Development of epitope-based immunotherapy for the treatment of Chronic Myeloid Leukaemia

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This Thesis is dedicated to my parents and my sister, who have supported me both emotionally and financially all through my PhD training.

Je dédie cette thèse à mes parents et à ma soeur adorée. Merci du fond du coeur de m'avoir toujours soutenu moralement et financièrement tout au long de ces interminables études à l'étranger....

Abstract

Haematopoietic stem cell transplantation, one of the major treatments for Chronic Myeloid Leukaemia (CML), provides an allogeneic Graft-versus-Leukaemia (GvL) effect mediated by the donor T cells infused with the graft. The antigens recognized by these T cells are not yet identified. One candidate antigen is the CML specific BCR/ABL oncogene. The aim of this thesis is to investigate immunotherapeutic strategies for the treatment of CML targeted to BCR/ABL epitopes.

We characterised BCR/ABL derived peptides that are naturally processed and presented on the surface of CML cells in the context of both HLA-A*0301 and HLA-B*0801 molecules. In order to screen for the presence of BCR/ABL specific T cells in CML patient samples, we generated HLA tetramer complexes, refolded with the appropriate BCR/ABL peptide. We detected the presence of low frequency (< 1%) BCR/ABL specific $CD8^+$ T cells circulating in the peripheral blood of CML patients.

The expansion of these BCR/ABL specific CTLs from healthy donors and CML patients was attempted using a number of different protocols, including peptide-pulsed dendritic cells. We generated leukaemia specific CTLs in some cases from healthy donors but not from patients. It was however possible in both groups to generate responses to viral antigens. We therefore investigated BCR/ABL immunogenicity, comparing CML patient responses to BCR/ABL antigens with other GvL associated tumour antigens.

To circumvent a potential Antigen Presenting Cell (APC) deficiency in CML patients, we generated a standardized and unlimited source of artificial soluble Antigen-Presenting Complexes by cross-linking BCR-ABL HLA/peptide monomers with costimulatory molecules. These sAPCs successfully generated functional $CD8^+$ BCR/ABL T cells from both healthy individuals and CML patients.

The data presented here demonstrates the feasibility of BCR/ABL based adoptive T cell immunotherapy for CML patients, at least in the context of HLA-A*0301.

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Abbreviations

AML	Acute Myeloid Leukaemia
ANRI	Anthony Nolan Research Institute
APC	AlloPhycoCyanine
APCs	Antigen-Presenting Cells
aAPCs	artificial-Antigen Presenting Cells
sAPCs	soluble Antigen-Presenting Complexes
ATP	Adenosine TriPhosphate
BCA	BicinChoninic Acid
BD	Becton Dickinson
BDCA	Blood Dendritic Cell Antigen
BSA	Bovine Serum Albumin
bsp	biotinylation substrate peptide
CD	Cluster of Differentiation
CDR	Complementary Determining Regions
cDNA	complementary DeoxyriboNucleic Acid
CFSE	Carboxy-Fluorescein diacetate Succinimidyl Ester
CI	Confidence Interval
CML	Chronic Myeloid Leukaemia
CMV	CytoMegaloVirus
Cpm	Count per minute
CR	Complete clinical Response
CTL	Cytotoxic T Cell
CTLA-4	Costimulation T Lymphocyte Antigen 4
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion
DMSO	DiMethylSulphOxide
DNA	DeoxyriboNucleic Acid
DTH	Delayed Type Hypersensitivity
EBV	Epstain-Barr Virus
EDTA	EthyleneDiamineTetra-acetic Acid
ELISA	Enzyme Linked Immuno-Sorbent Assay
ELIspot	Enzyme Linked Immuno-spot
EMF	Experimental Mean Fluorescence

ER	Endoplasmic Reticulum
ESI/MS	ElectroSpray Ionisation Mass Spectrometry
FACs	Fluorescent Activated Cells sorter
FCS	Foetal Calf Serum
FITC	Fluorescein IsoThioCyanate
FACs	Fluorescent activated cell sorting
FPLC	Fast Protein Liquid Chromatography
FSC	Forward Side Scatter
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Good Medical Practise
GvHD	Graft versus Host Disease
GvL	Graft versus Leukaemia
HA	minor Histocompatibility Antigen
HH	Hammersmith Hospital
HLA	Human Leucocyte Antigen
HPLC	High Performance Liquid Chromatography
HSCT	Haematopoietic Stem Cell Transplantation
HTERT	human Telomerase subunit antigenic protein
ICAM	InterCellular Adhesion Molecule
ICOS	Inducible co-Stimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	IsoPropylThio-b-D-Galactoside
IS	Immunological Synapse
ITAMs	Immunoreceptor Tyrosine based Activation Motifs
ITIMs	Immunoreceptor Tyrosine based Inhibition Motifs
KCH	Kings College Hospital
KIR	Killer-cell Immunoglobulib-like Receptor
LFA	Lymphocyte Function-associated Antigen
LRI	Liverpool Royal Infirmary
M	Molar or moles per litre
mDC	monocyte-derived Dendritic Cells
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MPa	Mega Pascal (pressure units)
MS	Mass Spectrometry

MW	Molecular Weight
MWCO	Molecular Weight Cut Off
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pDC	plasmacytoid Dendritic Cells
PE	PhycoErythrin
PerCP	Perididin Chlorophyll Protein
Ph	Philadelphia
PHA	PhytoHemAgglutinin
PMSF	PhenylMethylSulfonyl Fluoride
PR	Partial clinical Response
PR1	PRoteinase 3 antigen
RFH	Royal Free Hospital
RNA	RiboNucleic Acid
RPMI 1640	Roswell Park Memorial Institute, medium for lymphoid cell culture
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis
sMAC	supraMolecular Activator Cluster
SSC	Side Scatter
TAA	Tumour Associated Antigen
TAP	Transporter Associated with antigen Processing
TCD	T Cell Depletion
TCR	T Cell Receptor
TEMED	N-N-N'-N' TEtraMethylEthylene Diamine
TIL	Tumour Infiltrating Lymphocytes
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
Tr/Treg	Regulatory T cells
U	Unit
UCH	University College Hospital
UV	Ultra Violet
v/v	volume per volume
w/v	weight per volume
WT1	Wilms' Tumour antigen
X-gal	5-bromo 4-chloro 3-indolyl-b-D-galactosidase

CHAPTER 1
Introduction

1.1 Basis for immunotherapy development:

1.1.1 Evidence for cancer immune surveillance:

The first evidence that the immune system plays a role in the prevention and eradication of cancer cells arose from the observations of tumour regression in patients who contracted bacterial infection (Dr William B Coley, (Coley 1991)). Later, it was proposed that the immune system constantly seeks out and eliminates cells in the process of malignant transformation and that if this screening process fails, cancer will develop (Burnet 1970). This theory of "cancer immune surveillance" has, however, remained unproven and debated for some time (Pardoll 2003). Cancer development would be expected to occur only in situations where the immune system is compromised, yet not all immuno-compromised patients develop cancer (Ioachim 1990), and in the majority of cases cancer patients have been shown to have an intact immune system. Conversely, the occurrence of spontaneous tumour regression in cancer patients has been observed and sometimes associated with a detectable specific anti-tumour immune response (Rosenberg 1991).

Direct evidence for a role of the immune system in the elimination of cancer has come from animal models. The transfer of tumour cells from donor to recipient mice induced an allogeneic response, which resulted in the rejection of the transplanted tumour (Baldwin 1973; Globerson and Feldman 1964). In addition, while the implantation of tumour cells in mice resulted in tumour growth and eventually killed the animal, the surgical removal of the tumour followed by the implantation of irradiated tumour cells protected the mice against a secondary challenge with the same tumour. These experiments demonstrate that tumours can induce specific adaptive immune responses. Many efforts have thus concentrated on the elucidation and characterisation of the cellular and/or soluble components of the immune system in mediating these responses.

1.1.2 Immune cells involved in anti-tumour responses:

The relevance of cell-mediated immunity against tumours has been demonstrated in a number of murine models which lack genes for the generation of lymphocytes or for the generation of their effector functions. RAG, INFγ or INFγ receptor and perforin knock-out mice have been shown to have an increase incidence of spontaneous or carcinogen-induced tumour development (Kaplan et al. 1998; Kim et al. 2000b; Shankaran et al. 2001; Smyth et al. 2000; van den Broek et al. 1996). This was associated with the deficiency of Natural Killer cells (NK), T lymphocytes and/or NKT cells.

NK cells represent a lymphoid population which, unlike T or B lymphocytes does not express clonally distributed receptors for antigen. NK cells can discriminate between normal cells expressing self HLA molecules and cells that do not express adequate amount of HLA molecules, as is frequently the case in tumour or virus-infected cells (Moretta et al. 2000). The molecular mechanism for this discrimination resides in the expression of a set of receptors for HLA class I molecules, which exert an inhibitory effect upon recognition. These receptors belong to the immunoglobulin family and include the killer-cell Immunoglobulin-like receptors (KIRs) which recognize HLA-A, B and C alleles and CD94 which recognizes peptide derived from the non-classical HLA-E molecule (Braud et al. 1998; Lanier 1998). The interaction between KIRs and the corresponding HLA class I ligands prevents NK cells from killing target cells expressing self-HLA alleles (Ciccone et al. 1996). Conversely, the lack of KIR engagement will lead to the killing of the target cell. Such cytotoxic activity has been demonstrated to be beneficial for mediating NK cell-versus leukaemia responses in Acute Myeloid Leukaemia (AML) patients following haploidentical Haematopoietic Stem Cell Transplantation (HSCT) (Ruggeri et al. 1999). The donor NK cells lacking inhibitory KIRs for the host HLA class I molecule resulted in tumour eradication and consequently in a lower incidence of disease relapse in these patients. However, the impact of KIR mismatch on HLA-matched unrelated HSCT has been associated with an increased incidence of acute GvHD and lower survival (Davies et al. 2002). More recently, in addition to KIR/HLA mismatch, the presence of donor-activating KIR was shown to significantly reduce the patient overall survival (Cook et al. 2004). Thus the clinical relevance of KIR mismatch in HSCT remains to be clarified.

NK cells also express another set of receptors, referred to as activating receptors. Upon engagement with their respective ligand, NK cells become activated and mediate target cell lysis (Lanier 1998; Moretta et al. 2001). One of these receptors, NKG2D recognizes the stress-inducible non-classical surface MHC molecules, MICA and MICB (Bauer et al. 1999; Steinle et al. 2001). The expression of MICA and MICB molecules has been demonstrated in a wide range of tumours and virally-infected cells, thus rendering the transformed cells susceptible to NK cell lysis (Groh et al. 2001; Groh et al. 2002; Salih et al. 2003). As NKG2D is expressed not only by NK cells but also by αβ and γδ T cells, as well as NKT cells, all these cell types have been demonstrated to participate in anti-tumour immunity (Bauer et al. 1999; Girardi et al. 2001; Ho et al. 2002; Jamieson et al. 2002).

Additionally T lymphocytes, both $CD4^+$ and $CD8^+$ T cells have been detected in patient tumour lesions and these are referred to as tumour infiltrating lymphocytes (TIL) (Belldegrun et al. 1988; Topalian et al. 1987; Wong et al. 1989). The presence of TIL in cancer patients has been associated with a better clinical prognostic and in some cases, even with tumour regression *in-vivo* (Kawakami et al. 1995; Naito et al. 1998; Zhang et al. 2003).

The crucial role of T cells in mediating anti-tumour specific T cells has been clearly demonstrated with the successful clinical responses following Donor Lymphocyte Infusion (DLI) therapy. Patients with haematological malignancies, who relapse following HSCT have shown remarkable Graft-versus-Leukaemia (GvL) responses, mediated by the infused donor T lymphocytes (Dazzi et al. 2000; Kolb et al. 1990; Kolb et al. 1995; Mackinnon et al. 1995). Such responses have been shown to be most prominent in patients with Chronic Myeloid Leukaemia (CML) (Dazzi et al. 2000; Mehta et al. 1997), which renders CML an attractive candidate disease in which to assess the antigen-specific T cell responses involved in GvL and to examine immunotherapeutic approaches to eradicate the tumour. In this study, we will be focusing on anti-leukaemic $CD8^+$ T cell responses to CML antigens.

1.2: Generation of T lymphocytes:

T lymphocytes develop from an early lymphoid progenitor in the bone marrow and migrate to the secondary lymphoid organ, the thymus, to further mature and differentiate into $CD8^+$ or $CD4^+$ T cell subsets. The formation of the lymphocyte repertoire consists of a process by which T lymphocytes become educated to recognize foreign or abnormal antigenic peptides presented by pathogen-infected or tumour cells, while ignoring self-presented peptides. This recognition is mediated by the production of the antigen specific receptor, the T Cell Receptor (TCR).

1.2.1 T lymphocyte development:

T cell development occurs in the thymic specialized microenvironment through the interaction of thymocytes with the stromal cells and the signalling via growth factors. Thymocytes derive from a $CD34^+$ pluripotent haematopoietic stem cell progenitor and migrate from the bone marrow to the corticomedullary area of the thymus. The successive stage of thymocytes development into T lymphocytes is characterised by the ordered expression of several molecules, including the co-receptors CD4 and CD8 as schematized in **Figure 1.1** (Basson and Zamoyska 2001; Kruisbeek and Storb 1994).

