use of TKI in B cell mediated immune disorders such as rheumatoid arthritis or chronic graft-versus-host disease (Olivieri *et al*, 2009;Paniagua *et al*, 2006;Chen *et al*, 2011).

Polysaccharides stimulate B cells via cross-linking of multiple antigen receptors, resulting in activation of Btk, a critical enzyme in the TI-2 signalling cascade (Vos *et al*, 2000). Btk and its downstream substrate PLC-γ2 are involved in B cell signalling, survival and memory formation and maintenance (Breitkopf *et al*, 2010;Hantschel *et al*, 2007;Kurosaki & Hikida, 2009;Rix *et al*, 2007;Hikida *et al*, 2009). I hypothesized that through their off-target kinase inhibition, TKIs may impair

the intracellular phosphorylation of Btk and indirectly inhibit its downstream substrate PLC-γ2, resulting in impaired IgM responses to vaccination and a decrease in the memory B cell compartment. We found that co-incubation of plasma from CML patients on TKI with autologous B cells resulted in significant inhibition of Btk phosphorylation. Plasma from CML patients on dasatinib induced more profound suppression of Btk kinase activity compared to plasma derived from patients on imatinib or nilotinib, suggesting that dasatinib may have more potent off-target Btk inhibitory activity compared to the more specific BCR-ABL inhibitors such as imatinib or nilotinib. These findings are further supported by previous work showing dasatinib to be a strong inhibitor of Btk phosphotylation (Hantschel *et al*, 2007). Physiological concentrations of imatinib, dasatinib and nilotinib were also shown to inhibit Btk phosphorylation in activated B cells from healthy controls. Finally, we showed that all 3 TKI suppress Btk activity in memory B cell subsets, known to be critical to B cell memory development. Btk has also been shown to play an important role in class switching of B cells,(Halcomb *et al*, 2008) providing a possible explanation for our observation that a subset of CML patients on TKI fail to mount IgG responses to vaccination. These data are in keeping with a recent study in mice, reporting that imatinib impairs activation-induced class switching (Kawamata *et al*, 2012). Our study was not designed to address the underlying reasons for differential responses to pneumococcal vaccine in CML patients on TKI. A possible explanation could be variations in serum TKI levels due to inter-individual differences in drug metabolism(Di *et al*, 2011) or variations in adherence rates that may in turn impact on the in vivo B cell response to the vaccine (Marin *et al*, 2010).

In conclusion, treatment with TKIs is associated with loss of memory B cell subsets and impaired humoral immune responses to pneumococcal polysaccharide vaccine, likely driven by the off-target kinase inhibitory activity of these drugs. Our results call for close monitoring of patients on TKI to assess the long-term impact of impaired B cell function on immune surveillance and susceptibility to infection and cancer. The inhibitory effect of imatinib, dasatinib and nilotinib on memory B-cell expansion and antibody production provides further rationale for studies of selective tyrosine kinase inhibitors in the treatment of autoimmune diseases and cGVHD.

CHAPTER 5 IMPACT OF PHILADELPHIA-POSITIVE LYMPHOPOIESIS ON RESPONSE TO IMATINIB AND OUTCOME

V-1 INTRODUCTION

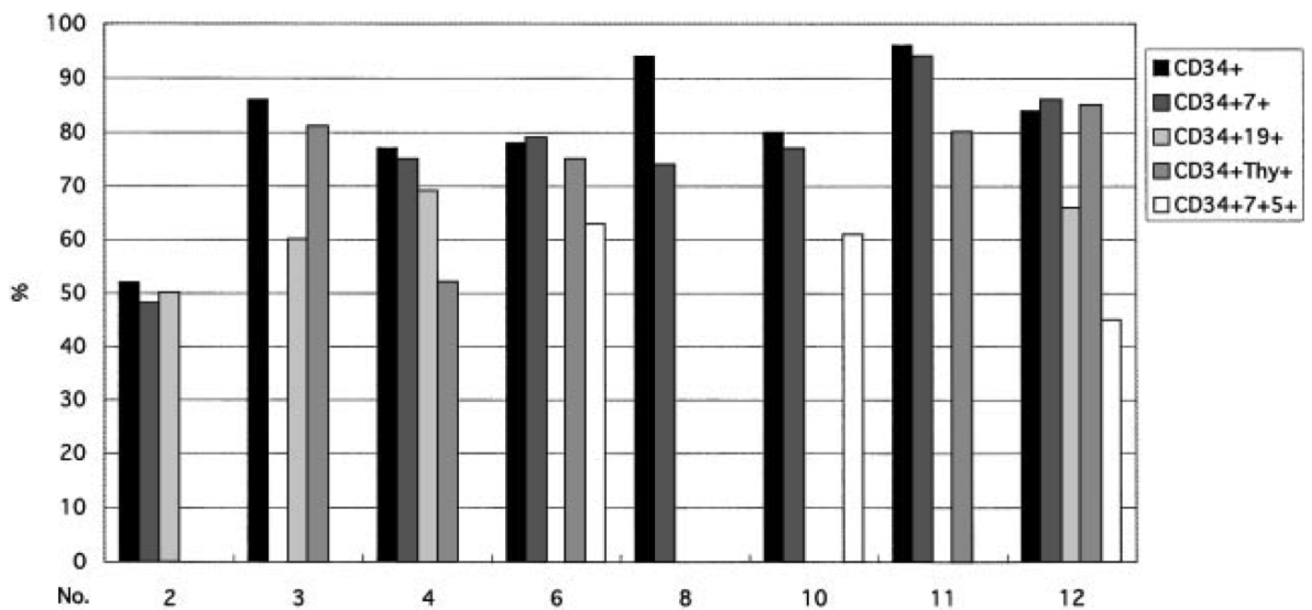
I demonstrated in the study outlined in the previous chapter that treatment with TKIs is associated with loss of memory B cell subsets and impaired humoral immune responses to pneumococcal polysaccharide vaccine, likely mediated by the off-target kinase inhibitory activity of these drugs. I next investigated if TKI could also impact on the function of other B cell subsets such as transitional and naive B cells.

To that end I performed a comprehensive analysis of B cell subsets in paired frozen samples obtained from CML-CP patients at diagnosis and after the achievement of a CCyR on imatinib, as well as in presentation samples from CML patients who did not respond to imatinib. The aim of this work was to further explore the effect of TKI on different B-cell subsets and to look for the presence of, and implications of Philadelphia positive lymphopoiesis on B cell function.

It is also possible that the impaired humoral response and loss of B cell subsets seen in our study is a consequence of CML itself, through BCR-ABL involvement in the B cell lineage. Indeed it has been shown that B cell progenitors are part of the leukaemic clone in a subset of CML patients. In the pre-imatinib era, Takahashi and collaborators reported the presence of the Philadelphia chromosome in B cell progenitors from CML patients at diagnosis or while on INF-alpha or hydroxycarbamide therapy (Takahashi *et al*, 1998), as shown in Figure V.1. It is

therefore possible that Ph+ B cells may be hypo- or dysfunctional and as a result fail to mount an effective humoral response to a pathogenic antigen. To investigate this possibility, I looked for the presence of BCR-ABL fusion gene in flow sorted B cell subsets.

Figure V.1: Cells with the BCR/ABL fusion signals inCD34+ subpopulations (Takahashi *et al*, 1998)



V-2 AIMS

The aims of this study were:

- to study changes in the phenotype of B cells (transitional, naïve, IgM memory and switched memory) in CML patients at diagnosis and following achievement of CCyR on imatinib
- to evaluate if the phenotype of B cells, and specifically involvement of B cell subsets by Ph+, predicts response to imatinib therapy

V-3 RESULTS

Patient characteristics are shown in Table V.1. Among the 39 chronic phase CML patients included in this study, extended B cell phenotyping were available in 18/25 in the responder group and in 12/14 in the non-responder group due to limitations in sample availability. Among the 14 non-responders, 9/14 transformed to blast crisis at a median time of 24 months (range, 5 to 57 months) following diagnosis.

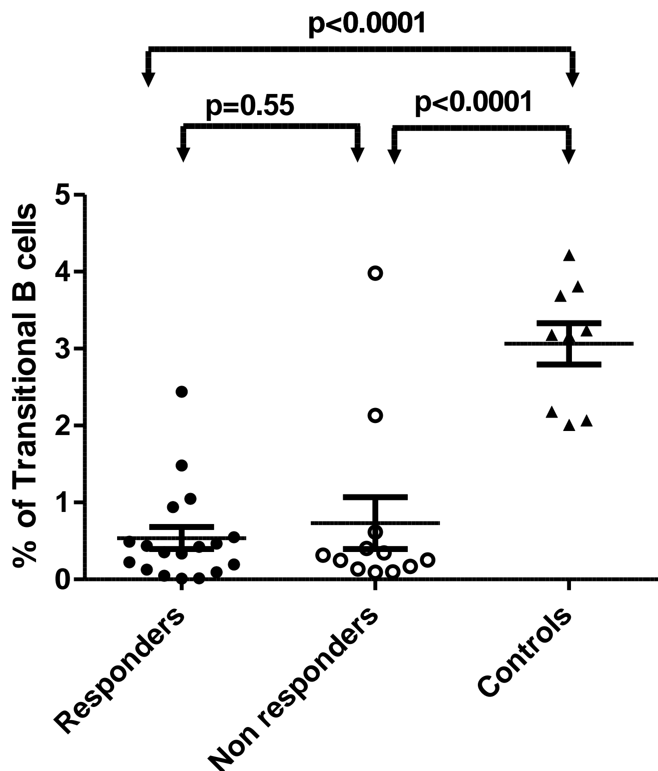
Table V.1: Patient characteristics. CCyR: complete cytogenetic response; NA: not applicable

Patient's group	Responders (n=25)	Non responders (n=14)
Age (median, range)	46 (24 to 65)	40 (21 to 68)
Sokal risk score at diagnosis -Low -Intermediate -High -unknown	 12 9 3 1	 2 6 6 0
Time to CCyR, months (median, range)	7 (3 to 17)	NA
Interval diagnostic-remission samples, months (median, range)	25 (15 to 56)	NA
Best CCyR response on imatinib	0.002% (0 to 0.184%)	NA
Time to transformation into blast crisis, Months (median, range)	NA	23 (5 to 57)
Blastic transformation (Lymphoid/Myeloid/Unknown)	NA	3/1/5
Transcript type (e14a2/e13a2)	14/11	7/7

V.3.1 Transitional B cells are reduced at diagnosis both in responders and non-responders compared to healthy controls

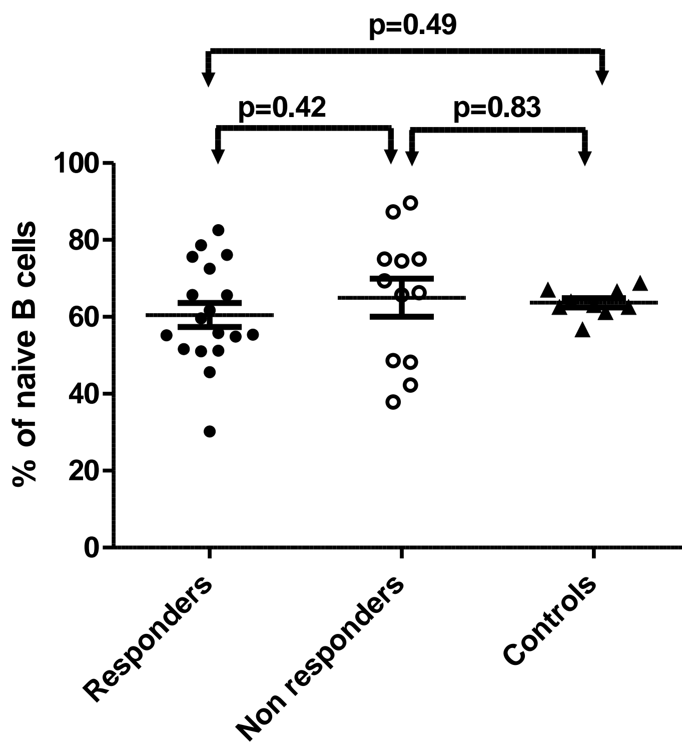
Among the 18/25 evaluable 'responder' group, the median percentage of transitional (T1 and T2) B cells at diagnosis (defined as CD24high CD38high) was significantly lower compared to healthy controls (0.39% vs 3.2% respectively, p<0.0001). Similarly the median percentage of transitional B cells at diagnosis was reduced among non-responders compared to the control group (0.28% vs. 3.2% respectively, p<0.0001) and was not different to the responder group (0.28% vs. 0.38%, p=0.55), Figure V.3.

Figure V.2: Transitional (T1 and T2) B cells in 18 CML responders and 12 non-responders at diagnosis compared to 8 healthy controls



I found no significant difference in the frequencies of naive B cells (defined as CD24int CD38int cells), when comparing CML responders, non responders and healthy controls (median, 57.7%, 67,8% and 63.1% respectively, means 60.5%, 65.0% and 63.7% respectively), suggesting that this subset may not be directly affected in CML patients (Figure V.4).