T cells expressing MHC class I restricted receptor are positively selected to the CD8 single positive lineage and programmed to become cytotoxic effector cells. In contrast T cells expressing MHC class II restricted receptor are positively selected to the single CD4 lineage and programmed to become cytokine-secreting cells (Basson and Zamoyska 2000). The positive selection results in the survival of lymphocytes bearing a TCR, which binds self MHC/peptide complexes with low avidity (antagonist peptide), thus ensuring T cell self-MHC restriction (Hogquist 2001; von Boehmer 1994). T lymphocytes expressing receptors that strongly react with abundant thymic self-antigen are clonally deleted from the mature T cell repertoire during the negative selection. This prevents T cells that recognise self-antigens with high affinity to induce auto-immune responses in the periphery. Together, positive and negative selections allow only T cells with functional TCRs that recognize self-HLA molecules presenting foreign antigens (only 5% of thymocytes) to migrate through the medulla and leave the thymus for the periphery (Jameson and Bevan 1998; Sebzda et al. 1999; Starr et al. 2003).

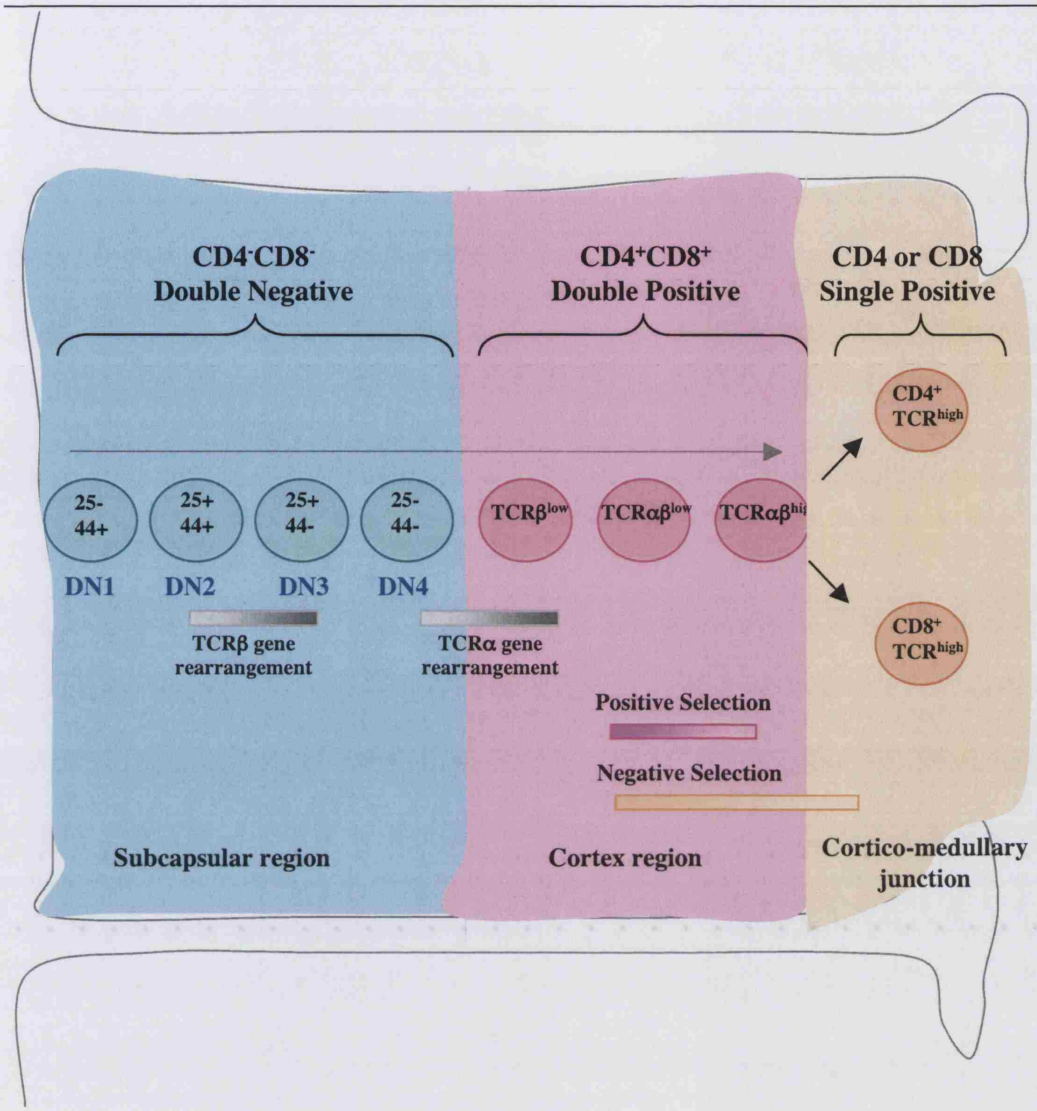


Figure 1.1: T lymphocytes development:

The development of thymocytes into T lymphocytes occurs in the secondary lymphoid organ, the thymus. Early double negative (DN1) $CD4^-CD8^-$ thymocytes expresses c-Kit (receptor for haematopoietic cytokines) and CD44 adhesion molecule but lack CD25 (α chain of the IL-2 receptor) expression (DN1). As thymocytes migrate from the subcapsular to the cortex areas of the thymus, they up-regulate CD25 and start down-regulating CD44 expressions (DN2 and DN3). At this stage γδ or β gene loci rearrangement occur and direct thymocytes to the γδ or the αβ differentiation pathway. The successful TCRβ rearrangement represents a crucial checkpoint, which induces thymocytes to either, proliferate and commit to the αβ T- cell lineage, or apoptose. It also induces the down-regulation of CD25 (DN4) followed by the up-regulation of both $CD4^+CD8^+$ expression. These double positive (DP) thymocytes undergoe a successive TCRα chain rearrangement until the generation of a functional αβ TCR. At this stage, the DP lymphocytes will be positively selected for recognition of self-restricted MHC class I ($CD8^+$) or class II ($CD4^+$) molecules. Thus, thymocytes down-regulate one of the co-receptor and migrate to the cortico-medullary junction for the elimination of T lymphocytes recognizing self-antigens/HLA complexes during the negative selection. Once T lymphocytes passe these checkpoints, they migrate to the medulla area (not shown) and leave the thymus for the periphery.

1.2.2 TCR gene rearrangement:

TCR germ-line DNA contain multigene families corresponding to the α, β, γ and δ chains of the receptor. Each of these genes comprises multiples gene segments called C (constant), V (variable), J (joining) and in the case of β and δ families, D (diversity). The δ encoding genes are entirely found within the α locus, thus the gene rearrangement will lead solely to the formation of either αβ (in most cases) or γδ T lymphocytes (Krangel et al. 1998). The TCR are produced in a process of random DNA translation, splicing and transcription of these VDJ segments that codes for the antigen-binding hypervariable region (CDR3) of the receptor (Bogue and Roth 1996; Krangel et al. 1998). The segments are cut out by nucleases, spliced together by VDJ recombinases, which are the product of recombination-activation genes (RAG-1 and RAG-2) and the resulting mRNA is finally translated into the polypeptide monomer (α or β) of the TCR (Oettinger et al. 1990). The clonal diversity of the TCR is the result of the multitude number of each VDJ regions and the multiplicity of combinational recombination, i.e. one of these genes can rearrange with any one other to form the final VDJ product. Besides, the gene splicing is inaccurate and the frameshift of base pairs leads to the production of different amino acids (junctional diversity) (Kim et al. 2000a). The VDJ sequence can be further altered by the terminal deoxyribonucleotidyl transferase, which insert N-nucleotides between the rearranged genes. Thus the VDJ rearrangement process results in the generation of a very large T cell repertoire estimated at over 10^{18} total T cells, which protect us against a wide range of pathogens.

1.3: Antigen presentation to T lymphocytes:

In order for a foreign protein antigen to be recognized by T lymphocytes, it must be degraded into small antigenic peptides and be presented associated with cell surface HLA molecules. The foreign antigen is presented on HLA class I and/or HLA class II molecules depending on the route of entry into the cell. Exogenous antigens are taken up by endocytosis or phagocytosis and presented on HLA class II molecules. The expression of HLA class II molecules, and thus the presentation of exogenous antigens, is limited to antigen presenting cells (APCs) such as macrophages, dendritic cells and B lymphocytes. On the other hand, endogenous antigens are expressed by infected or transformed (cancer) cells and are presented on HLA class I molecules. HLA class I molecules are expressed on the surface of nearly all nucleated cells, thus allowing them to alert the immune system of infection or abnormal functioning.

1.3.1 Structure of HLA molecules:

HLA molecules play a central role in the regulation of immune responses, as they bind and present antigenic peptides derived from pathogens to B and T lymphocytes (Townsend and Bodmer 1989). HLA molecules are encoded on chromosome 6, within the most polymorphic region of the human genome. Sequencing data and crystallographic studies of HLA class I molecule structures have demonstrated that these polymorphisms are concentrated in the peptide-binding groove and at sites in contact with the TCR (Colombani 1990; Parham et al. 1988; Rohren et al. 1993). The HLA class I molecule is comprised of an α polypeptide chain (or heavy chain) organized into three domains, α1 α2 and α3, which are non-convalently bound to the β-2 microglobulin (Bjorkman et al. 1987; Saper et al. 1991). The α1 and α2 chains interact and form a platform of eight anti-parallel β strands to create the peptide-binding groove. In contrast, the HLA class II molecule contains two different non-covalently associated polypeptide chains, α1α2 and β1β2 (Brown et al. 1993). The α1 and β1 chains interact and constitute the peptide-binding groove of the HLA class II molecule. The α3β (HLA class I) and the α2β2 (HLA class II) chains are similar in sequence and structure to immunoglobulins, and contains the hydrophobic transmembrane segment as well as the hydrophilic cytoplasmic tail. These domains are non-polymorphic and constitute the portion recognized by $CD8^+$ (HLA class I, α3 domain) or by $CD4^+$ T lymphocytes (HLA class II, β2 domain) (Gao et al. 1997; Wang et al. 2001).

The crystallographic analysis of an HLA class I molecules' 3-D structure showed that the peptide binding groove contains clefts and ridges through which the backbone of the peptide binds via hydrogen bonds to form the classic "pocket" shape as described by Fremont et al. (Fremont et al. 1996; Fremont et al. 1992). In HLA class I molecules the binding sites are predominantly clustered at both N-terminal and Carboxy-terminal ends of the peptide-binding groove, whereas in HLA class II molecules these sites are distributed throughout the binding site (Madden 1995). As a results, HLA class I molecules can accommodate 8-11 mer peptides and HLA class II molecules can accommodate much longer peptides (10 to 30-mer long) (Engelhard 1994; Rotzschke and Falk 1994). Accordingly, HLA-class I-bound peptides have conserved residues at both ends (called anchor residues), while for HLA-class II-bound peptides conserved residues are situated in the central core. Each HLA molecule will therefore bind a set of peptides demonstrating similar composition, while different HLA molecules will bind a very different set of peptides. The complementary structure required between an antigenic peptide and the HLA peptide-binding groove accounts for the selection and HLA restriction of epitopes presented by a particular HLA class I or class II molecule (Rohren et al. 1993). Thus, HLA polymorphism influences both the type of epitope presented by a particular HLA molecule and their interaction with T cells, which in turn determines the type of immune response generated.

1.3.2 Presentation of endogenous antigens:

The processing and presentation of endogenous antigens on HLA class I molecules has been well characterised (Pamer and Cresswell 1998; Rock and Goldberg 1999). Infected or transformed cells synthesize foreign, self or mutant proteins in the cytosol. These proteins are chaperoned by ubiquitin and targeted for rapid degradation to the proteasome (**Figure 1.2**, panel **A**). The proteasome is a cytoplasmic multicatalytic enzyme complex comprised of a multicatalytic complex (20S core) and regulatory complexes (19S and 11S). Proteasomes have a housekeeping function to degrade cytoplasmic proteins into peptides. In response to immune inflammation, three proteasome sub/units are replaced with the IFNγ induced Low Molecular weight Protein 2 (LMP 2), LMP 7 and Multicatalytic Endopeptidase Complex-Like 1 (MECL-1) (Fruh et al. 1994; Rock and Goldberg 1999). This immuno-proteasome has been shown not only to have an enhanced degradation kinetic but also to modify the substrate specificity so that smaller peptides (8-30 mer long) are generated containing hydrophobic carboxyl terminal residues.