Figure V.3.: Naive B cells in 18 CML responders and 12 non responders at diagnosis compared to 8 healthy controls

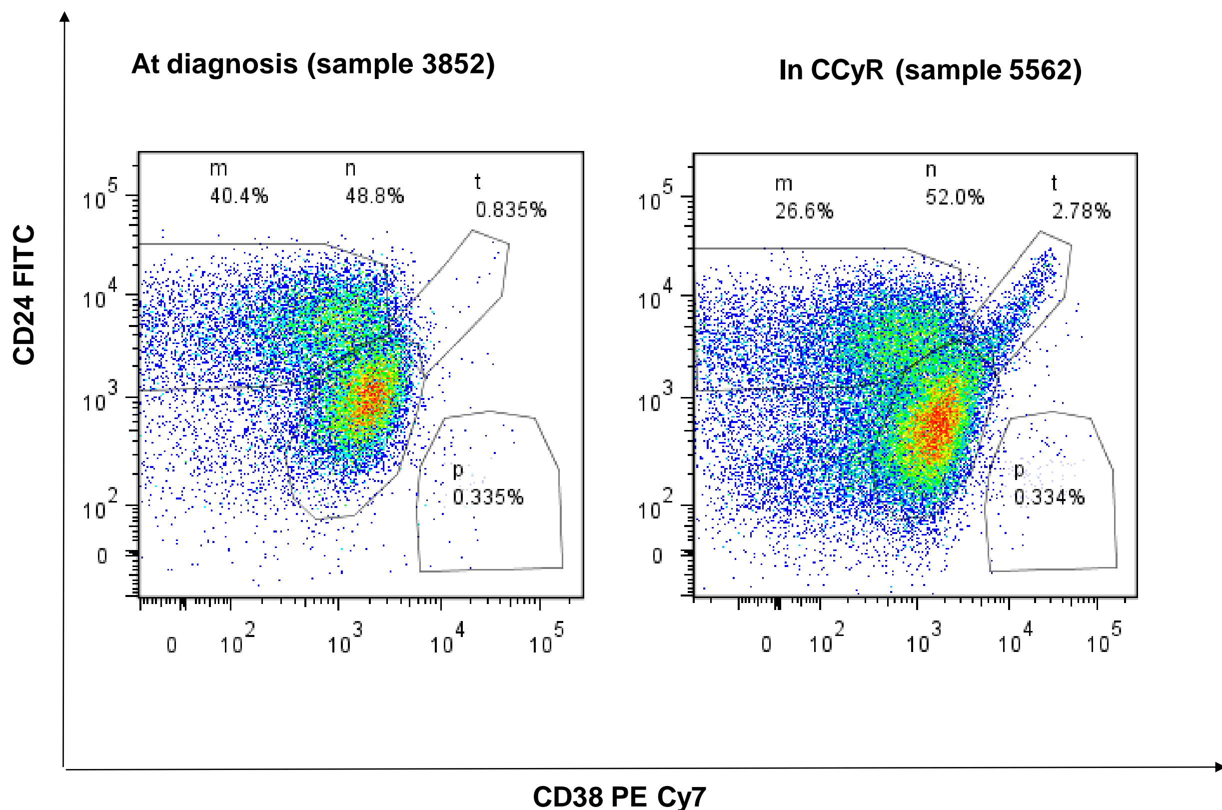


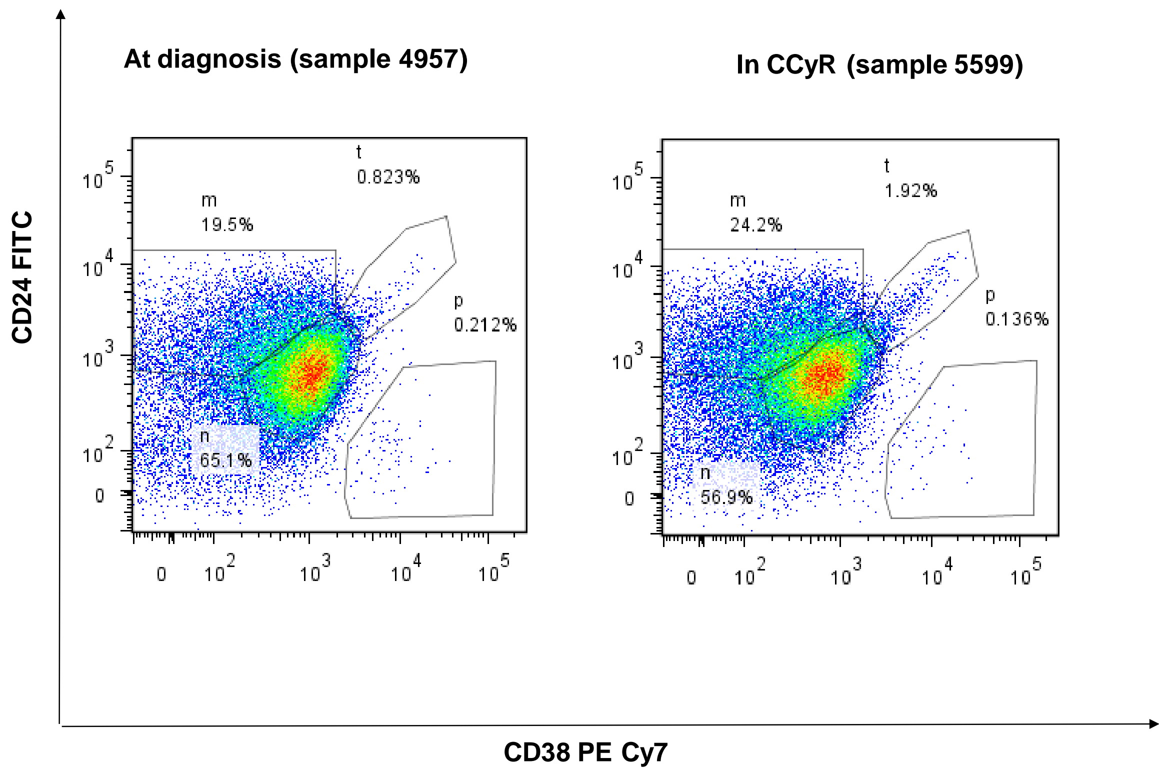
V.3.2 Transitional B cell frequencies partially normalise in CML patients who achieve CCyR on imatinib

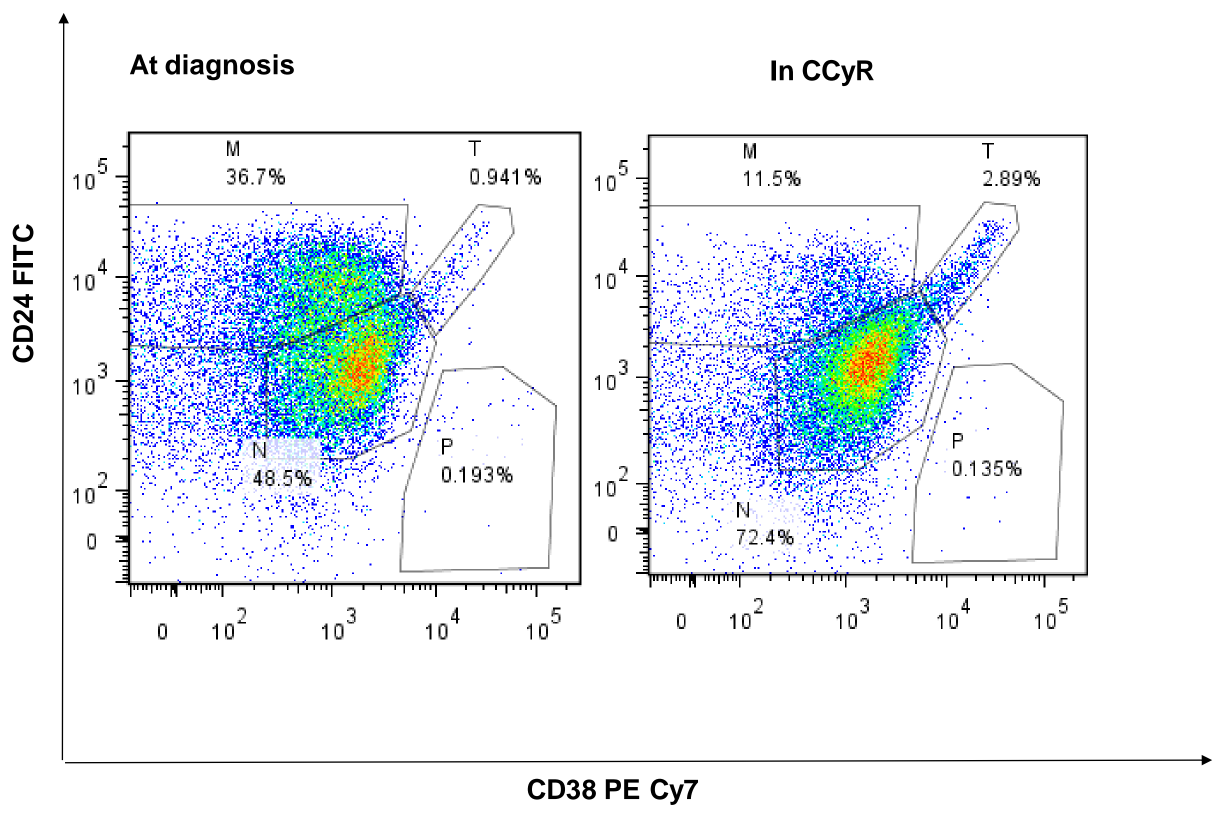
In paired samples taken from CML patients at diagnosis and following CCyR on imatinib, the percentage of transitional (T1 and T2) B cells increased while on treatment, suggesting that imatinib may restore normal transitional B cell frequencies in CML patients who achieve CCyR. Figure V.5 shows FACS plot examples of 3 representative patients at diagnosis and at CCyR.

Figure V.4: FACS plot examples of 3 representative patients at diagnosis and while in CCyR (paired samples)

t: transitional B-cells; n: naive B-cells; m: memory B-cells; p: plasmablasts

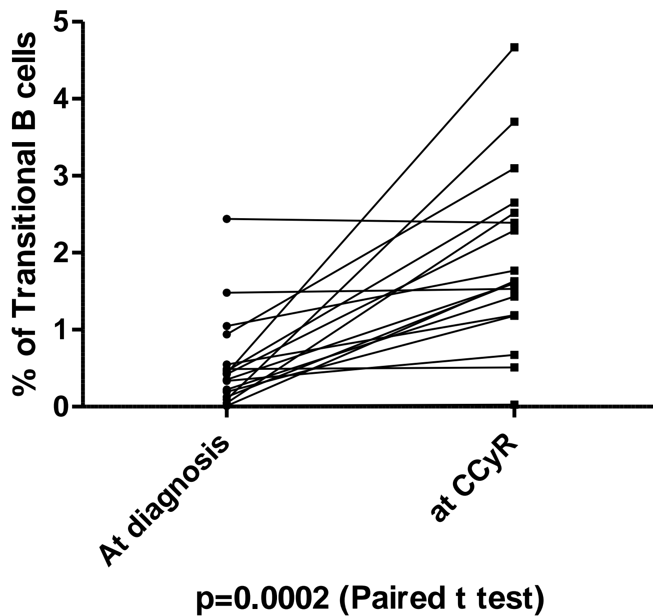



At diagnosis (sample 4957)
In CCyR (sample 5599)
m
19.5%
t
0.823%
p
0.212%
n
65.1%
m
24.2%
t
1.92%
p
0.136%
n
56.9%
CD24 FITC
CD38 PE Cy7


At diagnosis
In CCyR
M
36.7%
T
0.941%
P
0.193%
N
48.5%
M
11.5%
T
2.89%
P
0.135%
N
72.4%
CD24 FITC
CD38 PE Cy7

Analysis of 18 paired samples from diagnosis and at CCyR demonstrated significant increases in the frequencies of transitional B cells (median, 0.38% vs 1.62%, mean vs 0.54%, vs.1.91% p<0.001 paired t test) as shown in Figure V.6.

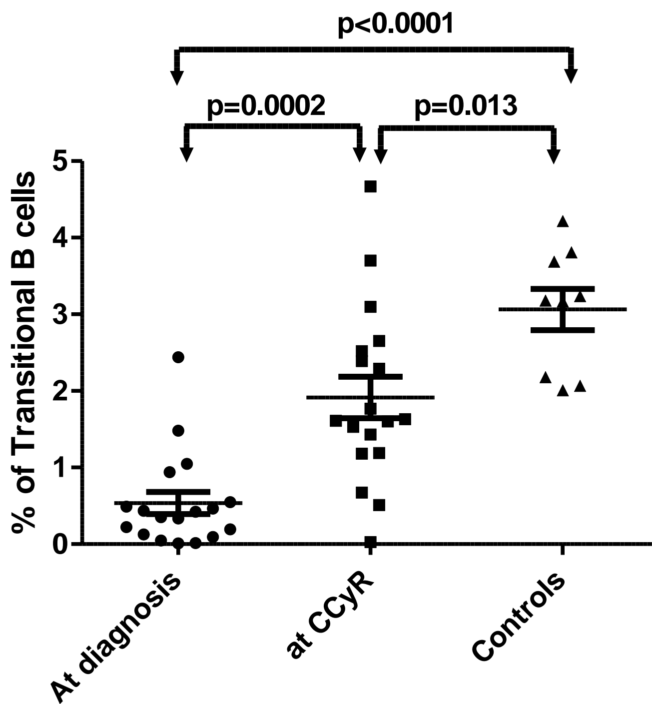
Figure V.5: Transitional B cell frequencies in paired samples from CML responders at diagnosis and while in CCyR



When comparing the transitional B cells between the paired samples and samples taken from 8 healthy controls, we found a statistically significant difference between diagnostic CML samples and healthy controls (median 0.38% vs 3.18% respectively, mean 0.53% vs 3.06%, p<0.0001) as shown in figure V.4.5. When comparing the transitional B cell frequencies in CML patients in CCyR with controls, there was still a statistically significant difference, although less pronounced than at diagnosis (median 1.62% vs 3.18% respectively, mean 1.91% vs 3.06%, p=0.013), suggesting that, although treatment with imatinib restores

transitional B cell frequencies, the recovery of this subset might not be complete (Figure V.7).

Figure V.6: Transitional B cells in paired samples from CML patients at diagnosis, following CCyR and compared to controls

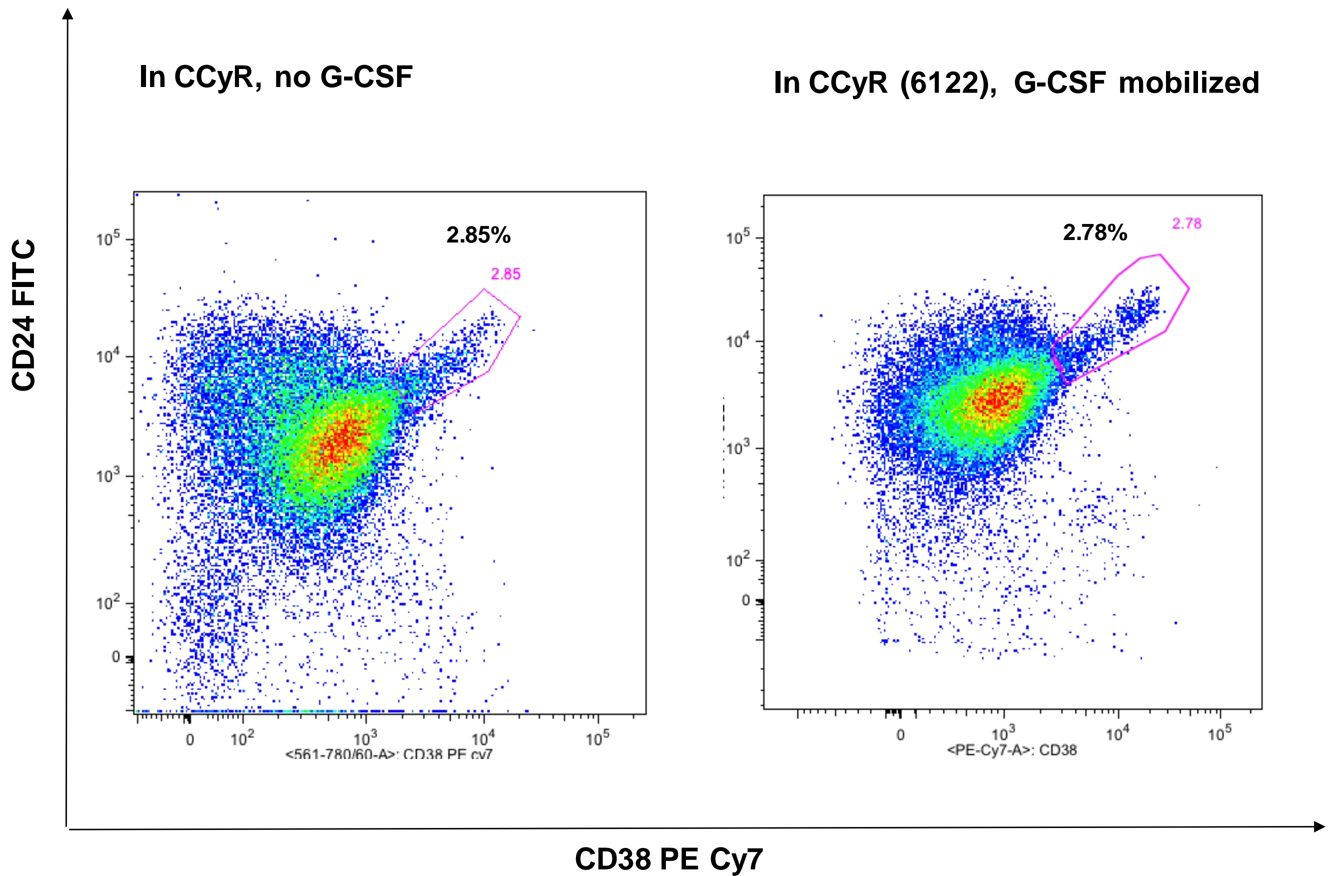


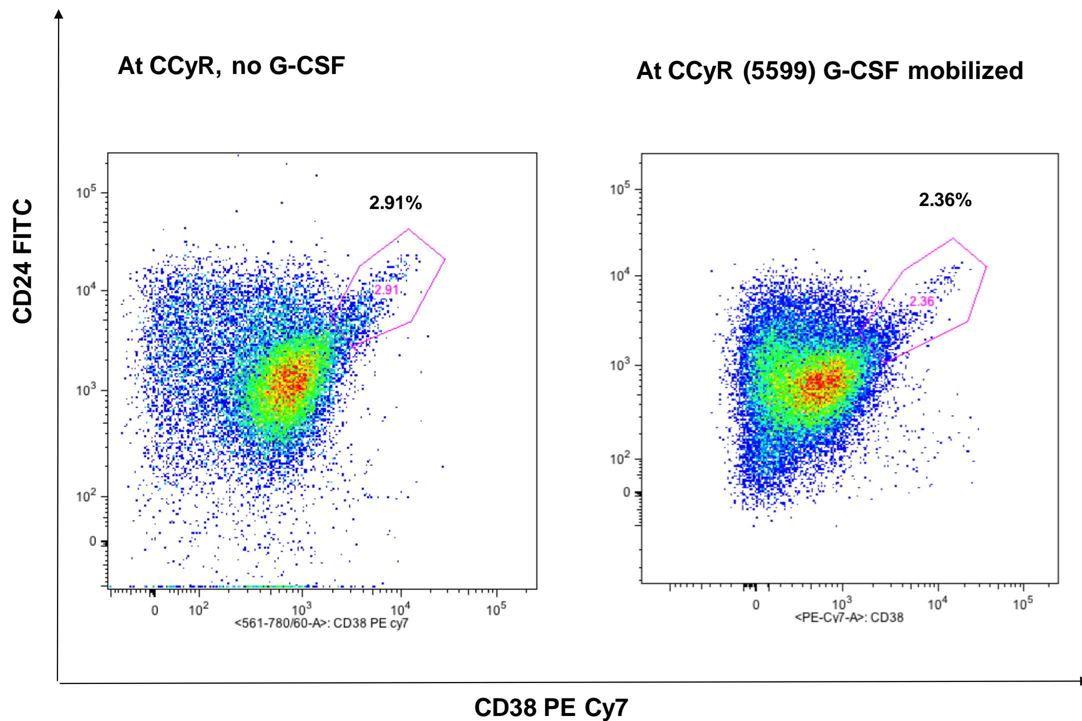
We next investigated if this difference could be attributed to the effect of G-CSF since the leukapheresis products from patients at CCyR were collected following G-CSF mobilisation. We compared the frequencies of B cell subsets in G-CSF mobilised leukapheresis products from 2 patients at CCyR and paired peripheral blood samples collected from the same patients a minimum of 12 months (12-36 months) following G-CSF therapy.

We found no significant impact of GCSF on the frequencies of transitional,

suggesting that the G-CSF may not be the underlying reason for the higher frequencies of transitional B cells observed at CCyR. Examples of FACS plots from two patients whose B cells were collected in the absence or presence of G-CSF are shown in Figure V.8.

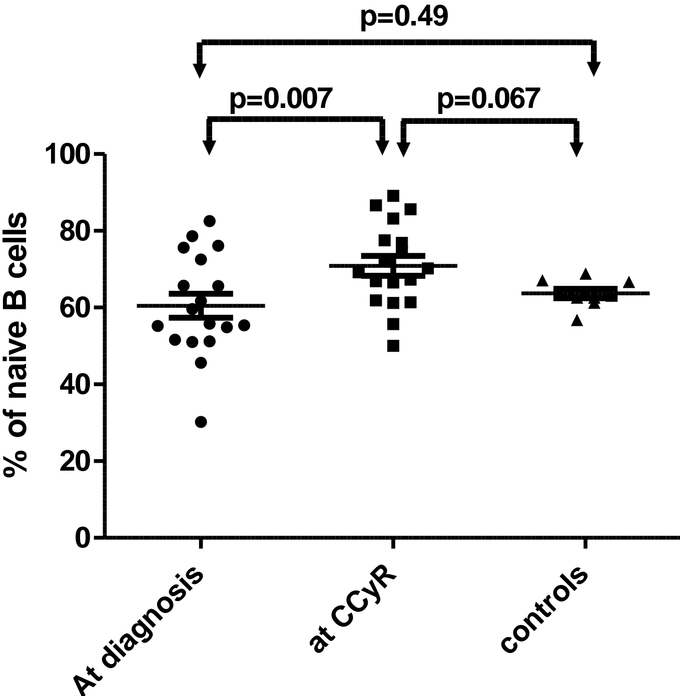
Figure V.7: B cell subsets with or without G-CSF mobilisation in two patients in CCyR (PBMCs)





We next compared the frequencies of naive B cells in paired samples collected from CML patients at diagnosis and following achievement of CCyR. Interestingly, we observed a small but significant increase in the percentage of naive B cells before and after imatinib (median 57.7% vs 69.7%, p=0.007 paired t test), Figure V.9. Of note there was a trend towards higher percentage of naive B cells in remission samples from CML patients compared to healthy controls (median 69.7% vs 63.1% respectively, mean 70.1% vs 63.7%, p=0.067), as shown on Figure V.9.

Figure V.8: Naive B cell subsets in paired samples from responders at diagnosis and in CCyR

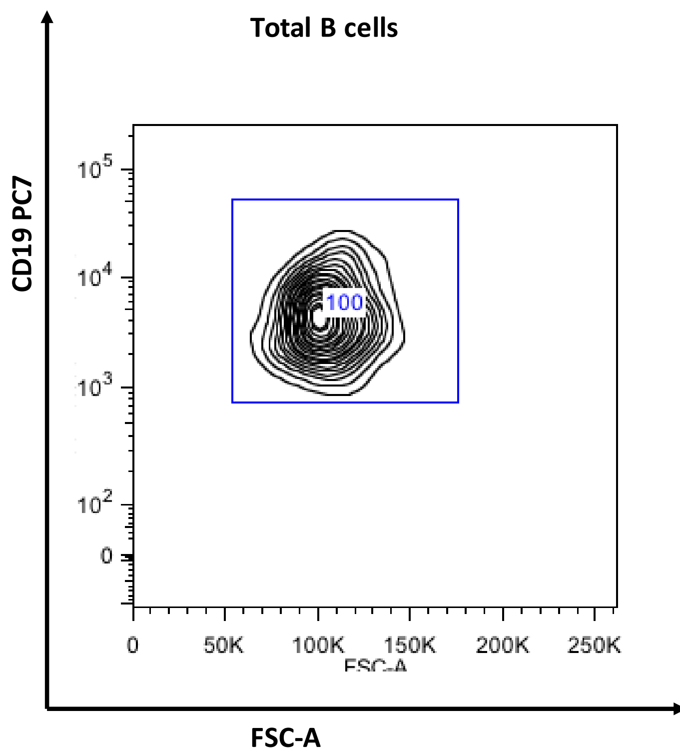


V.3.3 Purity of B cell subsets after FACS sorting

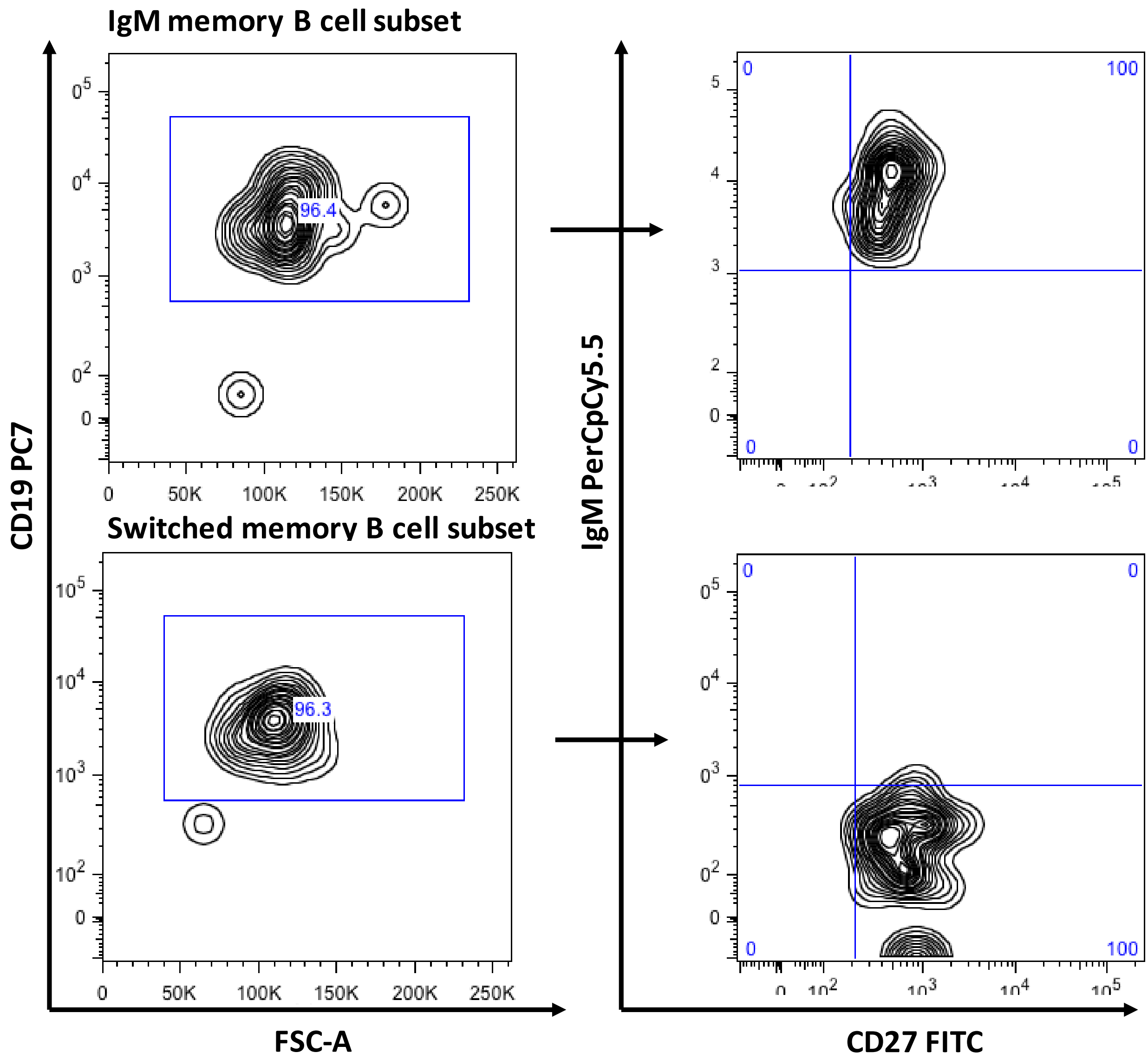
I next evaluated the purity of the B cell subsets after cell sorting on the FACS ARIA and before performing FISH analysis to look for the presence of BCR-ABL1 fusion gene. The median purity for CD19+ total B cells (out of live cells) was 99.4% (range 97% to 100%), while the median purity for IgM memory B cells, switched memory B cells, naive B cells and transitional B cells were 97.7% (range 86%-100%), 96.8% (range 96.6%-100%), 93.9% (range, 92.8%-96.8%) and 94.1% (range, 84.2%-100%) respectively. Examples of representative purity checks in 3 samples are shown in Figure V.10.