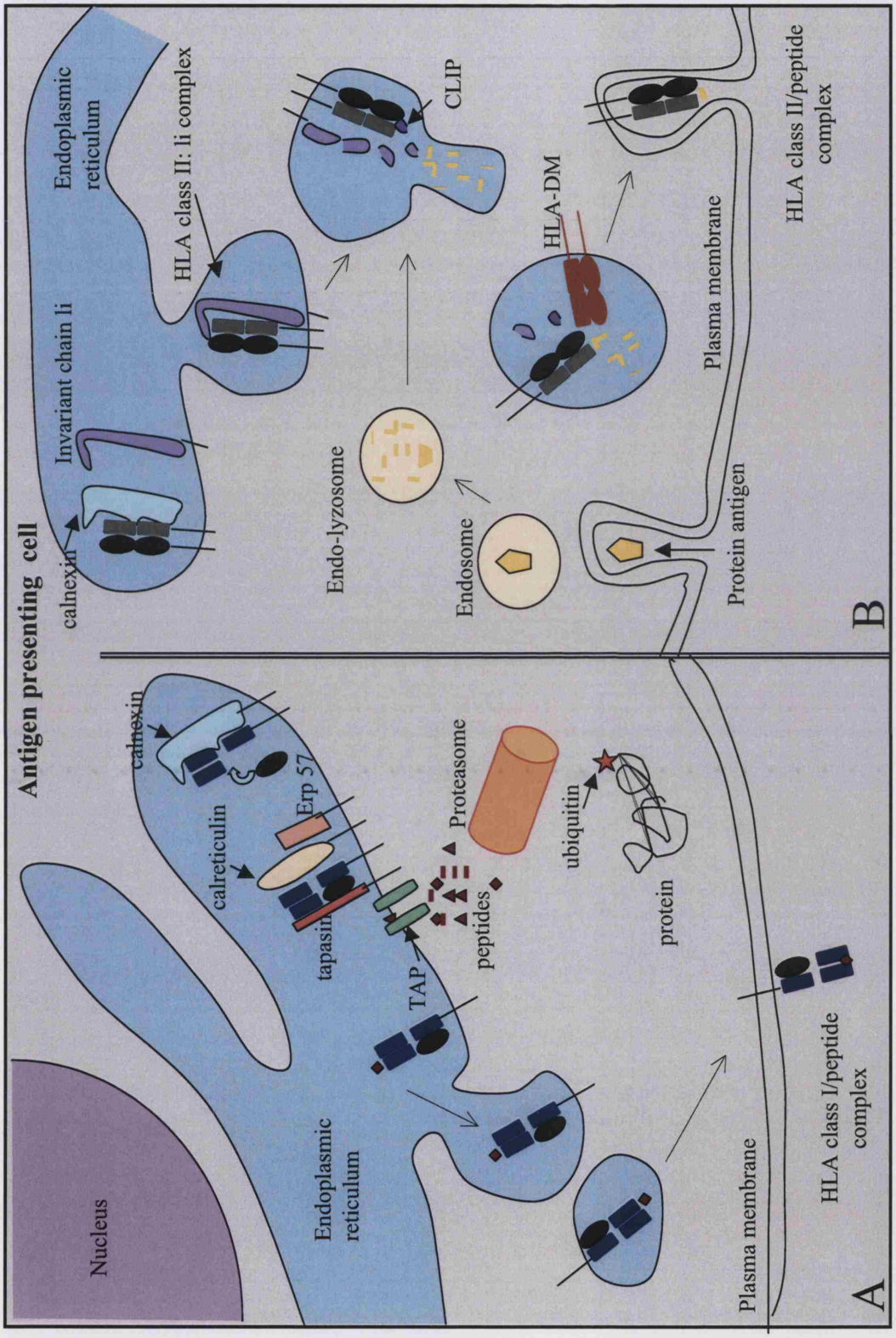


Figure 1.2: Antigen processing and presentation on HLA molecules:

The processing and presentation of endogenous antigen on HLA class I molecules (panel **A**) and exogenous antigens on HLA class II molecules (panel **B**) are represented. TAP: Transporter associated protein; Ii: Invariant chain; CLIP: class II-associated invariant chain peptide.

The crucial role of the proteasome in generating HLA-class I antigenic peptides has been demonstrated using a number of specific proteasome inhibitors. One such inhibitor, lactacystin, was demonstrated to considerably reduce the amount of antigenic peptides presented on cell surface HLA class I molecules (Cerundolo et al. 1997; Schwarz et al. 2000). The NH_2 terminal trimming of antigenic peptides has been shown to rely on other cytolytic proteases (Rock et al. 2004). These amino peptidase proteases are required for the generation of correct length precursor peptides that will fit the HLA class I peptide groove (Stoltze et al. 2000). A number of ER resident amino peptidases have been characterised, such as puromycin-sensitive aminopeptidases, bleomycin hydrolases and ER aminopeptidase 1 (ERAP1), and have been shown to act downstream of the proteasome and to increase the supply of peptides for MHC class I antigen presentation (Stoltze et al. 2000; York et al. 2002). Cytosolic peptides are then delivered to the lumen of the Endoplasmic Reticulum (ER) by Transporter associated with Antigen Processing proteins (TAP) (**Figure 1.2**, panel **A**). TAP preferentially transport 8-13 mer peptides, which contain hydrophobic Carboxy terminal residues, the preferred anchor residues for HLA class I molecules. Its function has been therefore solely associated with the transport of peptides for HLA class I molecule loading (Suh et al. 1994).

Although a number of antigenic peptides presented on HLA class I molecules have been shown to be derived from native self, mutated or viral proteins, a major source of antigenic peptides have been suggested to derived from newly synthesized non-native proteins (Yewdell et al. 1996; Yewdell et al. 2001). Such proteins are the result of defective ribosomal particles, which generate prematurely terminated polypeptides or misfolded polypeptides also referred to as DRiPs (defective ribosomal products). DRiPs are targeted for degradation by cytosolic proteases and are believed to represent the major source of antigenic peptides presented on HLA class I molecules (Schubert et al. 2000).

Nascent HLA class I α chains are synthesized and retained in the ER by the calnexin chaperone. The partial folding of the heavy chain with β-2 microglobulin releases the calnexin and the HLA class I molecule associates with the calreticulin and the Erp57 proteins, which chaperone the complexes to bind to the tapasin. Tapasin creates a bridge between the HLA class I molecules and TAP, which favour and regulate the loading of antigenic peptides (Grandea and Van Kaer 2001), **Figure 1.2**. The Erp 57 protein breaks and reforms the disulfide bond during peptide loading. Peptides, with enough affinity for the HLA class I molecule, dissociate from TAP, bind and stabilise the HLA class I complexes. While unstable HLA class I molecules are degraded in the ER, stable HLA class I complexes dissociated from the chaperone proteins and are transported to the cell surface

via the golgi apparatus. Endogenous antigens can therefore be presented at the cell surface to inform the immune system of infection or abnormality.

HLA class I restricted T cell responses have been shown to display dominance towards a particular part of the antigenic protein. Such immuno-dominance has been shown to be dependant not only on the peptide binding affinity for the HLA molecule, but also on the immuno-proteosome activity as this latter is responsible for the generation of peptide epitopes (Chen et al. 2001; Yewdell and Bennink 1999). More recently, tapasin has also been demonstrated to define antigenic peptide immunodominance, by promoting the preferential loading of higher affinity peptides (Howarth et al. 2004).

1.3.3 Presentation of exogenous antigens:

The processing and presentation of exogenous antigenic peptides on HLA class II molecules, similarly to the endogenous antigenic proteins, involves a number of proteases (Pieters 2000). Exogenous pathogen derived proteins are captured by professional APCs via phagocytosis and/or endocytosis. These exogenous antigens are then internalised in "early" endosomes, which then fuse with a lysosome (**Figure 1.2**, panel **B**). The increased amount of proteases and acidic environment in the endo-lysosome promotes the degradation of antigenic proteins into smaller peptides. A number of hydrolases, including proteases, nucleases and phospholipases reside in the lysosome, but the cathepsins have been demonstrated to be the major proteases for the generation of antigenic epitopes.

Nascent HLA class II molecules are synthesised in the ER and stabilised by calnexin as in the case of HLA class I molecule. To prevent ER derived peptides from binding non-specifically to HLA class II molecules, an invariant chain (Ii) associates and occupies the peptide-binding groove. The invariant chain also targets the HLA class II complexes to the endocytic compartment via the golgi apparatus (Brachet et al. 1997). Here in the endosomes, endo-lysosomes and then lysosomes; the invariant chain is cleaved by the acid-dependent proteases and leaves a short fragment bound to the peptide-binding groove called CLIP (class II-associated invariant chain peptide). In addition, two non-classical HLA class II molecules, HLA-DM and HLA-DO, play a role in the regulation of antigenic peptides loading (Alfonso and Karlsson 2000). HLA-DM catalyses the removal of the CLIP from the peptide-binding groove, necessary for the loading of antigenic peptides. In contrast, HLA-DO is a negative regulator of HLA class II antigen presentation and prevents the peptide loading by binding HLA-DM molecules. The removal of CLIP by HLA-DM allows the loading of antigenic peptides. Antigenic peptides with enough affinity bind and stabilize

HLA class II molecules and the stable HLA class II/peptide complexes are then transported by secretory vesicles to the cell surface membrane (**Figure 1.2**, panel **B**). Thus, pathogen derived antigens are presented to the immune cells for the activation and the generation of a specific immune response.

1.3.4 Professional antigen-presenting cells: Dendritic cells (DCs):

Dendritic cells represent the most potent APCs, capable of linking the innate immunity with the adaptive immunity. Dendritic cells derive from $CD34^+$ haematopoietic stem cell precursors, which differentiate into common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) in the bone marrow (Banchereau and Steinman 1998; Liu 2001). *In-vivo*, the growth and differentiation factors Fms-like tyrosine kinase receptor ligand 3 (Flt-3) and GM-CSF were shown to induce the CMP to further differentiate into $CD11c^+$ DCs, which reside in the skin (Langherhans DCs) or other tissues (Interstitial DCs) (Arpinati et al. 2000; Maraskovsky et al. 2000; Pulendran et al. 2000). In contrast, the CLP further differentiate into $CD11c^-$ DCs (Pulendran et al. 2000) also called plasmacytoid DCs (pDCs) because they were shown to share lymphocyte-like morphology and expressed lymphoid pre-T cell α chain markers (Res et al. 1999; Spits et al. 2000). These pDCs are also referred to as pre-DC2, in comparison with another type of $CD11c^-$ DCs called pre-DC1, the monocytes-derived DCs (Liu 2001). Dendritic cells are differentiated as immature cells (prior to encountering antigens) and circulate through the peripheral blood and tissues, surveying for pathogen entry. Immature dendritic cells (imDCs) have been shown to express low levels of surface HLA class I, HLA class II and costimulatory molecules, but to contain abundant levels of synthesised HLA class II molecules sequestered in a late endosomal compartment awaiting for antigen loading (Mellman and Steinman 2001). Additionally, high expression of receptors mediating endocytosis of pathogen derived proteins as well as pattern-recognition receptors have been characterised on the cell surface of imDCs (Aderem and Ulevitch 2000; Medzhitov and Janeway 1998; Sallusto et al. 1995). These receptors confer the imDCs with a high capacity to take up foreign antigens from the periphery. Upon encountering pathogens or inflammatory cytokines, imDCs are undergoing the process of maturation.

Mature DCs have been shown to have reduced endocytic activity but an increased proteases activity and cell surface expression of HLA class I, HLA class II, adhesion and costimulatory molecules (Chow et al. 2002; Mellman and Steinman 2001; Trombetta et al. 2003). Thus the maturation signals considerably increase the activity of the machinery for

antigen degradation and presentation. Additionally, mature DCs extend their dendrites, which promote the interaction and subsequent activation of lymphocytes. A recent microarray analysis of DC genes expression upon pathogen stimulation have clearly demonstrated the down-regulation of genes involved in antigen capture, in parallel to the up-regulation of genes involved in antigen processing and presentation (Huang et al. 2001). The nature of the genes expressed was shown to be tightly regulated by the maturation signals. Whilst bacterial signals induced the expression of inflammatory cytokines (such as IL-1β), which serve to attract innate immune cells, viral signals induced the expression of anti-viral cytokines, such as INFα and β. The maturation process has also been shown to induce the up-regulation of the CCR7 chemokine receptor, which drives DCs to migrate to the lymphoid organs for the induction of immune responses (Huang et al. 2001; Sallusto and Lanzavecchia 2000).

The restriction of HLA class I presentation to endogenous antigen is critical in preventing healthy cells taking up soluble antigens from the periphery and becoming targets for cytotoxic T cells. It is unlikely, however, that only infected or transformed DCs are able to present antigens in the context of HLA class I molecules. Professional APCs, including DCs are also able to induce an HLA-class I restricted response via a process called "cross-presentation" or "cross-priming" (Ackerman and Cresswell 2004). The first evidence for cross-presentation dates from the mid-1970s, when the minor histocompatibility antigens were shown to be capable of being transferred from donor cells to the host APCs (Bevan 1976). DCs have also been shown to acquire "endogenous" antigens from apoptotic infected or transformed neighbouring cells, which results in the efficient priming of antigen specific $CD8^+$ T cell responses (Albert et al. 1998; Nowak et al. 2003; Watts 1997). Soluble antigens were also shown to be taken up by DCs via the endocytosis mediating receptors or via heat shock proteins (Brossart and Bevan 1997; Fonteneau et al. 2003; Rodriguez et al. 1999). Although, these acquired "endogenous" antigens were thought to follow the classical HLA class I processing and presentation pathway, recently specialized ER-phagosome compartments have been characterised in DCs (Ackerman and Cresswell 2004; Guermonprez et al. 2003; Houde et al. 2003). These contain all the necessary machinery for antigen processing and presentation of endogenous antigens acquired by phagocytosis. The cross-priming capacity of DCs has been demonstrated to be essential for the generation of functional $CD8^+$ T cell specific immune responses but also for the induction of the cross-tolerance (Banchereau and Steinman 1998; Heath et al. 1998). DCs can therefore been seen as professional APCs crucial for the activation of antigen specific T cell responses and also for the tolerization of allo-reactive T cells, which have escaped the positive and negative selection (this will be discussed in section1.6).