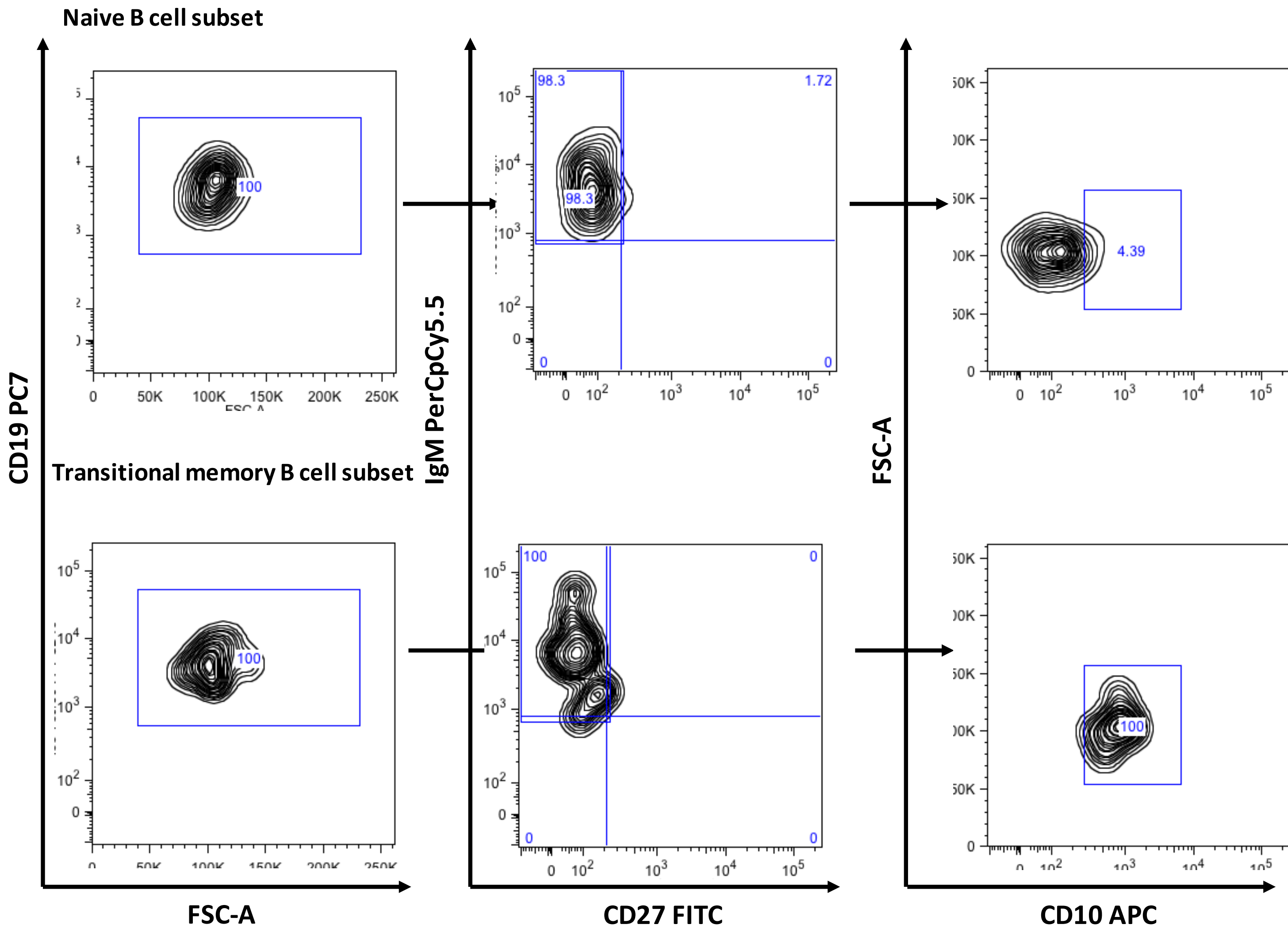
Figure V.9: Examples of representative purity checks in 3 samples