Dendritic cells can be generated *in-vitro* from $CD34^+$ bone marrow derived progenitor cells (Lardon et al. 1997). The recent discovery of blood DC antigen (BDCA) markers has enabled the rapid purification of $CD11c^+$ (BDCA-3) and $CD11c^-$ plasmacytoid DCs (BDCA-2 and –4) (Dzionek et al. 2000). However the most *in-vitro* studied human DCs have been obtained from monocytes ($CD14^+$ cells), since a larger quantity of cells can be generated and further analysed. Monocytes have been shown to differentiate *in-vivo* and *in-vitro* to imDCs with GM-CSF and either IL-4 or INF-α cytokines (Fonteneau et al. 2001; Romani et al. 1996; Santodonato et al. 2003; Veeraswamy et al. 2003). $CD11c^+$ derived DCs were also shown to be maintained with these cytokines while pDCs have been shown to be maintained *in-vitro* with IL-3 cytokines (Grouard et al. 1997; Kohrgruber et al. 1999; Osugi et al. 2002). These DCs were then matured with bacterial derived proteins, such as LPS and/or with inflammatory molecules derived from the TNF family, such as TNFα, RANKL and/or CD40L (Cella et al. 2000; De Smedt et al. 1996; Lardon et al. 1997; Larsson et al. 2000; Yu et al. 2003b).

1.4: T lymphocyte activation:

The cellular and molecular mechanisms necessary for the generation of antigen-specific T cell responses involve the recognition of HLA/peptide complexes presented by APCs and the subsequent formation of an immunological synapse, which brings both the T cell and the APC close together. This is necessary for T cell co-stimulation and activation, and provides cellular signals for the subsequent proliferation and acquisition of effector functions, which lead to the removal of the pathogen and the eradication of the pathogen-bearing cell.

1.4.1 T Cell Receptor structure and recognition of HLA/peptide complexes:

The crystal structure of TCR fragments, from a few αβ TCR chains, and finally of TCR/HLA-peptide complexes have resolved the TCR structure and demonstrated its interaction with the HLA/peptide complex (Bentley et al. 1995; Garboczi et al. 1996; Garcia et al. 1996; Kersh and Allen 1996; Rudolph and Wilson 2002). TCRs are composed of two polypeptide chains, αβ or γδ, linked together by disulfide bonds. Each chain contains an amino terminal variable region and a carboxy terminal constant region, similar to the immunoglobulin structure (Bentley and Mariuzza 1996). The carboxy terminal constant region encloses a positively charged transmembrane domain, which anchors each chain in the plasma membrane, and a cytoplasmic tail. The TCR is cross-linked to the CD3 molecule and forms the TCR/CD3 membrane complex (Jorgensen et al. 1992). CD3 molecules are composed of five invariant chains forming two extracellular heterodimers, γε and δε, and an intracellular ζζ homodimer (**Figure 1.3**). Each CD3 chain contains a cytoplasmic Immunoreceptor Tyrosine based Activation Motifs (ITAM), which has a crucial role for the signal transduction upon TCR-CD3/HLA-peptide complex recognition.

The TCR domain that is known to recognize and bind HLA/peptide complexes consists of three hypervariable complementarity-determining regions (CDR1, CDR2 and CDR3), analogous to the immunoglobulins CDRs. The CDR1 and CDR2 domains have been demonstrated to bind to the conserved helical elements of the HLA molecules, namely the α1α2 domains of HLA class I (Gao et al. 1997), and the α1β1 domains of HLA class II molecules (Wang et al. 2001). The CDR3 domain has been shown to be the most hypervariable region and represents the dominant loop for interaction with antigenic peptide bound on HLA molecules (Davis et al. 1998; Garcia et al. 1999; van der Merwe and Davis 2003).

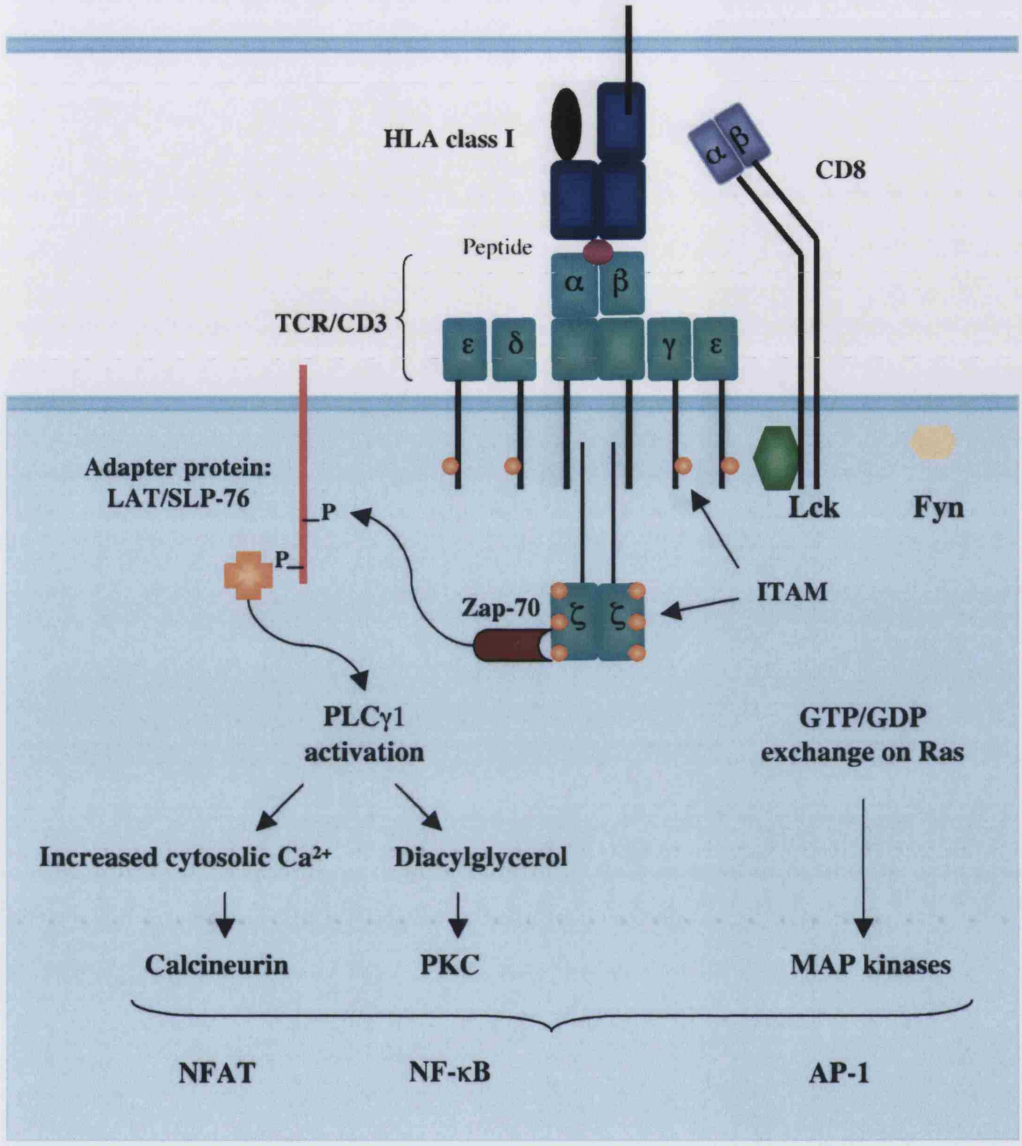


Figure 1.3: T cell receptor recognition and signalling:

The T cell recognition of HLA-class I molecules and the resulting cellular signaling events are represented for a $CD8^+$ T cell. TCR/CD3 complex recognizes and binds to the HLA/peptide complex presented by antigen presenting cell (APC). The CD8 co-receptor restricts and strengthens the interaction by binding to the α chain of the HLA molecule. This brings the cytoplasmic associated Lck protein kinase close to the immuno-receptor Tyrosine-based activating motif (ITAM) of the TCR/CD3 complex. The phosphorylation of ITAM recruits and activates Zap-70 kinase, which in turn phosphorylates adapter proteins, such as LAT and SLP-76. The major signalling pathways are represented, with the activation of the phospholipase C (PLC γ1) and Ras kinases. The downstream signalling activation of the calcineurin, the protein kinase C (PKC) and MAP kinases induces the deposphorylation and translocation of transcription factors in the nucleus for the expression of functional genes. The NF-κB, AP-1 and nuclear factor of activated T cells (NFAT) leads to the transcription of genes required for cellular proliferation, differentiation and effector functions. P= Phosphorylated residues.

Crystallographic studies have demonstrated a diagonal orientation or docking between the TCR and HLA/peptide complexes, which maximizes the contact and subsequently the specificity of the individual TCR for the presented antigenic peptide (Ding et al. 1998; Garcia et al. 1996; Teng et al. 1998). The CDR3 has been shown to slightly readjust at the TCR/HLA-peptide interface to accommodate the different peptides, some of which may have upward pointing side elements (Rudolph and Wilson 2002). This flexibility of the CDR3 loop is in fact crucial for enabling the TCR repertoire to recognize a much larger range of peptide/HLA complexes and contributes to the high degree of T cell alloreactivity.

1.4.2 Formation of the immunological synapse:

Circulating T lymphocytes become alerted to tissue injury by released mediators, such as chemokines. Chemokines not only attract T cells to the site of inflammation but also induce T cell polarization. Polarized T cells reorganize cytoplasmic organelles and recruit adhesion molecules, TCR and co-receptors at one edge of the cell surface (Bromley et al. 2001a). The initial contact between T cells and APCs is antigen-independent and mediated by the adhesion molecules LFA-1 and CD2, which interact with ICAM-1 and CD58 molecules on APCs respectively. This adhesion brings the membranes into close proximity and allows T cells to scan for appropriate HLA/peptide complexes presented by the APC. The CD2-CD58 interaction has been shown to recruit lipid raft-associated TCR, as well as other adhesion and costimulation molecules into clusters at the cell surface of T cells and initiate the formation of the Immunological Synapse (IS) (Dykstra et al. 2003; Grakoui et al. 1999; Krummel and Davis 2002; Langlet et al. 2000). Upon TCR engagement, the CD2-CD58, TCR/HLA-peptide complexes and the CD8 or CD4 co-receptor segregate at the centre of the IS and form the supramolecular activation cluster (sMAC) (Dustin and Shaw 1999). This effectively pushes LFA-1/ICAM-1 complexes outwards as well as larger molecules CD43 and CD45, which could disturb the binding and signalling of smaller molecules. The interactions between these key receptors and ligands are indeed initiating the formation of the IS, but also are believed to collectively contribute to T cell recognition and signalling. The activation signal has been demonstrated to be highly dependent upon the identity of the antigenic peptide being presented and on the strength of TCR/HLA-peptide binding (Grakoui et al. 1999; Lyons et al. 1996). Weak peptides have been shown to induce the formation of IS and in some cases the initial signals. However these weak signals were not sustained and failed to fully activate T cells. These interactions are also believed to contribute to the survival of mature T cells in the periphery (Takeda et al. 1996). Upon antigen stimulation, the clustered raft domain organization of lymphocytes is maintained and probably accounts for the faster immune response in the event of a subsequent antigen

exposure. Naïve and memory T cells have indeed been demonstrated to differ both quantitatively and qualitatively in membrane associated rafts distribution, which correlates with the observation that memory cells are more responsive than naïve cells (Tuosto et al. 2001).

1.4.3 T cell signalling:

The T cell signalling events upon TCR engagement are summarized in **Figure 1.3**. The specific recognition and binding of the TCR with HLA-peptide complex occurs at the sMAC. Additionally the CD8 and CD4 co-receptors restrict the TCRs interaction to HLA class I α3 domain or HLA class II β2 domain respectively (Gao et al. 1997; Wang et al. 2001). The single polypeptide CD4 chain and the CD8 heterodimer αβ chains are both non-convalently associated with the protein tyrosine kinase Lck. Thus the interaction of these co-receptors with the HLA molecules strengthens the TCR/HLA-peptide complex and chaperons the Lck tyrosine kinase close to the signalling components of the TCR/CD3 membrane complex (Daniels and Jameson 2000). The requirement of these co-receptors for stabilization, and hence activation, has been shown to depend on the T cell avidity for the HLA/peptide complex (Cawthon et al. 2001).

Upon TCR and CD8 or CD4 co-receptor engagement with an HLA/peptide molecule, the active Lck kinase phosphorylates tyrosine residues on ITAMs present in the TCR-associated CD3 chains (**Figure 1.3**). The phosphorylated ITAMs then recruit the major kinase protein ZAP-70, which in turn binds and phosphorylates adapter proteins such as LAT and SLP-76 (Burack et al. 2002). These activated adapter proteins recruit other downstream signalling proteins, including phospholipase C (PLCγ) and growth factor receptor binding protein 2 (Grb2) in the lipid rafts of the plasma membrane. The downstream signalling cascade results in the increase of cytosolic calcium level, the activation of protein kinase C (PKC) and the phosphorylation cascade of the Ras and MAP (Mitogen-activated protein kinases) pathway. These biochemical events triggered at the cell membrane lead to the dephosphorylation and activation of transcription factors, namely the nuclear factor of activated T cells (NFAT), NFκB and AP-1. These factors bind and induce gene transcription, which results in cellular proliferation, cytokines and/or effector molecules expression and secretion (Cantrell 1996; Germain and Stefanova 1999; Isakov and Altman 2002).

The crucial role of these signalling adapter proteins for the full activation and function of T cells has been shown in a number of mutant T cell line models. The deficiency in Zap-70,

SLP-76 or LAT signalling proteins have in fact been demonstrated to result in a lack of downstream activation of the PLC and Ras kinases (Finco et al. 1998; Herndon et al. 2001; Matsumoto et al. 2002; Yablonski et al. 1998). The ensuing absence of calcium mobilization, NFAT and/or NFκB activation consequently resulted in the immune unresponsiveness of these mutant cells.

The formation of the IS as well as the T cells signalling is also dependent on the nature of the co-stimulatory signals provided by the APCs in parallel to the HLA/peptide signals (Lee et al. 2002).

1.4.4 T cell activation and co-stimulatory signals:

Although T cell activation with the minimal TCR/CD3 and CD8 molecules engagement has been described (Reich et al. 1997), there are an increasing number of costimulatory molecules that have been demonstrated to be essential for the modulation of T cell responses. These costimulatory molecules fall into two families of receptors, the immunoglobulin-like receptor family that is comprised of CD28, Costimulation T Lymphocyte Antigen 4 (CTLA-4), Programmed Death 1 (PD-1) and Inducible COStimulator (ICOS); and the TNF receptor family, which include the CD27, CD40L (CD154), 41BB (CD137), OX40 (CD134) and Fas (CD95) molecules. These molecules can also be separated into two categories, depending on their T cell activation or suppression capacity and are represented in **Figure 1.4**. The majority of these costimulatory molecules promote T cells activation, expansion and acquisition of effector functions. Conversely, negative costimulation can prevent T cell activation (CTLA-4) or induce programmed T cell death (PD-1 and Fas). These negative co-stimulations are essential for immune regulation by preventing unwanted responses or continuous activation of T cells.

The CD28 costimulatory molecule is thought to be the second major signal necessary for T cell activation and is required for the activation of naïve T cells (Bachmann et al. 1997; Lucas et al. 1995). CD28 is constitutively expressed on T cells and binds the B7 receptor family, B7.1 (CD80) and B7.2 (CD86) expressed on APCs. CD28 is part of the early costimulatory signal and has been shown to promote the formation of the IS (Viola et al. 1999). However, CD28-independent T cell activation has also been described and correlated with TCR binding affinity and avidity for HLA/antigen complexes (Bromley et al. 2001b; Kundig et al. 1996).

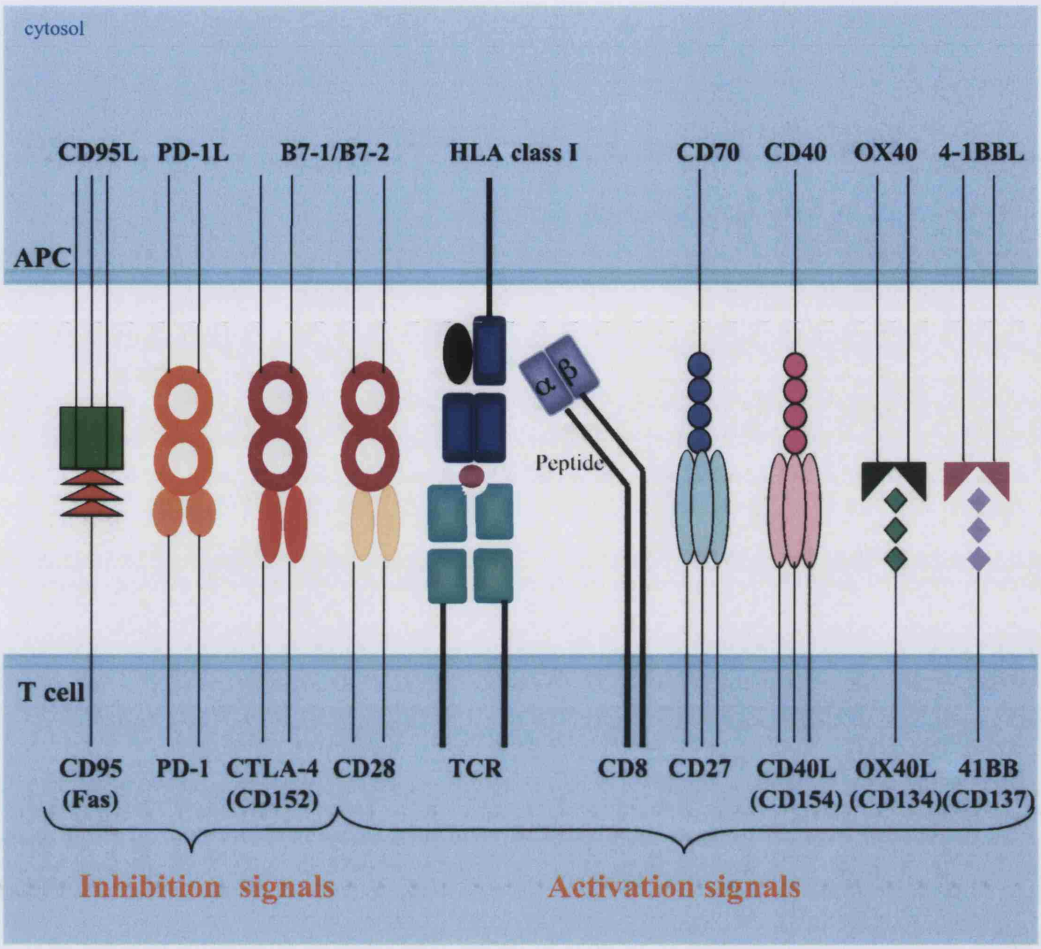


Figure 1.4: Costimulatory and inhibitory signals to T lymphocytes:

The major costimulatory and inhibitory mediators of T lymophocytes activation are represented. The recognition and binding of the **TCR** with the HLA class I/peptide complexes and the **CD8** co-receptor brings the T cell close to the APCs. The major early signals are provided by binding to the **CD28** molecules (major costimulatory signal required for the priming of naïve T cells). Other important costimulatory signals are provided by the binding of the TNF receptor family, including **CD27** (early signal), **CD40L** (late signal), **OX40L** and **41BB** receptors (signals for T cell survival and maintenance of immune response). The negative signals are provided by binding to the **CTLA-4** molecule (direct competitor of the CD28 molecules for binding to the B7.1 and B7.2 receptor on APCs). The **PD-1** (programmed death-1) and the **CD95** (Fas) signals both activate the caspases pathway and lead to the cell death. These co-stimulatory signals collectively contribute to the activation or inhibition of antigen specific T cell responses.

The expression of CD28, as well as B7.1 and B7.2 molecules, is highly upregulated upon antigen activation (Lenschow et al. 1996). CD28 ligation in conjunction with TCR engagement has been demonstrated to enhance the magnitude and the duration of the T cell response by inducing IL-2 cytokine and anti-apoptotic Bcl-xL gene expression (Coyle and Gutierrez-Ramos 2001; Jenkins et al. 1991). Although upon ligation, the cell surface CD28 receptor down-regulates, it also induces the cell surface expression of other costimulatory molecules, such as ICOS, CTLA-4 and/or PD-1.

CTLA-4 is stored in intracellular vesicles and expressed a few days following T cell activation (Egen and Allison 2002). This receptor shares a high level of homology with the CD28 molecule and directly competes for binding to the same ligands B7.1 (CD80) and B7.2 (CD86) (Coyle and Gutierrez-Ramos 2001). The affinity and binding avidity of CTLA-4 for these ligands is 20 times higher than of the CD28 molecule, thus favouring the delivery of a negative signal. CTLA-4 has been shown to prevent IL-2 gene transcription, thus arresting cell cycle progression (Brunner et al. 1999). CTLA-4 knock-out mice have been demonstrated to develop a lethal lymphoproliferative disorder (Tivol et al. 1995). Thus, the blockade of T cell activation (also called anergy) by CTLA-4 feedback signalling is crucial for the regulation of the immune responses.

CD27 costimulatory molecules are constitutively expressed on resting naïve T cells and can be upregulated upon TCR/CD3 engagement. Its ligand, CD70, is expressed on APCs but also on activated T cells (Croft 2003). Thus CD27/CD70 signalling not only provides activating signals from APCs but also contributes to the cellular communication between T cells. CD27 ligation has been shown to induce strong T cell proliferation, especially of naïve T cells, at the same time or just after CD28 signalling (Hintzen et al. 1995; Kobata et al. 1994). The cross-linking of CD27 has been demonstrated to increase the magnitude of antigen-specific T cell responses, especially in a secondary or recall immune response (Hendriks et al. 2000; Rowley and Al-Shamkhani 2004). The CD27 co-stimulation signalling is independent of IL-2 cytokine transduction but has been suggested to promote T cells survival by the induction of anti-apoptotic gene expression (Hendriks et al. 2000).

In contrast to CD28 and CD27 costimulatory molecules, ICOS, CD40L (CD154), 41BB (CD137), and OX40 (CD134) are not expressed on resting T cells but become upregulated from 24 hours to a few days following T cell activation (Croft 2003). CD40L (CD154) is expressed on activated T lymphocytes and has been demonstrated to be of major importance in $CD4^+$ T cell help for DC and B cells maturation, and to a lesser extent for cytotoxic $CD8^+$ T lymphocytes clonal expansion and differentiation (Bennett et al. 1998; Bourgeois et al.

2002; Schoenberger et al. 1998). CD40L knock-out mice have been shown to be defective in their ability to prime antigen-specific lymphocytes, thus resulting in a higher susceptibility to parasite infections (Moodycliffe et al. 2000; Noelle 1996). These observations demonstrate the crucial role of CD40L costimulation in both cell-mediated and humoral immunity.

Finally, 41BB and OX40 are expressed on activated lymphocytes and bind 41BBL and OX40L respectively, which are expressed on professional APCs (Croft 2003). Both costimulatory signals have been demonstrated to act downstream to the early CD28 activation signal and to promote antigen specific T cell survival by inducing anti-apoptotic Bcl-xL and Bfl-1 protein expression (Bertram et al. 2004; Diehl et al. 2002; Rogers et al. 2001). Both 41BBL and OX40L are also expressed on activated T cells and participate in the cellular communication between lymphocytes. While 41BB costimulation has been shown to principally affect $CD8^+$ T cell responses (Bertram et al. 2004; Bertram et al. 2002; Croft 2003), OX40 has also been shown to affect $CD4^+$ T cell responses (Gramaglia et al. 2000).

The different costimulatory molecules act collectively to activate, sustain or suppress antigen specific T cell responses.

1.5: T cell differentiation and effector functions:

Antigen stimulated T cells undergo a large degree of expansion and acquire the ability to differentiate into effector cells with the capacity of eliminating the pathogen-bearing cells, which provided the original stimulus. Activated T cells upregulate cell surface markers, such as CD69 receptor, early after stimulation. As CD69 is expressed within a few hours following TCR signalling, it has become a standard marker for recent T cell activation events (Hamann et al. 1993).

1.5.1 Cytokine secretion:

As mentioned previously, T cell costimulation results in the expression of a number of genes coding for cytokine production, which sustain antigen-activated T cell proliferation and induce cell differentiation. The major cytokine produced upon activation is IL-2. IL-2 binds to the IL-2 receptor expressed on T cells and induces a signalling cascade resulting in the progression of T cells through cell cycle division (Minami et al. 1993). Thus, IL-2 strongly participates in the amplification of an antigen specific T cell response.

Another cytokine promoting antigen specific T cell survival and proliferation is IL-15 (Becker et al. 2002; Judge et al. 2002; Schluns et al. 2002). In contrast to IL-2, IL-15 is not produced by T lymphocytes but by other cell types, including monocytes (Fehniger and Caligiuri 2001). While IL-2 regulates the immune response by promoting activation-induced cell death of antigen specific T cells, IL-15 does not induce this signalling pathway and actually prevents it (Marks-Konczalik et al. 2000). Similarly to IL-15, IL-7 has been shown to be crucial for the survival and maintenance of lymphocyte homeostasis (Prlic et al. 2002). Additionally, IL-7 has been shown to increase antigen specific cytotoxic activity and has been widely introduced in protocols for culturing antigen-specific T cells *in-vitro* (Abdul-Hai et al. 1997; Lalvani et al. 1997).

These cytokines contribute to both the proliferation and survival of antigen-activated T cells, as well as the acquisition of effector functions, which are crucial for the elimination of the pathogen-infected cells or transformed cells.

1.5.2 T cell-mediated cytotoxicity:

Activated T cells recognise foreign antigens presented on infected or transformed cells in the context of HLA class I and/or class II molecules and mediate their destruction. There are two major lymphocyte-mediated killing pathways, the trans-membrane death-signalling via Fas (CD95)/FasL (CD95L) interaction and the calcium-dependent perforin/granzyme pathway (Kagi et al. 1996; Russell and Ley 2002).

The activation of T cells induces the expression of the CD95L, which bind to CD95 present on target cells and subsequently induce the trimerisation of the receptor. The CD95 trimeric-cytoplasmic death domains initiate the caspases cascade, which lead to DNA fragmentation and apoptosis of the target cell. The FasL pathway is believed to be the major cellular mediated cytotoxicity of $CD4^+$ activated T cells. Besides, this pathway has been demonstrated to also be involved in the maintenance of peripheral tolerance by inducing programmed cell death of activated T cells (Ju et al. 1999; Lynch et al. 1995).

Alternatively, activated T cells release perforin and granzyme granules to the site of contact with target cells. The secretion of perforin damages cell membrane plasma, thus creating pores in the target cell. This leads to the entry of the second cytotoxic granules, the serine proteases namely granzymes and mainly granzyme B, which lead to the caspases cascade as described above (Trapani and Smyth 2002). The perforin/granzyme pathway is believed to be the major Cytotoxic T Lymphocytes (CTLs) mediated killing pathway.

Apoptotic target cells are then ingested by phagocytes and eliminated from the tissue or circulation. The acquisition of effector functions and maintenance of an antigen-specific T cell memory pool upon antigen encountering is a characteristic of T cell mediated immunity.