Purity check 5450

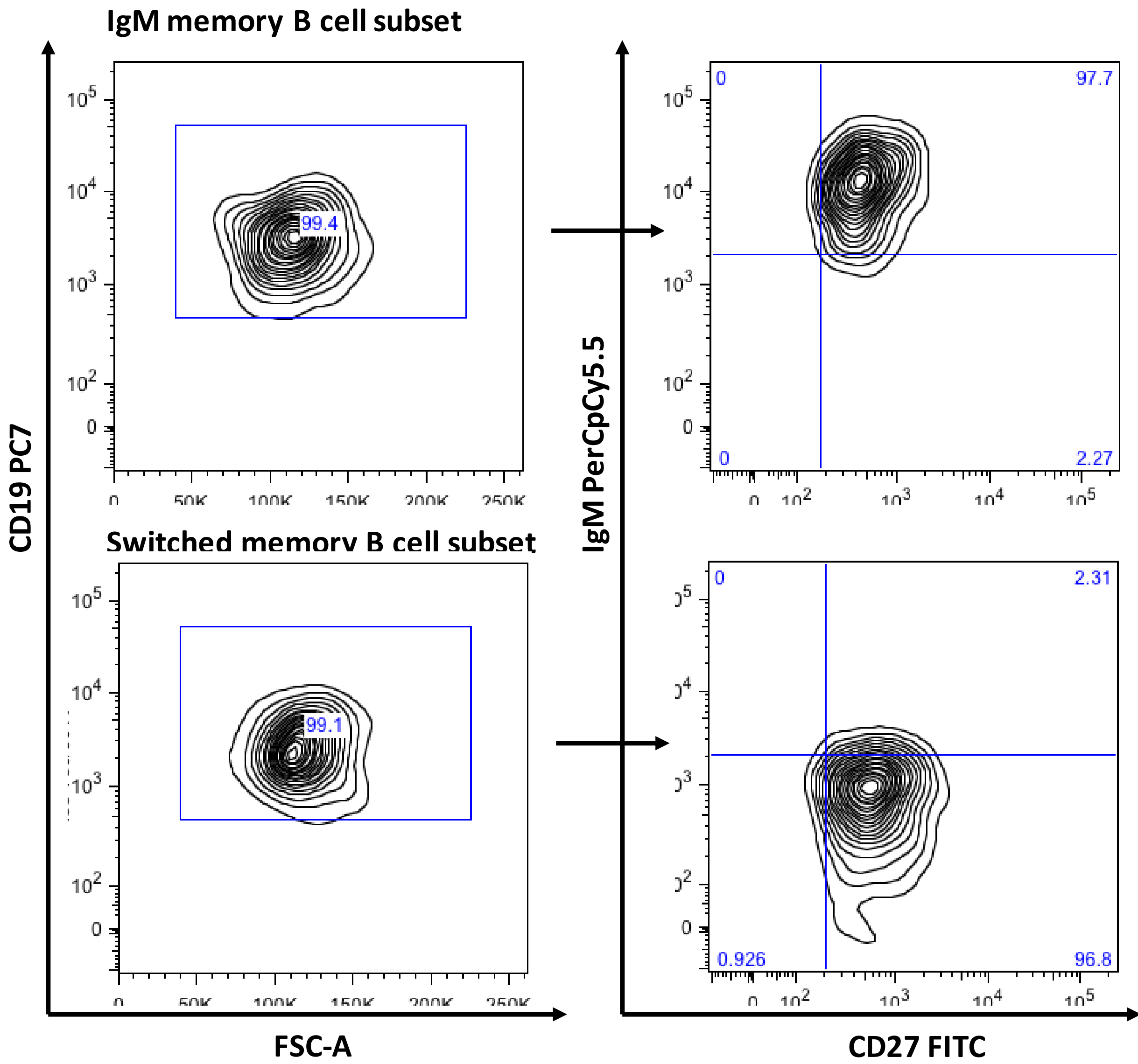


Purity check 5450

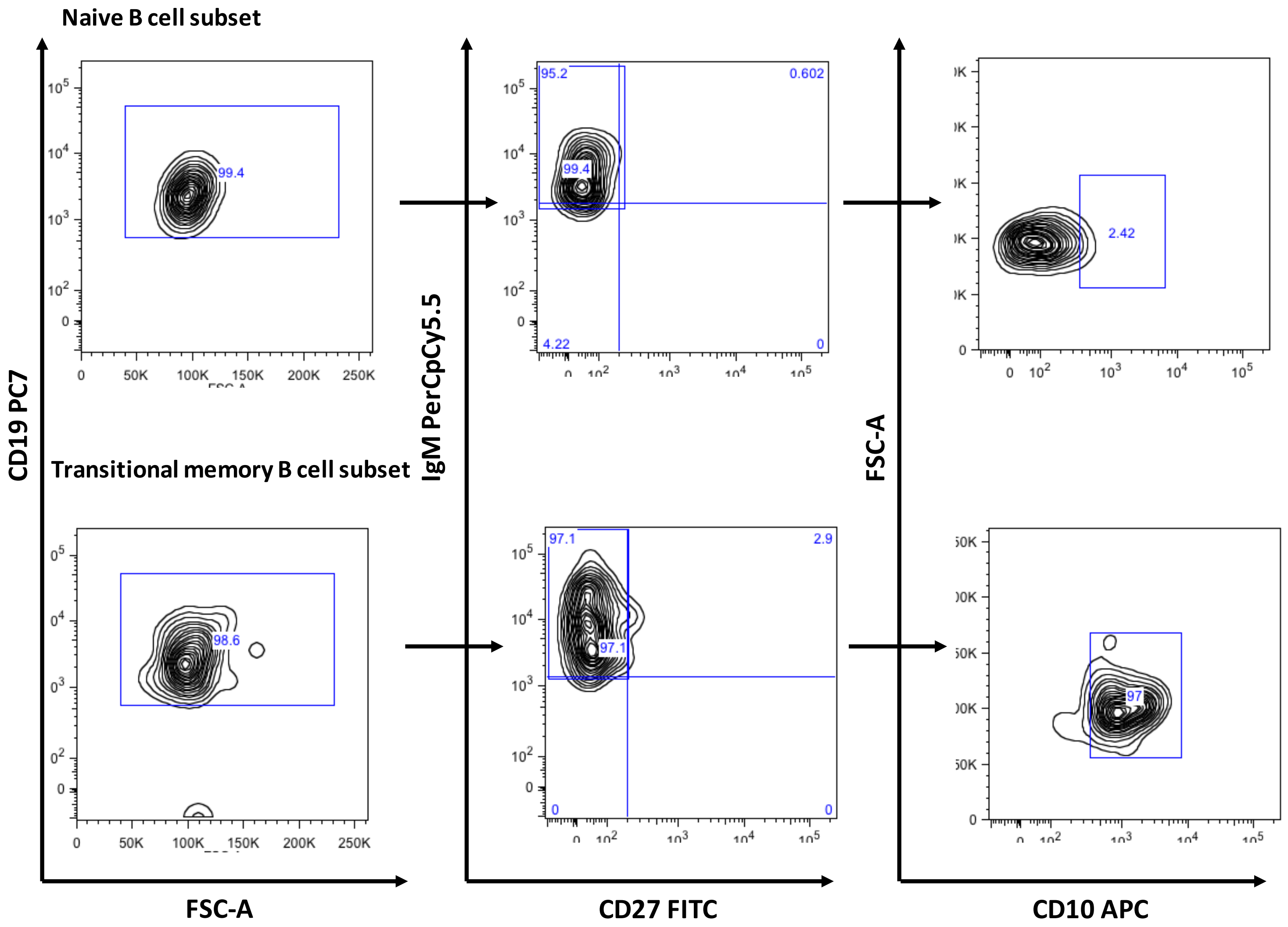


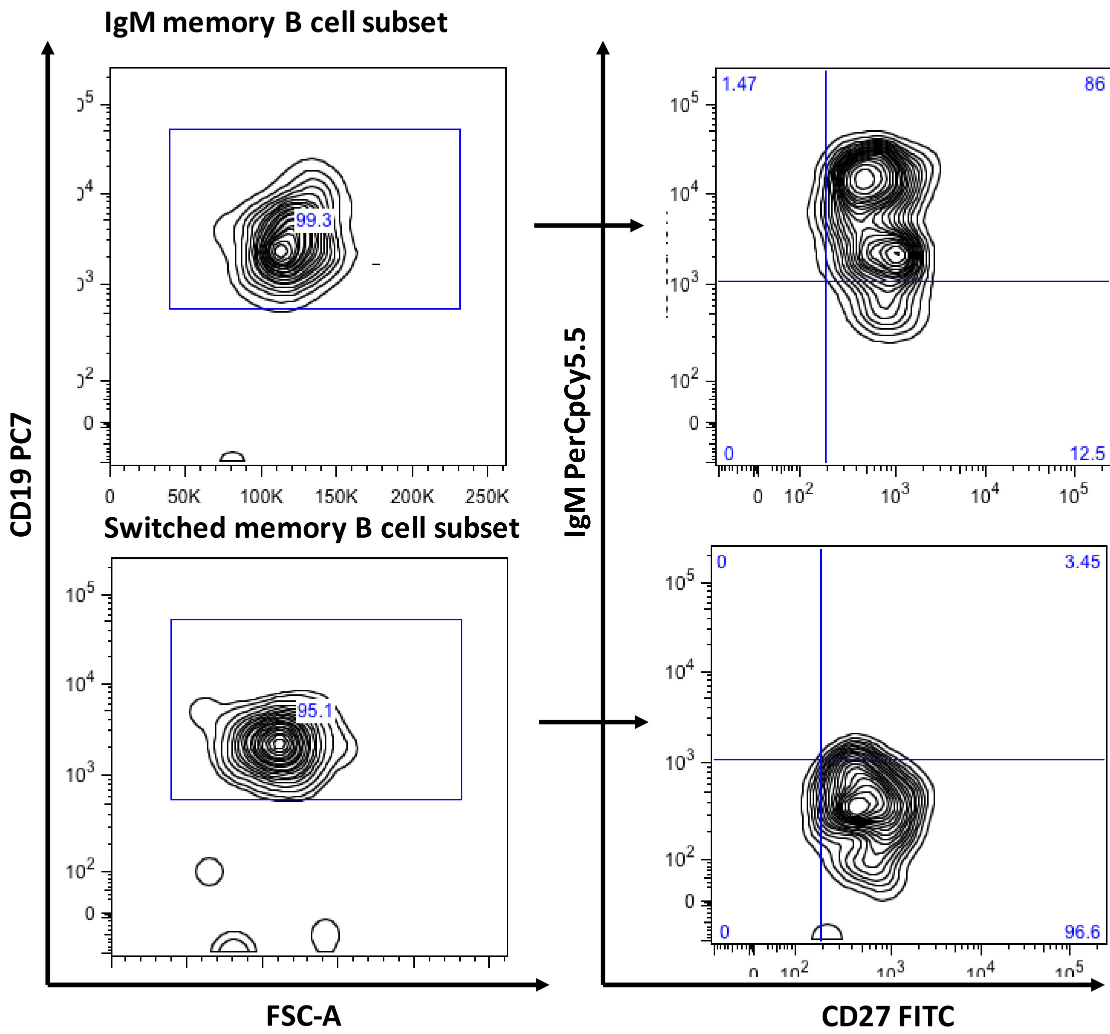


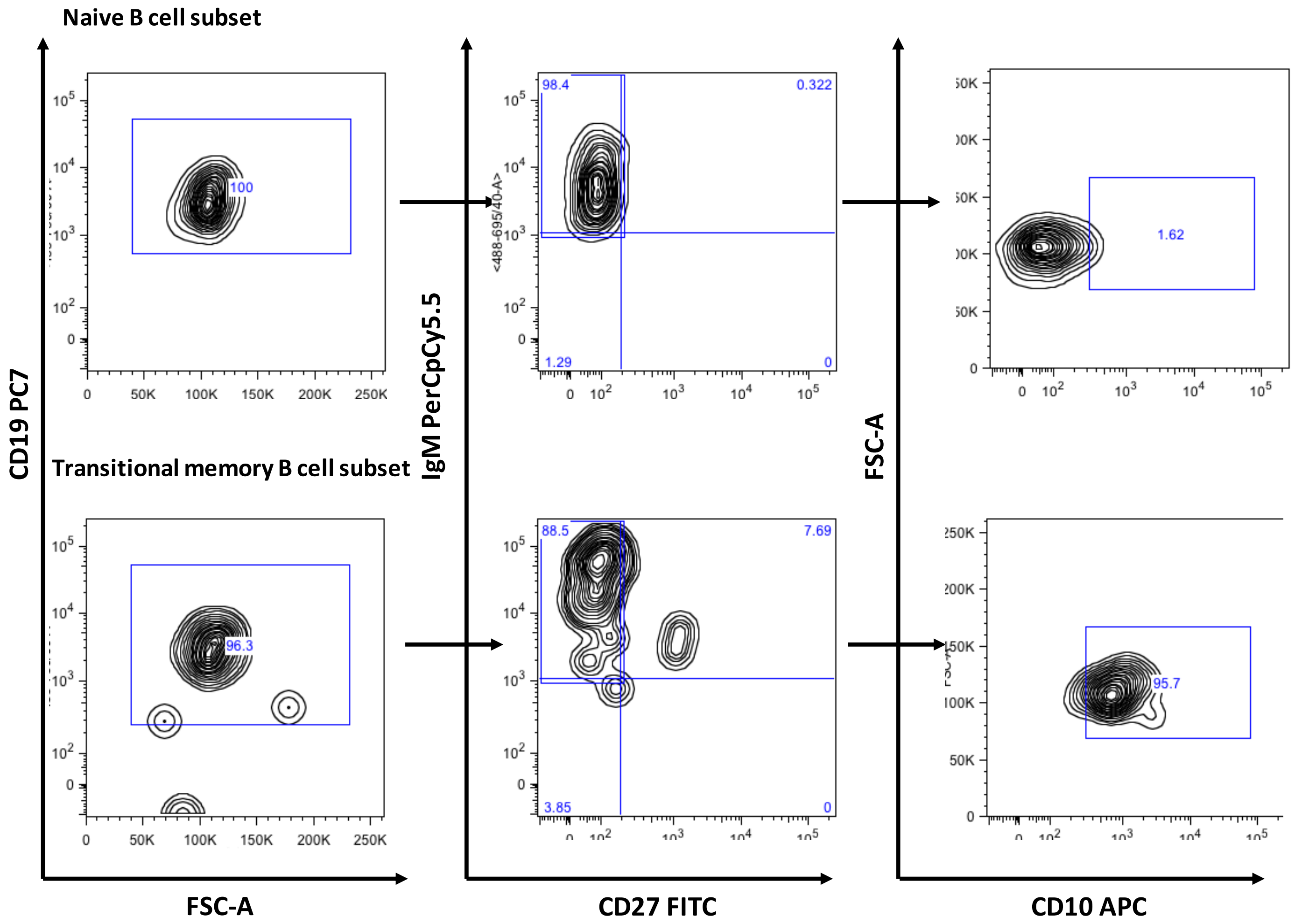
Purity check 5450
Naive B cell subset
Transitional memory B cell subset
CD19 PC7
IgM PerCpCy5.5
FSC-A
FSC-A
CD27 FITC
CD10 APC
100
98.3
1.72
98.3
0
0
4.39
100
100
0
0
0
100



Purity check 5494
IgM memory B cell subset
Switched memory B cell subset
CD19 PC7
IgM PerCpCy5.5
FSC-A
CD27 FITC
99.4
99.1
0
97.7
0
2.27
0
2.31
0.926
96.8


Purity check 5494
Naive B cell subset
Transitional memory B cell subset
CD19 PC7
IgM PerCpCy5.5
FSC-A
FSC-A
CD27 FITC
CD10 APC
99.4
95.2
0.602
99.4
4.22
0
2.42
98.6
97.1
2.9
97.1
0
0
97


Purity check 4997
IgM memory B cell subset
Switched memory B cell subset
CD19 PC7
IgM PerCpCy5.5
FSC-A
CD27 FITC
99.3
95.1
1.47
86
0
12.5
0
3.45
0
96.6
0
50K
100K
150K
200K
250K


Purity check 4997
Naive B cell subset
Transitional memory B cell subset
CD19 PC7
FSC-A
IgM PerCpCy5.5
CD27 FITC
FSC-A
CD10 APC
100
98.4
0.322
1.29
0
1.62
96.3
88.5
7.69
3.85
0
95.7

V.3.4 The presence of BCR-ABL positive B cells at diagnosis predicts for poor response to imatinib

Among the 39 chronic phase CML patients, FISH for BCR-ABL was performed in 26/39 patients, including paired samples from 12/25 patients in the responder group and in 14/14 samples in the non responder group.

The presence of BCR-ABL fusion gene was found in B cell subsets from 13/26 (50%) diagnostic samples. Approximately 5.5% (range, 1% to 27%) of B cells had evidence of BCR-ABL fusion gene by FISH, Table V.2. Among these13 cases, transitional B cells were involved in all (10/10) evaluable samples (median 53.5% of BCR-ABL+/ total transitional B cells, range 21% to 89%), Naive B cells in 9/12 (75%) evaluable samples (median 11%, range 4% to 29%), IgM memory B cells in 4/12 (33%) evaluable samples (median 11.5%, range 7% to 23%) and switched memory B cells in 5/13 (38%) evaluable patients (median 14%, range 4% to 25%), as shown in table V.2.

I then investigated if there was a correlation between BCR-ABL involvement of B cell subset and the B cell subsets frequencies. I found no significant difference in the frequencies of transitional B cells at diagnosis between patients with or without evidence of Ph+ lymphopoiesis (median 0.34% vs 0.34% respectively, mean 0.69% vs 0.50%, p=0.49, unpaired t test). Similarly there was no significant difference in the frequencies of naive B cells in the two groups (median 72% vs 60% respectively, mean 65% vs 60%, p=0.37, unpaired t test)

Table V.2: Percentage of BCR-ABL positivity in sorted B cell subsets from diagnostic samples collected from 13 CML patients with BCR-ABL positive B lymphopoiesis (3 responders and 10 non responders)

NA: not available

Patients	Total B cells	Transitional B cells	Switched memory B cells	Naive B cells	IgM memory B cells	Blastic transformation from diagnosis, Months, type
Responder, Sample 5450	6%	39%	20%	0%	0%	No
Responder, Sample 5086	NA	21%	25%	5%	23%	No
Responder Sample 4997	2%	42%	0%	0%	0%	No
Non responder Sample 6021	20%	NA	0%	29%	7%	No
Non responder Sample 4902	5%	69%	0%	11%	0%	24 Myeloid
Non responder Sample 6950	3%	NA	4%	7%	NA	No
Non responder Sample 5887	2%	80%	0%	4%	0%	8 Unknown
Non responder Sample 6144	6%	77%	0%	8%	0%	No
Non responder Sample 5337	13%	NA	14%	12%	13%	No
Non responder Sample 5394	27%	50%	0%	13%	0%	5 Lymphoid
Non responder Sample 3958	1%	57%	0%	NA	0%	35 Lymphoid
Non responder Sample 3298	1%	36%	0%	0%	0%	57 Unknown
Non responder Sample 4980	14%	89%	9%	16%	10%	22 Unknown

I next investigated the impact of BCR-ABL positive B cells at diagnosis on response to imatinib. Significantly higher proportion of non-responders had evidence of BCR-ABL positive B lymphopoiesis compared to responders (10/14 vs 3/12 respectively, p= 0.047, Fisher exact test), suggesting that the presence of BCR-ABL positive B cells at diagnosis may be a maker of poor response to imatinib. Among the 14 non-responders, 9/14 had secondary transformation to

blast crisis and 5/14 had primary resistance to imatinib but did not transform to accelerated phase. Of note 6/9 patients with secondary blast crisis and 2/5 patients with primary resistance had evidence of BCR-ABL positive B lymphopoiesis at diagnosis.

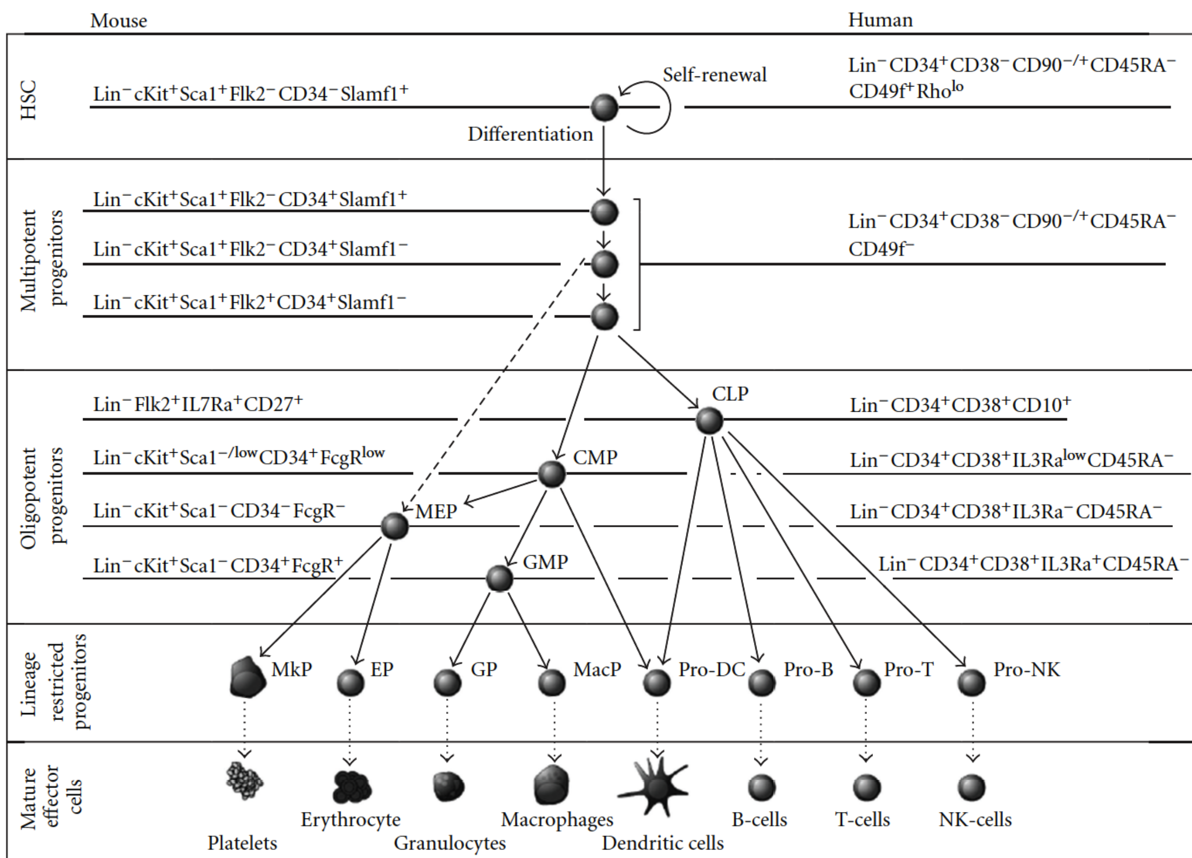
V.3.5 Imatinib eradicates BCR-ABL positive B cell clones in CML patients who achieve CCyR

Among the 3 patients who had BCR-ABL positive B cells at diagnosis and who subsequently achieved CCyR (samples 5450, 5086 and 4997, Table V.4.4), FISH for BCR-ABL at remission (CCyR) was available in 2/3 (paired samples from samples 5450 and 4997). There was no detectable BCR-ABL fusion gene in the B cells in the remission samples, suggesting that imatinib is capable of eradicating the BCR-ABL positive B cell clone.

V.3.6 BCR-ABL positive CD34+CD38+CD19+ and CD34+CD38+CD19- cells are detected in a patient with BCR-ABL positive B cells subsets

An additional panel was performed in one of the patients with BCR-ABL positive B cells (sample 5394, see Table V.3). This panel was designed to sort CD34+CD38- leukaemia stem cell and multipotent progenitor (MPP), CD34+CD38+CD19- common myeloid progenitor (CMP) and granulocyte-macrophage progenitor (GMP), and CD34+CD38+CD19+ common lymphoid progenitors (CLP), Figure V.11. The gating strategy is shown in Figure V.4.7. CD34+CD38- represented 1.7% of the MNCs while the CD34+CD38+CD19- and CD34+CD38+CD19+ subsets were 4.6% and 4% respectively.

Figure V.10: hierarchy of haematopoiesis



BCR-ABL by FISH was present in 100% of the CD34+CD38+CD19+ (CLP) and

CD34+CD38+CD19- (CMP and GMP) subsets, as shown in table V.12. Unfortunately the number of CD34+CD38- sorted cells was not sufficient to perform FISH analysis.

Figure V.11: Gating strategy for myeloid and lymphoid progenitors

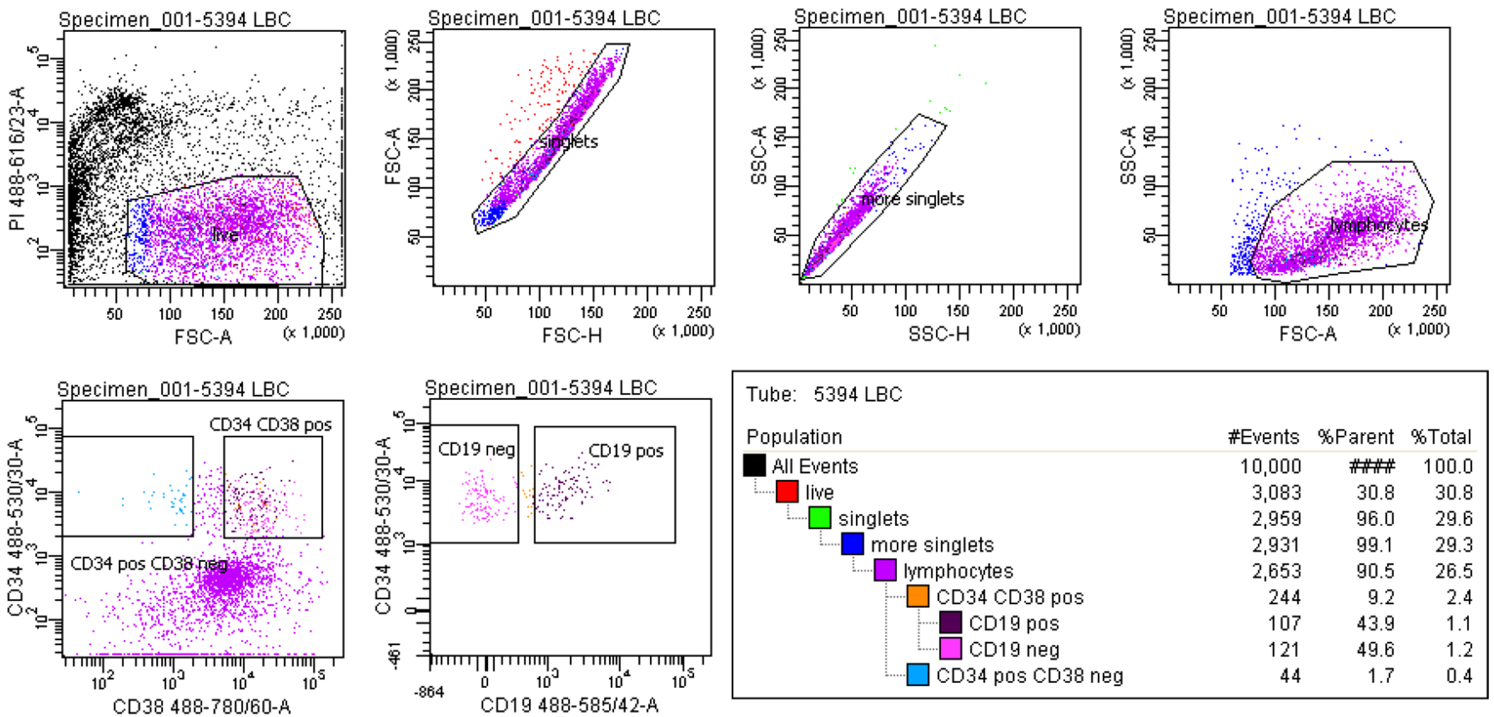


Table V.3: Frequencies of BCR-ABL positive cells (by FISH analysis) in B cell subsets in the diagnostic sample 5394

Total B cells	CD34+CD38+CD19-	CD34+CD38+CD19+	Transitional B cells	Switched memory B cells	Naive B cells	IgM memory B cells
27%	100%	100%	50%	0%	13%	0%

V-4 DISCUSSION

The first aim of this study was to perform a comprehensive analysis of B cell subsets in CML patients at presentation and, where possible, following achievement of CCyR on imatinib treatment. I found that transitional B cells are significantly lower in CML patients at diagnosis compared to healthy donors. There was no significant difference in the frequencies of transitional B cells in patients who failed to subsequently respond to imatinib (non-responders) compared to those who achieved CCyR (responders). Interestingly, I observed a partial normalisation of transitional B cell frequencies in patients who had a complete cytogenetic response to imatinib (median, 1.62% vs 0.38% at diagnosis, p<0.001).

In the setting of allogeneic haematopoietic stem cell transplantation (allo-SCT), transitional B cells have been shown to be a necessary developmental intermediate for mature B cell generation in humans. Indeed, after allo-SCT transitional B cells are the first B cell subset to reconstitute (Marie-Cardine *et al*, 2008). Similarly, it is possible that B cell lymphopoiesis is suppressed or restrained by the expansion of the myeloid CML clone and that eradication of the CML clone by TKI therapy may facilitate B cell reconstitution, including transitional B cell recovery. It is also possible that the increase in the transitional B cell frequencies at CCyR could be attributed to a direct effect of imatinib, although my previous study does not support this hypothesis, since I showed that TKIs, through their off target kinase inhibition, impair Btk activity and memory B cell response. Analysis of B cell subsets in paired samples from non responders (i.e. CML patients with significant residual disease after treatment with imatinib) might have provided further insights into the possible role of the CML clone in suppressing B lymphopoiesis;

unfortunately this study was not possible as paired samples from non-responders were not available.

Transitional B cell frequencies restored only partially following therapy and continued to remain significantly lower than normal levels, even in patients who achieved CCyR (median 1.62% vs 3.18% in healthy controls respectively, p=0.013). The failure of transitional B cell normalisation may at least be partly related to the off target inhibition of Btk in this subset. This supposition is further supported by my observation, summarised in Chapter 4, that imatinib inhibits Btk phosphorylation not only in IgM and switched memory B cells but also in CD27+IgM+ B cells (naive and transitional B cells), as shown in Figure IV.16 (Chapter IV), although I did not specifically investigate the effect of imatinib on Btk in transitional (CD10+CD27+IgM+) compared to naïve B cells (CD10-CD27+IgM+). Finally I also found a small but significant increase in the percentage of naive B cells after imatinib therapy (median 69.7%, vs. 57.7% prior to therapy p=0.007 paired t test). One possible explanation could be that B cell reconstitution in CML patients on TKI therapy mirrors B cell recovery after allo-SCT, where transitional B cell frequencies decrease secondarily, while the mature naive B cell compartment expands at around 9 months post allo-SCT (Marie-Cardine *et al*, 2008). In our study the median interval between collection of the diagnostic samples and the remission samples was 25 months (range, 15 to 56 months). It is therefore possible that by 25 months post TKI therapy, transitional B cells have already started declining in numbers while the naive B cell compartment is recovering. It is however also possible that imatinib may exert differential effect on transitional and naive B cell recovery.

I next looked for the presence of Philadelphia positive B lymphopoiesis at diagnosis and its implications on disease response. Although in all patients with Ph+ B cells, transitional B cells were involved, I found no difference in the frequencies of transitional B cells in patients with or without Ph+ B lymphopoiesis. Interestingly in patients with Ph+ B lymphopoiesis, transitional and naive B cell were BCR-ABL+ cells by FISH in 10/10 and 9/12 evaluable samples respectively compared to only 4/12 and 5/13 evaluable samples for IgM memory and switched memory B cell subsets. This indicates that BCR-ABL is present in the most immature B cell subsets, with possibly a maturation arrest. Although other groups have looked into the presence of a Philadelphia chromosome in B cell progenitors (Takahashi *et al*, 1998) or total B cells (Primo *et al*, 2006), my study is the first to selectively look at the presence of the Philadelphia chromosome in the different B cell subsets. Primo reported the presence of BCR-ABL positive B cells in 43% of CML patients, which is comparable to the 50% I found in my study (Table V.4.shows the clinical characteristics of patients reported in the study by Primo and colleagues).

Table V.4: patients characteristics of the study reported by Primo and colleagues (Primo *et al*, 2006)

Table I. Chronic myeloid leukaemia patients ($n = 15$): clinical and laboratory data at the moment of entering this study together with the distribution of *BCR/ABL*$^+$ cells within the different cell subpopulations.

Case no.	Age (years)	Sex	Stage of the disease	Months after diagnosis	Therapy	Sample	BCR/ABL transcript	FISH pattern	% of BCR/ABL$^+$ cells* Whole sample	CD34$^+$ HPC	Neutrophils	Monocytic cells	Eosinophils	Erythroblasts	B-cells	T-cells	NK-cells
1	25	Female	Diagnosis	0	None	BM	p190	t(9;22), mBCR	94	NA‡	NA	NA	NA	NA	80	0	81
2	71	Male	Diagnosis	0	None	BM	p190	t(9;22), mBCR	95	NA	NA	NA	NA	NA	45	0	77
3	63	Female	Chronic phase	28	Imatinib	BM	p190	t(9;22),mBCR	60	75	90	91	92	89	22	0	51
4	45	Male	Diagnosis	0	None	BM	p210	t(9;22), MBCR	95	NA	99	95	NA	NA	0	0	NA
5	70	Male	Diagnosis	0	None	BM	p210	t(9;22), MBCR	93	97	99	93	97	98	0	0	0
6	55	Female	Diagnosis	0	None	PB	p210	t(9;22), MBCR	94	82	100	98	96	NA	0	0	0
7	57	Female	Diagnosis	0	None	BM	p210	t(9;22), MBCR	96	NA	NA	NA	NA	NA	0	0	0
8	45	Male	Diagnosis	0	None	BM	p210	t(9;22), MBCR, del(9q$^+$)	94	NA	NA	NA	NA	NA	0	0	0
9	63	Male	Diagnosis	0	None	BM	p210	t(9;22), MBCR, del(9q$^+$)	95	NA†	100	95	96	84	NA	NA	NA
10	70	Male	Diagnosis	0	None	PB	p210	t(9;22), MBCR	95	NA	NA	NA	NA	NA	35	0	0
11	75	Female	Chronic phase	42	Imatinib	BM	p210	t(9;22), MBCR	22	95	24	7	10	28	0	0	0
12	75	Female	Chronic phase	NA	HU	BM	p210	t(9;22), MBCR	85	95	95	98	96	97	46	0	0
13	74	Female	Chronic phase	44	Imatinib	BM	p210	t(9;22), MBCR	60	89§	NA	NA	NA	NA	0	49	70
14	39	Male	Myeloid blast crisis	30	Imatinib	BM	p210	t(9;22), +Ph, MBCR	72	100	99	98	95	64	0	0	0
15	55	Male	Lymphoid blast crisis	37	Imatinib	PB	p210	t(9;22), +Ph, MBCR	60	90	0	0	0	NA	97	0	0

NA, not analysed; BM, bone marrow; PB, peripheral blood; HU, hydroxyurea; HPC, haematopoietic stem and progenitor cells.

*Results expressed as percentage of *BCR/ABL*$^+$ nuclei by interphase FISH from the whole sample studied or from each FACS-purified cell population.

†Percentage of *BCR/ABL*$^+$ CD7$^+$ /CD33$^+$ /CD34$^+$ cells of 98% and % of *BCR/ABL*$^+$ CD19$^+$ /CD34$^+$ cells of 8%.

‡Percentage of *BCR/ABL*$^+$ CD19$^+$ /CD34$^+$ cells of 96%.

§Percentage of *BCR/ABL*$^+$ CD7$^+$ /CD33$^+$ /CD34$^+$ cells of 93% and % of *BCR/ABL*$^+$ CD19$^+$ /CD34$^+$ cells of 0%.

Involvement of the neutrophil, monocytic and eosinophilic lineages was systematically observed, except in case 15 who relapsed with a B-cell precursor acute lymphoblastic leukaemia.