1.5.3 T cell differentiation:

As mentioned previously, T cells bearing a functional TCR leave the thymus and circulate through the tissue in their surveillance role. These T cells are referred to as naïve lymphocytes, as they have not yet encountered their cognate antigen. Upon antigen stimulation, T cells differentiate into effector/memory subsets. This encompasses both lymphocytes that expand for the control of the infection (effector) and those that acquire receptors for homing into the lymph node, to provide a more rapid response in subsequent potential antigen exposure (memory). Effector cells can further differentiate into "terminal effectors" with a lower capacity to expand but a greater cytotoxic ability to eradicate pathogen-infected cells. These distinct T lymphocytes differentiation states can be characterised by the expression of cell surface markers, including phenotypic markers, costimulatory markers and effector molecule secretion as represented in **Figure 1.5**.

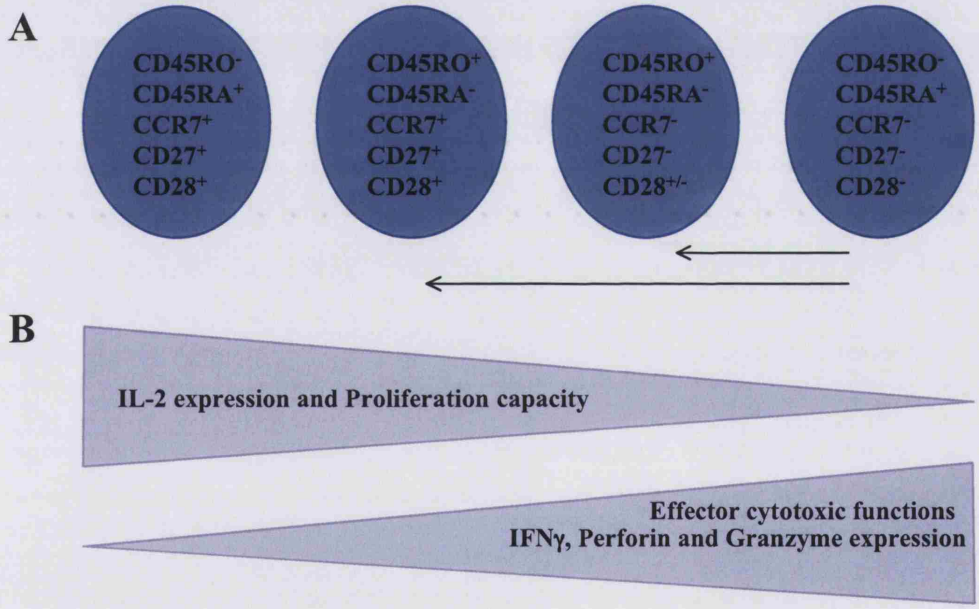


Figure 1.5: T lymphocyte differentiation pathways:

$CD8^+$ T lymphocytes, upon antigen stimulation differentiate from Naïve cells to different population subsets, including Central memory, effector memory and terminal effectors. The differentiation of antigen specific T cells can be characterised by the expression of cell surface phenotypic markers CD45RA/CD45RO, homing chemokine receptor CCR7 and costimulatory molecules CD27 and CD28 (panel **A**). The differentiation of activated T cells induces the modification of proliferation capacity, as well as the acquisition of cytotoxic functions such as expression of perforin and granzyme cytolitic proteins (panel **B**). The arrows in panel A represent the conversion of effector cells into a less differentiated memory phenotype.

Initially, the high molecular isoform of leukocyte common antigen CD45RA was associated with naïve T cells, while the CD45RO antigen was associated with antigen-experienced cells (Akbar et al. 1988; Michie et al. 1992). It has now been demonstrated that the $CD45RO^+$ memory population revert to $CD45RA^+$ and acquired higher cytolytic functions, such as perforin and granzyme molecule up-regulation (Hamann et al. 1997). In their study, the costimulatory marker CD27 and CD28 were shown to distinguish between the $CD45RA^+$ naïve population and the terminally differentiated cytotoxic $CD45RA^+$ reverted population. The down-regulation of these markers correlated with the progressive loss of proliferative capacity and the shortening of telomerase length in parallel to the progressive acquisition of effector functions as shown in **Figure 1.5** (Appay et al. 2002; Hamann et al. 1997). Furthermore, the chemokine receptor CCR7, which controls lymphocytes homing to the secondary lymphoid organs, has been shown to further distinguish two different populations within the memory T cell subset (Champagne et al. 2001; Sallusto et al. 1999; Wills et al. 2002). The naïve $CD45RA^+$ lymphocyte population differentiated into $CD45RA^-CCR7^+$ cells, also referred to as central memory, and $CD45RA^-CCR7^-$ T cells, referred to as effector memory. The central memory population has been demonstrated to have a higher proliferation capacity, lack effector functions and represent the population, which expands and generates a rapid effective immune response upon antigen rechallenge.

In contrast, the effector memory population has a lower proliferation capacity but expresses a higher level of both perforin and granzyme cytolitic proteins. The rate and proportion of differentiation from naïve cells into $CD45RA^-CCR7^+$ central memory, or $CD45RA^-CCR7^-$ effector memory cells, has been suggested to depend on the load of antigen, as well as duration and strength of TCR stimulation (Champagne et al. 2001; Sallusto et al. 1999; Wherry et al. 2003). Although the terminally differentiated lymphocytes ($CD45RA^-CCR7^-$) were thought to irreversibly commit to this status and undergo activation-induced cell death, it has been demonstrated that these cells can revert to a less differentiated $CD45RO^+$ memory population (Wills et al. 2002).

1.6: Regulation of the Immune system:

The regulation of cell-mediated immunity is crucial for the suppression of unwanted T cell responses, such as those targeted to self-antigens or harmless environmental antigens. The major regulatory mechanism to suppress self-antigen specific T cells is mediated by positive and negative selection during T cell development in the thymus. Some auto-reactive T cells have, however, been shown to be capable of escaping from this selection and it is believed that some self-proteins do not access the thymus or are expressed later in life, after the formation of the T cell repertoire (Bouneaud et al. 2000; Stockinger 1999). Thus additional regulatory mechanisms must occur in the periphery. Dendritic cells have been widely documented to mediate both innate and adaptive immune responses, but they are also capable of inducing T cell unresponsiveness or tolerance in the central lymphoid organs and the periphery (Jonuleit et al. 2001; Steinman et al. 2003).

As mentioned earlier, DCs have the capacity to take up antigens from their surroundings and generate a potent antigen-specific T cell response. It has, however, been demonstrated that the uptake of antigens from apoptotic cells failed to induce DCs maturation. The resulting lower costimulatory efficiency of immature DCs was associated with the induction of T cell anergy or tolerance to the presented self-antigens (Hawiger et al. 2001; Liu et al. 2002; Steinman et al. 2000). The delivery of antigen to immature DCs *in-vitro* or *in-vivo* has been demonstrated to induce antigen-specific T cell tolerance (Bonifaz et al. 2002; Dhodapkar and Steinman 2002; Dhodapkar et al. 2001; Jonuleit et al. 2000). These so-called regulatory T cells (Tr) have been shown to produce a significant amount of the suppressive IL-10 cytokine and to inhibit the proliferation of lymphocytes in an antigen-specific manner. Alternatively IL-10 treated DCs, also referred to as regulatory DCs, have also been demonstrated *in-vitro* to induce the generation of Tr cells (Steinbrink et al. 2002; Wakkach et al. 2003). Both CD4 and CD8 Tr populations were described in these studies. The $CD8^+$ restricted Tr were IL-10-dependent and similar to the previously described Tr generated in the periphery by immature DCs.

In contrast, $CD4^+$ suppressor T cells represent a professional regulatory population also referred to as Treg, which arise from the thymus (Sakaguchi 2004; Shevach 2001). These cells have been characterised to have a low proliferative capacity and to constitutively express cell surface IL-2 receptor α chain (CD25), CTLA-4 and glucocorticoid-induced TNF receptors (Annunziato et al. 2002; McHugh et al. 2002; Shimizu et al. 2002). Another characteristic of the $CD4^+CD25^+$ Tr cells is the expression of the forkhead transcription

factor Foxp3, which has been demonstrated to be crucial for both their development and their functions (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003). In contrast to the $CD8^+$ Tr, the Treg ($CD4^+CD25^+$) have been demonstrated to suppress immune responses in an antigen non-specific but TCR-dependent manner (Thornton and Shevach 1998). Mice deficient in $CD4^+CD25^+$ Treg have been shown to develop multiple organ auto-immune disease and the infusion of Treg has been demonstrated to prevent autoimmunity in different models (Asano et al. 1996; Hoffmann et al. 2002; Taylor et al. 2002). Thus, these $CD4^+CD25^+$ Tregs are believed to be essential for the maintenance of lymphocyte homeostasis and for the prevention of auto-immune responses targeted to self-antigens, including tumour antigens (to be discussed later).

1.7: Chronic Myelogenous Leukaemia:

Chronic Myeloid Leukaemia (CML) is a clonal disease that results from an acquired genetic abnormality in a pluripotent haematopoietic stem cell. This stem cell proliferates and generates a population of differentiated cell that gradually replaces the normal marrow with a leukaemic myeloid mass. CML incidence is constant worldwide and occur in 1 per 100 000 of the population, accounting for 15-20% of all leukaemias in adults (Enright and McGlave 2000). The incidence is constant in all decades, above the age of 20 years, with a slightly higher incidence in males than females. No predisposing factors have been associated with the development of CML, but an increased incidence has been observed in individuals exposed to the atomic bomb in Hiroshima and Nagasaki during the Second World War, implicating radiation as a contributory factor. The identification of the chromosomal abnormality associated with CML, the Philadelphia (Ph) chromosome by Nowell and Hungerford in 1960 has provided a valuable marker for disease diagnosis (Nowell and Hungerford 1960). In addition as the Ph chromosome represent the first genetic abnormality associated with a specific human malignancy and is present in 95% of patients, CML is an ideal candidate disease for assessing targeted therapies.

1.7.1 Cytogenetics of CML:

The hallmark Ph chromosome in CML is the reciprocal translocation t(9;22)(q34;q11) between the *c-abl* oncogene on chromosome 9 and the *bcr* region on chromosome 22 (Rowley 1973). The juxtaposition of variable 5' sequences from the *bcr* gene on chromosome 22 with the majority of 3' *abl* sequence derived from chromosome 9 produces a chimeric bcr/abl gene, which is transcribed into an 8.5kb mRNA and translated into a 210kDA protein with a greater tyrosine kinase activity than its normal *abl* gene counterpart (Groffen et al. 1984; Shtivelman et al. 1985). The position of the genomic breakpoint in the *abl* gene is variable but always occurs upstream to the a2 exon. There have been three major genomic breakpoints described in the *bcr* gene (or breakpoint cluster region). Most commonly in CML, breakpoints occur within the Major region (M-bcr) between the exons b1 and b5. Two of the major breakpoints within the M-bcr gene have been described to occur between the exons b2 and b3 or between the exons b3 and b4 and give rise to the b2a2 and b3a2 bcr/abl fusion gene respectively, as schematized in **Figure 1.6** (Kurzrock et al. 1988; Melo 1996). The mRNA b2a2 transcript has been observed in 40% of CML patients and the mRNA b3a2 transcript in another 40% of patients. Alternative splicing has been shown in the remaining 20% of CML patients, which possess both b2a2 and b3a2 mRNA

transcripts. Both b2a2 and b3a2 mRNA translate into an active 210kDa BCR/ABL protein, also referred to as p210 BCR/ABL.

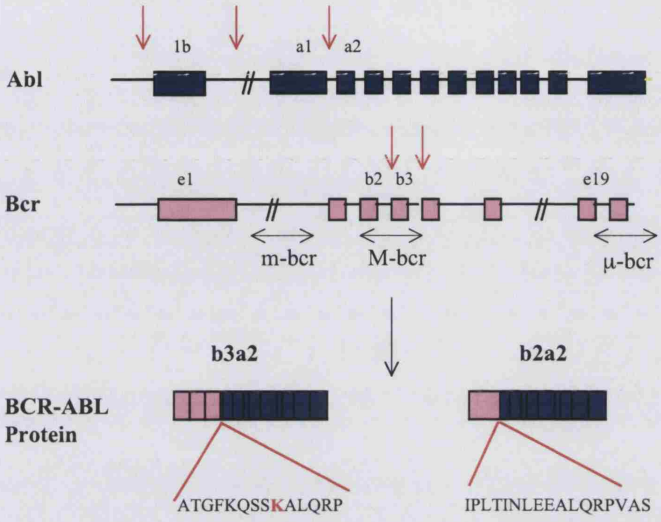


Figure 1.6: Bcr/abl gene translocation:

The Philadelphia chromosome is the result of the translocation between the c-abl gene on chromosome 9 and the bcr gene on chromosome 22. The breakpoints can occur at different locations and are represented with the red arrows. The most frequent breakpoints in the Abl gene occur upstream of the a2 codon. Breakpoint in the Bcr gene can occur within the minor (m-bcr), the Major (M-bcr) or the micro (μ-bcr) breakpoint regions, as represented. The two major breakpoints occur within the M-bcr region, upstream of the b2 or b3 codon. These fusion genes are then transcribed into an active 210kDa protein, the b2a2 and b3a2 BCR/ABL protein respectively. The characteristic amino sequences of these fusion proteins are represented, with the new lysine (K) amino acid in the b3a2 fusion protein shown in red.