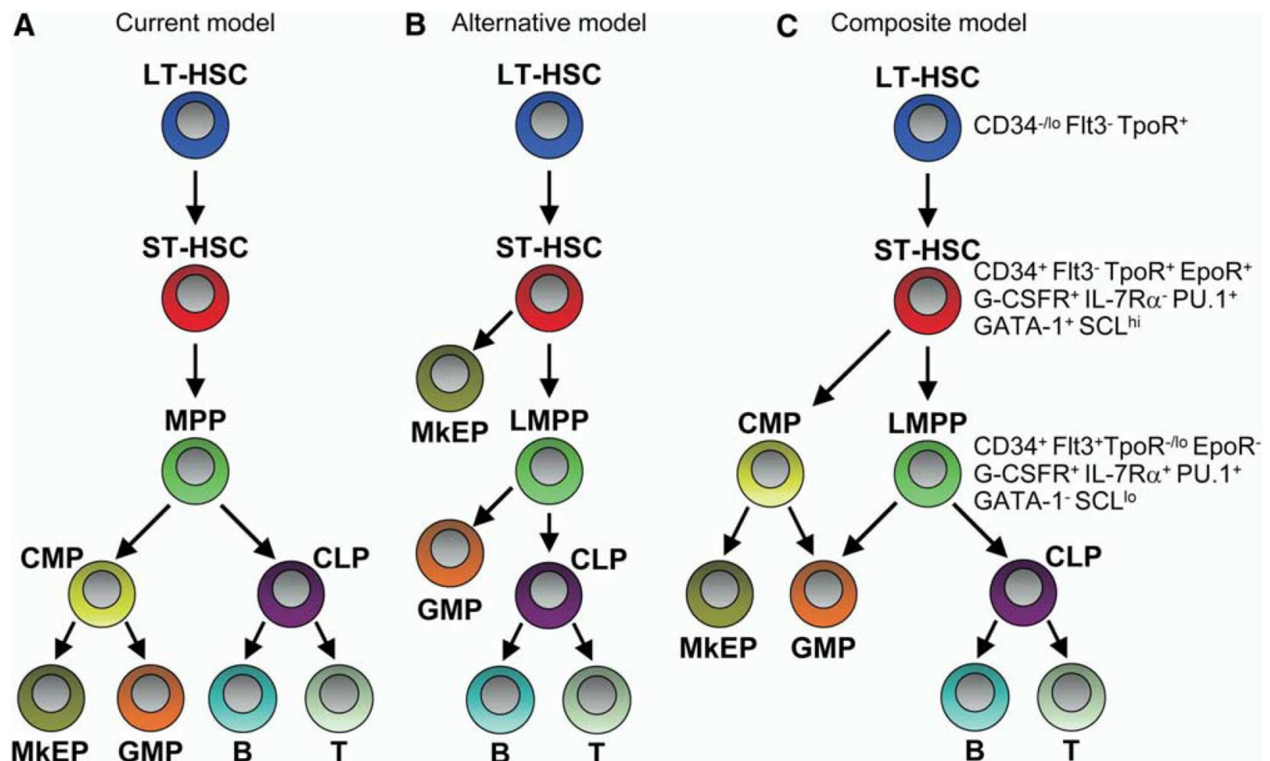
Note that in all CML cases with p190 transcripts both B- and NK-cells were always *BCR/ABL*$^+$ positive, while M*BCR/ABL*$^+$ cases only showed B- and NK-cell lineage involvement in 27% and 10% of the cases, respectively.

Notably, the presence of Ph+ B lymphopoiesis was a poor prognostic marker. A significantly higher proportion of patients who failed to respond to imatinib (non-responders) had Ph+ B cell involvement compared to the responder group (10/14 vs 3/12 respectively, p= 0.047, Fisher exact test). To my knowledge, this is the first study demonstrating that the presence of BCR-ABL positive B lymphopoiesis at diagnosis predicts for poor response to imatinib. Although this study was not originally designed to look at the B cell progenitors, BCR-ABL was found by FISH in 100% of the CD34+CD38+CD19+ common lymphoid progenitors (CLP) and CD34+CD38+CD19- (common myeloid progenitor, CMP and granulocyte-macrophage progenitor, GMP) subsets in one evaluable patient. One possible explanation for the observation that patients with Ph+ B lymphopoiesis have a worse prognosis may be related to a relative insensitivity of the BCR-ABL positive B cell clone to imatinib. However, my data do not support this hypothesis as in the two patients with Ph+ B cells who subsequently responded to imatinib, BCR-ABL positive B cell subsets were no longer detectable at CCyR. However it is still possible that B cell subsets may have different levels of sensitivity to imatinib depending on the disease biology.

Another possible explanation may be that the presence of BCR-ABL positive B-cells could be a marker of a downstream progenitor with self-renewal capacity. Although haematopoietic stem cells (HSC) are the only normal progenitors that renew themselves and are therefore considered to be the only cells in the marrow in which preleukaemic changes can accumulate, it is also possible that a downstream progenitor can acquire self-renewal capacity. There is growing evidence that whereas leukaemic progression could occur in self-renewing clones

in the HSC compartment, frank AML and blast crisis CML occur also in their clonal progeny at a non-HSC stage. Indeed it has been shown that the leukaemic stem cells (LSCs) giving rise to blast crisis CML are granulocyte-macrophage progenitor (GMP)-like (Jamieson *et al*, 2004), challenging the notion that LSCs reside solely in the CD34+CD38- compartment. More recently Goardon and colleagues have added another candidate LSC, a lymphoid-primed multipotential progenitor (LMPP)-like LSC coexisting with a GMP-like LSC (Goardon *et al*, 2011). Building on previous models of normal HSC development, they found that the LMPP-like and GMP-like LSCs were hierarchically organised, whereby the LMPP-like cells gave rise to the GMP-like cells. Alternative models for normal HSC are shown in Figure V.13.

Figure V.12: current and alternative models for HSC commitment (Adolfsson *et al*, 2005)
LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage pronenitor; MkEP, megakaryocyte-erythroid progenitor; B, B cell; T, T cell.



The authors, through the investigation of a large number of AML patient samples found two predominant populations within CD34+ cells, a Lin-CD34+CD38-CD90-CD45RA+ LMPP-like population (CD38-CD45RA+) and a Lin-CD34+CD38+CD123+CD110-CD45RA+ GMP-like population. Both of these populations possessed LSC activity (Figure V.14). This previously uncharacterised LMPP-like population was shown to be able to produce in vitro granulocytes, monocytes/macrophages and lymphocytes, but not erythroid cells or megakaryocytes. LMPP-like cells gave rise to GMP-like cells but not vice versa. Figure V.15 shows the phenotype of the different populations and their ontogeny based on Goardon's findings. These results implicate normal hematopoietic progenitors, LMPP, and/or GMP as the cell of origin for AML LSC in the majority of cases. Our data would be consistent with a similar hypothesis in CML whereby a LMPP-like LSC would be the candidate LSC giving rise to both a GMP-like LSC (as reported by Jamiesson and colleagues (Jamieson *et al*, 2004)) and B cell subsets (through a common lymphoid progenitor). Our data suggest that the presence of BCR-ABL positive B cells may be a marker for the presence of an LMPP-like LSC in CML, from which a GMP-like and blast crisis CML could derive.

In keeping with this hypothesis, work on acute lymphoblastic leukaemia (ALL) using NOD/SCID mice have demonstrated that the CD34+/CD38-/CD19+ as well as CD34+/CD38+/CD19+ cells are leukaemia-initiating cells in human B-precursor ALL (Kong *et al*, 2008). Moreover le Viseur and colleagues described that the leukaemia-initiating ability of standard-risk ALL was found in both the CD34+CD19+ and CD34-CD19+ populations, and that the CD34-CD19+ population was able to serially transplant leukaemia in high-risk ALL (le *et al*,

2008). These data raise the intriguing possibility that a more lineage-committed B cell subset could give rise to a LSC in CML, as also suggested in a recent study in philadelphia positive ALL or blast crisis CML (Tanizaki *et al*, 2010).

Figure V.13: Coexistence of LMPP-like and GMP-like Leukaemia Stem Cells in Acute Myeloid Leukaemia (Goardon *et al*, 2011)

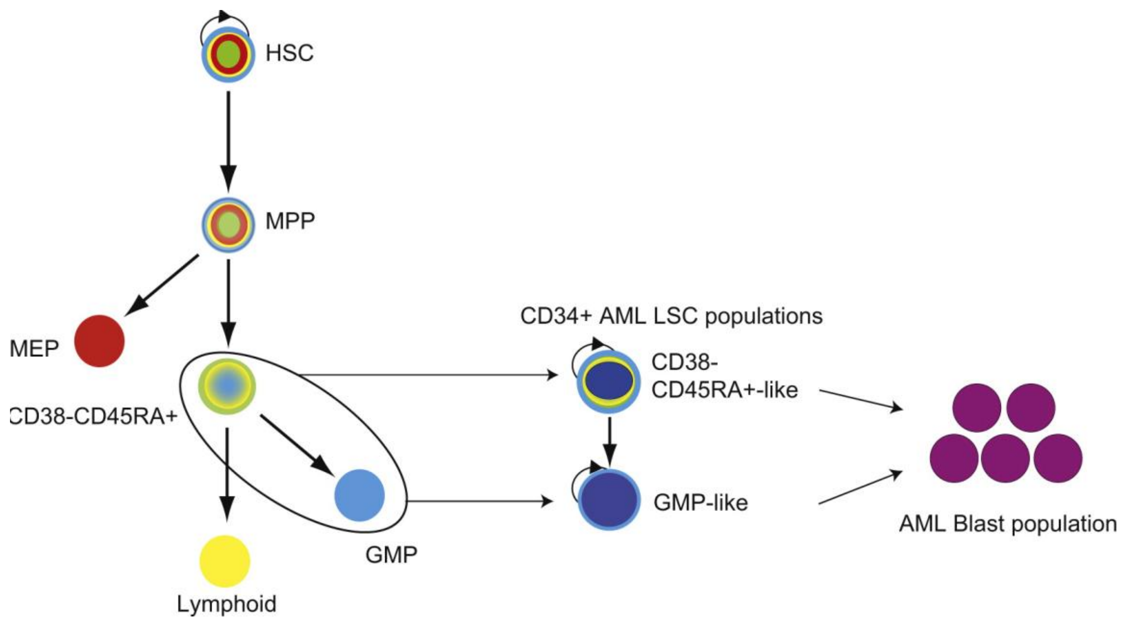
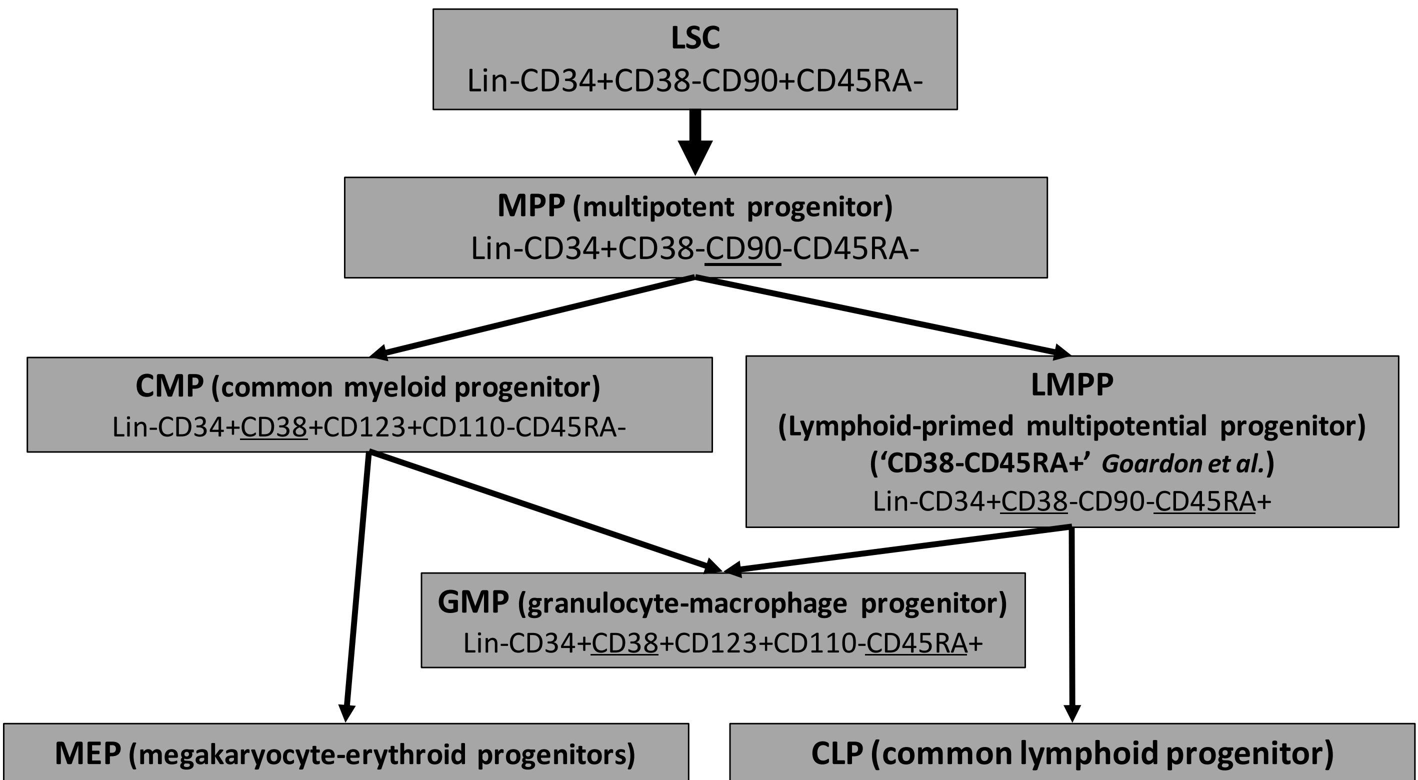


Figure V.14: Model for LSC commitment in AML and CML



CHAPTER 6 CONCLUSIONS AND FUTURE WORK

VI.1 CONCLUSIONS

Because of their lower toxicity and impressive efficacy, tyrosine kinase inhibitors (TKIs), notably imatinib, have replaced allo-SCT as first-line therapy for CML. Although over 85% of imatinib-treated patients with chronic phase CML (CML-CP) achieve a complete cytogenetic response (CCyR), the majority of patients have persisting molecular disease as assessed by q-PCR for BCR-ABL transcripts. Functional leukaemic CD34+ progenitor cells have been identified in such patients in CCyR, suggesting the presence of a reservoir of leukaemic cells resistant to TKIs. Therefore future developments in CML therapy seem likely to combine different approaches to integrate and reinforce the patient's immune response against residual leukaemic stem cells. The immunological GVL effect seen after allo-SCT suggests that an approach based on the amplification of the patient's own immune response to the disease might increase the depth of the responses seen after treatment with TKIs. One such approach would be to vaccinate the patient with leukaemia derived antigens. I hypothesised that vaccination against leukaemia antigens will be most effective once a state of minimal disease is achieved on imatinib.