Another breakpoint has been described to occur within the first *bcr* exon in the minor region (m-bcr), which results in the translation of the e1a2 fusion gene and transcription of a 190kDa chimeric BCR/ABL protein. Although very rarely present in CML patients, the e1a2 fusion gene is very common in Ph positive Acute Lymphoid Leukaemia (ALL) (Hermans et al. 1987; Saglio et al. 1996). Finally another genomic breakpoint has been described to occur in Ph positive chronic neutrophilic leukaemia within the micro region of the *bcr* gene (μ-bcr), resulting in the transcription of the e19a2 bcr/abl fusion gene into a 230kDa chimeric protein (Pane et al. 1996). The expression of the reciprocal translocated abl/bcr fusion gene has also been characterised in CML patients (Melo et al. 1993).

1.7.2 BCR/ABL fusion protein pathogenesis in CML:

The ability to transform myeloid cells and induce a CML-like disease in transgenic mice bearing the bcr/abl fusion gene clearly demonstrated the role of the bcr/abl translocation in the pathogenesis of CML (Daley et al. 1990). The p210 BCR/ABL protein has a deregulated abl tyrosine kinase activity and was defined as the pathogenetic cause of the development of CML (Lugo et al. 1990). In contrast to the endogenous normal p145 c-ABL protein located in the nucleus, the translocation of the p210 BCR/ABL protein has been shown to induce its relocation in the cytoplasm (Wetzler et al. 1993). This subcellular location is needed for the malignant related activity of the BCR/ABL protein. The induction of BCR/ABL protein accumulation in the nucleus with the drug leptomycin B (inhibitor of protein nuclear transport) was in fact shown to lead to the apoptosis of the cells (Vigneri and Wang 2001). Under the bcr promoter, the p210 BCR/ABL fusion protein has also been shown to be over-expressed in leukaemic cells, compared to the expression level of the c-ABL protein in normal cells and this has also been correlated to the markedly increased tyrosine kinase activity in transformed cells.

The c-ABL nuclear protein plays a major role in regulating cell cycle progression (Pendergast 2002). The overexpression of c-ABL has been shown to induce cell cycle arrest suggesting that it is a negative regulator of cellular growth. Additionally, c-ABL protein has been demonstrated to signal through integrins and to transmit information about the cellular environment to the nucleus (Van Etten 1999). The activity of the abl tyrosine kinase is regulated by the SH3 inhibitory domain (Mayer and Baltimore 1994). As the bcr/abl translocation induces the deletion of the abl SH3 domain, it also abrogates the physiological suppression of the abl kinase activity, which results in the constitutive tyrosine kinase activation of the BCR/ABL fusion protein.

The coiled-coil domain on the N-terminal BCR protein has been shown to induce the oligomerization of the BCR/ABL protein (He et al. 2002). The autophosphorylation of this oligomeric protein increases the phosphotyrosine residues on the BCR/ABL protein, which creates SH2 binding sites for a number of substrates involved in cellular functions and contributes to malignant transformation. Many of these substrates are intermediates of different signalling pathways, which lead to the transcription of genes involved in cell growth and differentiation. BCR/ABL protein substrates include adaptor molecules (such as Grb-2, Crkl and SHC), proteins associated with the organization of the cytoskeleton and adhesion (such as talin and paxillin) and catalytic proteins (Ras-GAP, PLC, ERK and PI3 kinase). The binding of these substrates result in the activation of a large number of cellular

pathways, including the Ras, MAP-kinase, Jak-Stat, PI-3 kinase and various CRKL signalling pathways, depending on the cellular context (Bedi et al. 1994; Gordon et al. 1987; Holyoake 2001; Salgia et al. 1997; Skorski et al. 1995; Tauchi et al. 1994). Thus the BCR/ABL fusion protein has been associated with a resulting enhanced cell growth, reduced apoptosis, reduced growth-factor dependency and altered adhesion to the marrow stroma of CML cells. The cellular transformations induced by the BCR/ABL fusion protein results in the development of CML in Ph positive patients.

1.7.3 Clinical manifestation of CML:

CML manifests itself by an initial chronic phase, which is symptomatic in some patients and relatively well controlled by the current therapies. This phase has been shown to last from 2 to 5 years before progressing into a short and more severe accelerated phase. Finally CML patients eventually progress to a terminal phase, known as blast crisis, which resembles acute leukaemias and is refractory to conventional therapies. During the initial chronic phase, myeloid progenitor cells retain their ability to differentiate and proliferate. However the progression of the disease induces a loss of differentiation resulting in accelerated growth of immature myeloid progenitors. Accordingly, an increased number of blast cells are present in the peripheral blood and in the bone marrow of CML patients in accelerated phase and blast crisis and their enumeration has been used as a diagnostic marker of disease status (Enright and McGlave 2000).

Additional chromosome abnormalities have been characterised in more advanced CML patients and may be responsible for therapy unresponsiveness. The duplication of the Ph chromosome translocation, isochromosome 17, trisomy 8 and trisomy 19 has been described in CML patients in blast crisis and is associated with a worse prognostic (Derderian et al. 1993). Molecular abnormalities in the p16, p53, Ras and retinoblastoma gene products have also been described to occur in 20% of CML patients in blast crisis (Guinn and Mills 1997; Sill et al. 1995). It is possible that the initial Ph chromosome translocation induces a genetic instability, which results in the occurrence of many other mutations. Subsequently, many therapies are now aiming to resolve the course of CML disease while in the chronic phase. The slow progression of the disease (2 to 5 years) also renders CML a good model disease for the assessment of immunotherapeutic treatments.

1.8: Management of CML:

1.8.1 Radio- and chemotherapies:

The initial use of arsenic for the treatment of CML patients in the 19th century demonstrated its efficiency in reducing the leukocyte cell count (Geary 2000). However due to its extreme toxicity, arsenic was replaced by the introduction of radiotherapy in 1903. The therapeutic effect of the X-rays was shown to prevent cell replication and induce disease remissions that lasted for months and occasionally years in some treated patients. The successive treatment of CML patients with radiotherapy eventually induced the disease to become refractory.

The introduction of the alkylating agent Busulphan and the less toxic anti-metabolite hydroxyurea in the 1950s for the treatment of CML and other haematological malignancies successfully achieved the normalization of the white blood cell count in treated patients. These cytotoxic drugs were found to control the chronic phase of CML by a relatively selective inhibition of DNA synthesis in haematopoietic tissues and particularly granulocytes (Enright and McGlave 2000). Busulphan has been replaced by hydroxyurea due to its association with severe bone marrow hypoplasia as a side effect and the prolonged survival observed in patients treated with hydroxyurea (Hehlmann et al. 1993). Hydroxyurea has not been shown to induce cytogenetic remission or change the natural history of CML disease, however it remains the palliative drug of choice, when other therapies are not available.

1.8.2 IFN-α:

Interferon-α (IFN-α) was introduced in the 1980s and has been demonstrated to increase the overall survival of treated patients on average by 20 months compared to the conventional chemotherapies (Hehlmann et al. 1994; Silver et al. 1999). IFN-α can induce haematological remission in up to 80% of cases and complete cytogenetic responses have been observed in 5 to 30% of CML patients treated in chronic phase. In addition, it has been suggested to delay the disease progression to accelerated phase (Bonifazi et al. 2001). Although the mechanism of action of IFN-α in CML remains unclear, the restoration of adhesion properties and subsequently cellular interactions of CML cells after IFN-α treatment has been demonstrated to inhibit their proliferation *in-vitro* (Cornelissen et al. 1998). Additionally, INF-α has been suggested to recruit cells of the immune system for

surveillance and increase the cytotoxicity of NK cells and T cells, which may in turn contribute to the anti-leukaemic response (Pfeffer et al. 1998).
IFN-α was until recently the standard treatment of newly diagnosed CML patients or those not eligible for, or who relapsed after HSCT (Higano et al. 1997).

1.8.3 Haematopoietic Stem Ccll Transplantation (HSCT):

Initially Haematopoiteic Stem Cell Transplantation (HSCT) had been used to correct or reconstitute the marrow failures experienced by patients who received high dose chemotherapies. Today HSCT constitutes the standard treatment option for many haematological malignancies and is currently the only curative treatment for CML. The general principle of HSCT consists of the eradication of tumour burden by a combination of radiotherapy and chemotherapy regimens, followed by the reconstitution of the bone marrow with the infusion of haematopoietic stem cells from a healthy donor. The first successful HSCT in humans was performed on identical twins (syngeneic transplant), but the patient demonstrated disease relapsed after few months (Thomas et al. 1959; Thomas et al. 1957). Since, HSCT has been applied broadly with the use of stem cells derived from related and unrelated HLA-matched donors (allogeneic transplant) as well as with stem cells derived from the patient themselves (autologous transplant) (Goldman 1992; James et al. 1989; McGlave et al. 1994; Thomas et al. 1977). Although initially HSCT were mostly unsuccessful and resulted in the rejection of the graft, especially these derived from allogeneic donors, much has improved since.

The first evidence of a graft versus leukaemia (GvL) response was demonstrated in animal models, by the eradication of leukaemia in mice receiving allogeneic, but not syngeneic, bone marrow transplant (Barnes et al. 1956). The confirmation of a similar GvL response post-HSCT in humans has also been provided by the observed higher rate of disease relapse in patients who received a syngeneic or a T cell depleted (TCD) graft when compared to those who received unmanipulated graft transplants from allogeneic donors (Drobyski et al. 1994; Horowitz et al. 1990; Jiang et al. 1991; Maraninchi et al. 1987; Weiden et al. 1979). In addition, donor lymphocyte infusion (DLI) has been demonstrated to be a successful salvage therapy for 70-80% of CML patients who relapse after HSCT (Collins et al. 1997; Dazzi et al. 2000; Kolb et al. 1990; Kolb et al. 1995; Mackinnon et al. 1995; Porter et al. 1994; van Rhee et al. 1994). Thus immuno-competent donor T cells transplanted with stem cells exert a potent allogeneic GvL effect. In addition to the GvL response, the immunocompetent cells from the donor can also mount an immune response against the recipient cells causing Graft

versus Host Disease (GvHD). This remains a significant cause of morbidity and mortality for 25-30% of patients following HSCT. Although GvHD can be prevented by the removal of T cells from the graft, the simultaneous depletion of the anti-leukaemic cells often leads to poor-engraftment and disease relapse (Ho and Soiffer 2001).

Following HSCT, the T cell repertoire reconstitution is primarily derived from the expansion of the mature T cells present within the stem cell graft, but also from the host cells that survived the chemotherapy (ie chimerism). Because of the reduced thymic output in adults, there is a slow recovery and a narrow diversity of the T cell repertoire (Roux et al. 2000). It has been demonstrated to take at least 12 months to recover a normal T cell repertoire and can even take up to 2 to 3 years for TCD HSCT, or if immunosuppressive drugs have been used to treat GvHD (Shilling et al. 2003; Verfuerth et al. 2000; Wu et al. 2000). This prolonged state of immune deficiency is responsible for a number of life-threatening opportunistic infections, including those due to viruses, bacteria and fungi.

HSCT has proved to be an undisputable curative therapy for 45-70% of treated CML patients (Enright and McGlave 2000). With the gained experiences in HSCT therapies, it has been established that a higher survival rate has been obtained in younger patients, receiving stem cell graft from a better HLA-matched, young and same gender donor; treated soon after diagnosis and in early chronic phase of the disease (Clift et al. 1994; Devergie et al. 1997; Enright et al. 1996; Goldman et al. 1993; Gratwohl et al. 1998; Petersdorf et al. 2001; Shaw et al. 2003; van Rhee et al. 1997). Additional factors, such as the transplant conditioning regimen, the source and the nature of the stem cell graft and the GvHD prophylaxis treatment have also been associated with a better or worse engraftment, disease relapse, GvHD and overall survival (Gratwohl et al. 2002; Korbling and Anderlini 2001; Lee 2000; Storek et al. 2001). HSCT settings remain in constant improvement with the application of reduced intensity conditioning and aim to prevent GvHD while retaining and/or stimulating GvL responses (Amrolia et al. 2003; Chakraverty et al. 2002; Craddock 1999; Koh et al. 1999; Niederwieser et al. 2003; Radich et al. 2003).

However, only 15 to 20% of patients are candidates for HSCT, due to the limited and low probability of having an HLA-matched suitable donor (Enright and McGlave 2000). The characterisation of the molecular events responsible for the transformation of CML cells, namely the bcr/abl signalling pathways have allowed the development and assessment of a number of active blocking molecules for alternative treatment modalities for these CML patients.

1.8.4 Molecular targeting of the BCR/ABL fusion protein:

As described earlier, the bcr/abl gene translocation induces the up-regulation of abl tyrosine kinase activity, which acts on various cellular signalling pathways and is responsible for the development of CML disease. A number of molecules targeted to BCR/ABL have been developed and assessed for their efficiency in the inhibition of its oncogenic activity.