Because previous studies have documented conflicting effects of TKIs on the immune response, ranging from impaired antigen-specific T-cell responses to enhanced stimulation of tolerant T cells, I aimed to determine the in vivo immunomodulatory effects of TKIs on T and B cell immune responses to antigens.

The specific aims of this thesis were:

1. To determine the in vivo effect of TKI on T and B cell responses in patients with CML on TKI. To address this question, I assessed the cellular and humoral immune response to a novel pandemic 2009 H1N1 vaccine in CML-CP patients on TKI, compared to patients with other haematological malignancies and healthy controls
2. To determine if TKI have a differential effect on IgM and IgG humoral responses to antigen. This goal was achieved by assessing T-cell and B-cell responses to vaccination against seasonal influenza virus and a polysaccharide pneumococcal vaccine in CML patients treated with tyrosine kinase inhibitors compared to healthy controls
3. To understand the potential mechanisms responsible for the immunomodulatory effect of TKIs on B cells by examining their impact on B cell receptor downstream pathway
4. To investigate any possible impact of Ph+ B lymphopoiesis on normal B cell function by comparing B cell responses to vaccination in CML patients with or without leukaemic B cell clones and to determine the effect of TKI on different B cell subsets

The achievements of this thesis can be summarised as follows. The purpose of chapter 3 was to determine the in vivo cellular and humoral immune responses to H1N1 influenza vaccination in CML-CP patients on TKI, compared to patients with other haematological malignancies and healthy controls. I found that the B cell responses to H1N1 vaccine was significantly better in patients with CML compared to patients with other haematological malignancies and was not significantly

different from healthy controls. We did not find a significant difference in the proportion of individuals with H1N1 specific T-cell response in CML patients and healthy controls. However this study did not address differences in the quality of the T cell response between CML patients and healthy controls and the differential impact of TKI on IgM and IgG humoral responses.

In chapter 4 I performed a more comprehensive analysis of T and B cell responses to vaccination with influenza and Pneumococcus in 51 CML-CP on imatinib, dasatinib or nilotinib compared to 24 healthy controls. The T cell response to influenza was defined both quantitatively and qualitatively, including analysis of the cytokine profile and effector function of vaccine-induced T cells responses. I did not find a significant difference in the T cell response to influenza vaccine in patients with CML on TKI and healthy controls. Furthermore, vaccine-induced T cells in patients with CML-CP on TKIs were functional and capable of producing effector cytokines as well as cytotoxicity, as assessed by CD107a degranulation when stimulated with the relevant antigen in vitro.

I also studied the B cell response to vaccination with Pneumococcus in more details, including titres of pneumococcal IgM and IgG antibodies and frequencies of IgM memory and switched memory B cells. I found that following vaccination, CML patients on TKI had significant impairment in their IgM humoral response to pneumococcus compared to controls. The impairment in IgM humoral response was associated with significantly lower frequencies of peripheral blood IgM memory B cells. To elucidate whether CML itself or treatment with TKI was responsible for the impaired humoral response, I assessed memory B-cell subsets in paired samples collected before and after imatinib therapy. Treatment with imatinib was

associated with significant reductions in IgM memory B-cells. To further elucidate the underlying mechanism for this observation, I studied the off target effect of TKI on kinases important in B cell activation and survival. In vitro co-incubation of B-cells with plasma from CML patients on TKI or with imatinib, dasatinib or nilotinib induced significant and dose-dependent inhibition of Bruton's tyrosine kinase and indirectly its downstream substrate, phospholipase-C-γ2, both important in B cell signalling and survival. I concluded that treatment with TKIs is associated with loss of memory B cell subsets and impaired humoral immune responses to pneumococcal polysaccharide vaccine, likely driven by the off-target kinase inhibitory activity of these drugs.

The impaired humoral response to vaccination and loss of memory B cell subsets observed in patients on TKI may also be a consequence of CML itself, through BCR-ABL involvement in the B cell lineage. In chapter 5, I further explored the effect of TKI on different B-cell subsets and the implications of Philadelphia positive lymphopoiesis on B cell function. I performed a comprehensive analysis of B cell subsets in paired frozen samples obtained from CML-CP patients at diagnosis and after the achievement of a CCyR on imatinib, as well as in samples collected from CML patients at diagnosis who subsequently failed to respond to imatinib. I found that CML patients at diagnosis have significantly lower frequencies of transitional B cells compared to healthy donors, and that following achievement of CCyR, there is a partial normalisation of transitional B cell frequencies. These data suggest that B cell lymphopoiesis may be suppressed or restrained by the expansion of the myeloid CML clone and that eradication of the CML clone by TKI therapy may facilitate B cell reconstitution, including transitional B cell recovery. The failure of

complete normalisation of transitional B cells at CCyR may be partly related to the off target inhibition of Btk in transitional B cells, as shown in chapter 4. Next, I looked for the presence of Philadelphia positive B lymphopoiesis in samples from CML patients at diagnosis. I found evidence of Ph+ B lymphopoiesis in 43% of CML patients at diagnosis. I then sort purified different B cell subsets and evaluated for evidence of BCR-ABL involvement by FISH analysis. I observed that nearly all patients with Ph+ B lymphopoiesis had BCR-ABL+ transitional and naive B cells. In contrast, only a minority of patients exhibited BCR-ABL+ IgM memory and switched memory B cells. These results suggest that Ph+ involvement of the B lymphoid lineage may result in maturation arrest. I found no difference in the frequencies of transitional B cells in patients with or without Ph+ B lymphopoiesis.

Overall BCR-ABL positive B cells were present in 43% of CML patients at diagnosis. Notably, the presence of Ph+ B lymphopoiesis was a poor prognostic marker as a significantly higher proportion of patients who failed to respond to imatinib (non-responders) had Ph+ B cell involvement compared to the responder group. In the two patients with Ph+ B cells who subsequently responded to imatinib, BCR-ABL positive B cell subsets were no longer detectable at CCyR, suggesting that the BCR-ABL positive B cell clone is sensitive to imatinib. The observation that patients with Ph+ B lymphopoiesis appear to have a worse prognosis is intriguing. BCR-ABL positive B cell involvement may indicate the presence of a downstream progenitor with self-renewal capacity, such as a lymphoid-primed multipotential progenitor (LMPP)-like subset that was recently described in acute myeloid leukaemia (Goardon *et al*, 2011). Our data support a similar hypothesis in CML, whereby a LMPP-like BCR-ABL+ LSC may give rise to both a GMP-like LSC (as reported by Jamiesson and colleagues (Jamieson *et al*,

2004)) and a common lymphoid progenitor resulting in Ph+ B lymphopoiesis. These data suggest that BCR-ABL positive B cells may be a surrogate for the presence of an LMPP-like LSC, which could potentially result in a GMP-like driven disease progression and blast crisis CML (Jamieson *et al*, 2004).

In summary these results indicate that CML patients in CCyR on TKIs can mount a normal T-cell response to vaccination, suggesting that in these patients, concomitant treatment with TKI may not hamper a T-cell response to LAA vaccination. Therefore the combined application of vaccination with LAA with TKI in chronic myeloid leukaemia may be a successful immunotherapeutic approach to improve the rate of CCyR. However TKIs induce significant functional alterations in B cell subsets in a subset of CML patients on TKI, likely related to their off-target tyrosine kinase inhibition. While the presence of Ph+ B cells at diagnosis predicts for worse prognosis, Ph+ B lymphopoiesis does not appear to correlate with impaired humoral immunity. Our finding that BCR-ABL positive B cell involvement is predictive for poor response to imatinib may indicate the presence of an LMPP-like LSC in these patients and warrants further studies.

VI.2 FUTURE WORK

To further confirm my hypothesis that BCR-ABL+ B cells may indicate the presence of a LMPP-like LSC, I propose to perform a comprehensive analysis of myeloid and lymphoid B cell progenitors and stem cells in samples from patients with and without Ph+ B cell involvement.

To that end, I will sort purify the following progenitors, based on the surface markers outlined below, for BCR-ABL FISH, DNA and RNA analysis:

- LSC: Lin-CD34+CD38-CD90+CD45RA-
- MPP: Lin-CD34+CD38-CD90-CD45RA-
- LMPP: Lin-CD34+CD38-CD90-CD45RA+
- CMP: Lin-CD34+CD38+CD123+CD110-CD45RA-
- GMP: Lin-CD34+CD38+CD123+CD110-CD45RA+
- CLP: CD34+CD38+CD19+

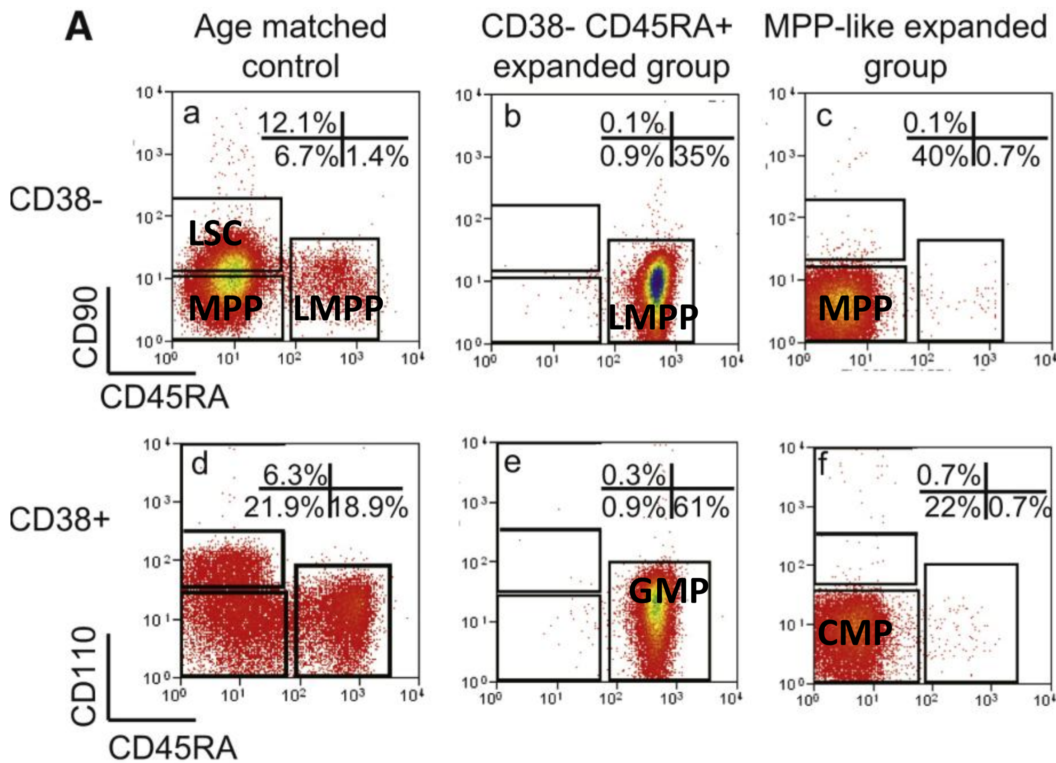
The hierarchy of LSC and progenitors are shown in Figure V.15 ‘Model for LSC commitment in AML and CML.

I will first enrich for CD34+ progenitors using CD34 Microbead kit and MACS separation columns (Miltenyi Biotec, Bisley UK), followed by cell sorting on a FACS ARIA cell sorter using a panel of surface antibodies consisting of CD38, CD45RA, CD90, CD110 and CD19. Cells will be gated on CD38, and the following subsets will be sort purified:

- LSC: CD38neg, CD45RAneg, CD90 pos
- MPP: CD38neg, CD45RAneg, CD90 neg
- LMPP: CD38neg, CD45RApos
- CMP: CD38pos CD45RAneg, CD110 neg
- GMP: CD38pos CD45RApos
- CLP: CD38pos, CD19pos

An example of cell sorting strategy employed by Goardon and colleagues to study LSCs and progeny cells is shown in Figure VI.1.

Figure VI.1: example of cell sorting of LSC, MPP, LMPP, CMP and GMP is AML patients (Goardon *et al*, 2011)



Finally, I will perform gene expression profiling and/or whole exome sequencing on the sort purified LSC and progenitors cells and B cell subsets collected from patients both with or without BCR-ABL positive B cells as outlined in Chapter V. Cryopreserved mononuclear cells, as well as DNA and RNA from sort purified B cells subsets are stored and available for these analyses.

Candidate genes such as deletion of IKZF1 (encoding the transcription factor Ikaros) will be of particular interest, since Ikaros was recently shown to be deleted in 83% of patients with Ph+ ALL but not in chronic phase CML (Mullighan *et al*, 2008). Expression of the B cell mutator AID, reported to be overexpressed in CML lymphoid blast crisis, will also be investigated (Klemm *et al*, 2009).

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