Imatinib mesylate (Gleevec):

The most succesfull molecule described thus far for targeting BCR/ABL tyrosine kinase activity is Imatinib mesylate (2-phenylaminopyrimidine) also referred to as signal transduction inhibitor 571 (STI-571), Gleevec, Glivec and formally CGP57148. Imatinib mesylate competes for the adenosine triphosphate (ATP) binding site on the BCR/ABL protein (Schindler et al. 2000). This binding blocks the autophosphorylation of the kinase thus preventing the phosphorylation of the BCR/ABL substrates required for downstream signal transduction. Imatinib mesylate has been demonstrated *in-vitro* to selectively induce growth-arrest and apoptosis of BCR/ABL positive cells without affecting normal cells (Deininger et al. 1997; Druker et al. 1996; Gambacorti-Passerini et al. 1997). The abrogation of cellular proliferation has been shown to be mediated through the inhibition of the abl tyrosine kinase activity, as well as the stem cell factor receptor c-kit and the platelet-derived growth factor receptor (PDGFR) tyrosine kinases (Druker and Lydon 2000; Druker et al. 1996).

Imatinib mesylate has been shown to induce long-term cytogenetic remission with minimal toxicity in more than 60% of CML patients in chronic phase, including patients refractory or resistant to IFN-α therapy (Braziel et al. 2002; Druker et al. 2001; Kantarjian et al. 2002a). Although Imatinib mesylate has been shown to exert some activity in accelerated or blast crisis CML patients and Ph positive ALL patients, only 10 to 24% of patients demonstrated cytogenetic responses (Druker et al. 2001; Kantarjian et al. 2002b; Sawyers et al. 2002; Talpaz et al. 2002). In these clinical studies, Imatinib therapy clearly was demonstrated to delay disease progression and to induce higher rates of cytogenetic responses and subsequently survival, when compared to other chemotherapies. Responses were also shown to be dose-dependent with the highest Imatinib mesylate dose inducing cytogenetic responses in 96% of chronic phase CML patients at 15 months (Cortes et al. 2003; Kantarjian et al. 2004; Talpaz et al. 2002).

However *in-vitro* insensitivity of quiescent Ph positive stem cells and disease relapse in long-term Imatinib mesylate treated patients, especially those in accelerated or blast crisis phase of CML, have been reported despite initial response to the drug (Graham et al. 2002; Kantarjian et al. 2002b). The development of Imatinib resistance has been shown to arise from various primary or acquired mechanisms, including increased expression of multi-drug-resistant protein MDR1 and/or BCR/ABL protein, bcr/abl gene amplification, clonal cytogenetic evolution and, most importantly, a point-mutation within the ABL-kinase domain (Branford et al. 2002; Gorre et al. 2001; le Coutre et al. 2000; Mahon et al. 2000; O'Dwyer et al. 2002). A number of point mutations have been described, within and outside the ABL active binding site and these have been shown to contribute to the resistance of Imatinib mesylate binding observed in patients who relapsed (Branford et al. 2002; Nardi et al. 2004; Shah et al. 2002; von Bubnoff et al. 2002). Some of the mutations have been associated with disease progression and poor prognostic.

Development of additional molecular targets:

Imatinib mesylate resistant clones have been shown to reactivate the BCR/ABL tyrosine kinase activity and signalling, thus the BCR/ABL protein itself and/or its downstream signalling protein remain a therapeutic target. A number of synthetic molecules targeting the binding of BCR/ABL to heat shock protein 90, Ras, Jak, Mek or PI-3 kinase pathway have been demonstrated to inhibit the proliferation of resistant clones *in-vitro* and their use in a clinic setting have been suggested in combination with Imatinib mesylate (Druker 2003; George et al. 2004; Gorre et al. 2002; Hoover et al. 2002; Nimmanapalli et al. 2001; Tipping and Melo 2003). In addition, other molecules such as arsenic trioxide, histone deacetylase inhibitor and the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) have also been shown to induce the selective apoptosis of Ph positive leukaemic cells (La Rosee et al. 2004; Nimmanapalli et al. 2003; Uno et al. 2003; Yu et al. 2003a). More recently newly developed tyrosine kinase inhibitors, such as adaphostin have been demonstrated *in-vitro* to selectively inhibit the proliferation of CML cells. These molecules inhibit the ABL tyrosine kinase via mechanisms other than Imatinib mesylate and have a dual Src/ABL kinase inhibitory activity. Thus, they may remain active for the inhibition of Imatinib mesylate resistant clones (Mow et al. 2002; Shah et al. 2004; Tipping et al. 2004). Their efficiency has yet to be determined in clinic but it would offer a rescue therapy for Imatinib mesylate resistant patients, especially those progressing in accelerating or blast crisis phase of CML disease and who become refractory to other conventional therapies including HSCT.

As described earlier, HSCT and DLI can eradicate the leukaemia via the GvL effect and remain the treatment of choice for young CML patients in early chronic phase that have an available HLA-matched donor (Goldman and Druker 2001). Unfortunately many patients suffer from other complications associated with HSCT such as graft-versus host disease, which account for one of the major cause of morbidity and mortality in transplanted patients. Both GvL and GvHD responses were initially believed to be mediated by the same, or at least a shared T cell population. However, GvL responses were isolated from GvHD in a number of mice models and CML patients demonstrated a clinical GvL response, without necessarily developing GvHD (Chen et al. 2002; Drobyski et al. 2003; Mackinnon et al. 1995; Rocha et al. 1997). Thus the characterisation of the antigen(s) recognized and initiating the GvL response would allow the development of more specific T cell therapies not only for post-HSCT patients, but also for those who are not candidates for HSCT.

1.9: Tumour antigens recognized by immune cells:

1.9.1 Characterisation of Tumour antigens:

The first attempt to identify antigens expressed by tumour cells and recognized by T cells was performed by culturing lymphocytes with autologous tumour cell lines and establishing specific cytotoxic T cell lines (CTL) (Traversari et al. 1992). Antigens recognized by these CTLs were characterised by constructing recombinant cDNA libraries derived from the tumour cell line. These cDNA libraries were then transfected into HLA-matched cells, which were tested for their ability to stimulate the CTL lines (Coulie et al. 1994; Van den Eynde et al. 1995). The cDNA library encoding the immunogenic antigen was then sequenced and synthetic peptides spanning the region of interest were synthesised and tested for recognition by CTLs. These methods allowed the identification of a large number of tumour melanoma antigens.

Another strategy for characterising target antigens is to acid-elute HLA-bound peptides and sequence them by tandem mass spectrometry. The resulting sequences are matched with the antigenic protein sequence and tested for sensitization of specific CTL lines (Castelli et al. 1995; Cox et al. 1994; Hunt et al. 1992). This strategy has also been used to confirm the presentation of antigenic peptides associated with HLA molecules, on the surface of CML cells and will be discussed later (Clark et al. 2001).

The understanding of the structural features of peptides bound to a specific HLA class I molecule by sequencing antigenic peptides eluted from the surface of different homozygous cell lines, allowed the characterization of the binding preference profiles for individual HLA molecules (Falk et al. 1994; Kubo et al. 1994). This resulted in the development of computer generated epitope prediction algorithms for a more rapid characterisation of antigenic peptides by "reverse immunology" (van der Bruggen et al. 1994). There are two major peptide prediction databases available to the public, Syfpeithi from the University of Tubingen in Germany (www.syfpeithi.de) and Bimas, BioInformatics and Molecular Analysis Section from the National Institute of Health in the United States of America (www.bimas.dcrt.nih.gov/molbio/hla_bind).

The Syfpeithi epitope prediction databases are based on a scoring system related to the presence of a particular amino acid at a specific position in the peptide sequence, which will favour or will not favour its binding to the HLA peptide-binding groove (Rammensee et al.

1999). A known dominant anchor residue is given an arbitrary value of 10, an auxiliary residue a value of 6, and a preferred residue a value between 1 and 4. Unfavourable residues are given values between –1 and –3. The final 8 to 11 mers peptide is given an overall value, which is the sum of the scores of each amino acid contained at the various positions.

The Bimas prediction database is also based on experimental data obtained by measuring the rate of dissociation of β-2 microglobulin from HLA/peptide complexes (Parker et al. 1992). Their data demonstrates that each amino acid component of the peptide contributes independently to the stability of the HLA complex. They quantified the contribution of every possible amino acid at each position to the binding affinity to HLA-A*0201 or other alleles and generated a theoretical coefficient table (Parker et al. 1994). Similarly to the Syfpeithi database, dominant anchor residues have a coefficient significantly different from 1, preferred anchors greater than 1, auxiliary close to 1 and unfavourable anchors lower than 1. Amino acids, which are not known to be either favourable or unfavourable for stable HLA class I binding are given a value of 1. The final score of a peptide is the product of the nine coefficients, multiplied by a final constant (an estimate of dissociation half time dependent on the selected HLA molecule). Both prediction databases state that the naturally presented epitopes from any antigenic protein are ranked in the top 2% of the listed peptides, in more than 90% of cases. However the epitope prediction algorithms reliability is only assured for the HLA class I (and some HLA class II) alleles for which a large number of natural peptide ligands are known.

More recently, prediction algorithms of proteosomal cleavages were developed to improve the definition of antigenic peptides. The C-terminal cleavage activity is crucial for the generation of HLA class I epitopes *in vivo*, as position 9 is, in most cases, an anchor motif for HLA binding affinity (Bouvier and Wiley 1994). Therefore, the prediction of proteosomal cleavage at the C-terminal can help by narrowing the screening process for antigenic peptides generated *in vivo*, especially in the case of a large antigenic protein. Three such algorithms are publicly available, Fragpredict, PAPROC (Prediction Algorithm for PROteosomal Cleavage) and NetChop. Fragpredict predicts the 20S subunit cleavage probability based on a statistical-empirical method developed by H.G. Holzhutter ((Holzhutter et al. 1999), www.mpiib-berlin.mpg.de/MAPPP). PaPROC was developed at the University of Tubingen and predicts the cleavage potential of a protein sequence as well as the individual cleavage strength based on experimental proteosomal cleavages obtained with wild type I, II and III 20S human erythrocyte proteosomes (ww.paproc.de). Finally NetChop predicts proteosomal cleavage sites by neural networks, which have been trained with a large database of publicly available human C-terminal MHC class I ligands

(www.cbs.dtu.dk/services/NetChop). Both epitope prediction and proteosomal cleavage algorithms can facilitate the pre-selection of a smaller set of peptides that matches the requirements of potential T cell epitopes and will be discussed in chapter 3.

Using these techniques, many tumour antigens that are recognized by T cells have been identified and classified into two different categories, namely tumour associated antigens or tumour specific antigens (Boon et al. 1994; Houghton 1994).

1.9.2 Tumour associated antigens:

The significant levels of genetic mutations that occur during cancer development induce genomic instability, which results in the over-expression or in the re-expression of individual proteins (Stoler et al. 1999). These proteins represent a source of tumour antigens, also referred to as tumour associated antigens (TAA), since their expression is not entirely restricted to the particular tumour cells. One major advantage of targeting these tumour-associated antigens in T-cell based immunotherapy is that they are often shared between different tumours. However their use remains restricted to cancer where the destruction of self is more tolerable, as it is the case for melanocytes.

A number of over-expressed proteins have been characterised from patients with haematological malignancies, including CML patients and defined as potential antigen targets of the GvL response. These include 1) the proteinase 3 antigen (PR1), a primary granule enzyme expressed in normal promyelocytes, 2) the Wilm's tumour antigen (WT1), a zinc finger transcription factor expressed in various normal tissues and 3) the human telomerase (hTERT), a ribonucleoprotein enzyme, which plays a role in cellular replicative life-span by maintaining telomere length (Inoue et al. 1994; Molldrem et al. 1996; Molldrem et al. 2000; Vonderheide et al. 1999).

Although these antigens are also expressed by normal cells, their expression in tumour cells is significantly increased. For example, the Proteinase 3 expression was shown to be abundant in normal myeloid cells but was over-expressed up to 5-fold in some leukaemias (Dengler et al. 1995). WT1 protein is a transcription factor essential for embryonic development and has been shown in adulthood to have a low level of expression restricted to hematopoietic stem cells, myeloepithelial progenitor cells, renal, testis and ovary tissues (Huang et al. 1990). Antigens derived from the human ribonucleoprotein telomerase (hTERT) have been described as an ideal target for immuno-therapy strategies as it was

shown to be expressed by more than 85% of all cancer cells and was shown to be absent in most normal cells (Kim et al. 1994; Vonderheide et al. 1999).

Peptides derived from these tumour associated antigens have been identified in the context of the HLA-A*0201 molecule (Molldrem et al. 1997; Oka et al. 2000; Vonderheide et al. 1999), as well as in the context of the HLA-A*2401 molecule for WT1 and hTERT (Arai et al. 2001; Ohminami et al. 2000). Antigen specific T cells targeted to these cited antigens have been generated *in-vitro* and demonstrated to recognize leukaemic cells and inhibit their proliferation and/or induce specific cytotoxic responses (Arai et al. 2001; Bellantuono et al. 2002; Gao et al. 2000; Molldrem et al. 1997; Ohminami et al. 2000; Vonderheide et al. 2001). Additionally, reactive $CD8^+$ T cells specific for these TAA have been detected from the peripheral blood of leukaemic patients, including CML patients and in some cases these were correlated with clinical responses (Molldrem et al. 2000; Nagorsen et al. 2003; Rezvani et al. 2003; Scheibenbogen et al. 2002; Vonderheide et al. 1999). However these responses have not mediated tumour rejection in other patients and the mechanisms by which tumour specific T cells are thought to become unresponsive will be discussed in the following section.

In the context of HSCT, the differences in the minor histocompatibility antigens (mHA), due to the genomic polymorphism between the donor and the recipients have been shown to play an important role in mediating GvL and GvHD responses (den Haan et al. 1995; Goulmy et al. 1996; Mutis et al. 1999b). Some mHA antigen expression, such as HA-1 and HA-2, are restricted to haematopoietic tissues and has been strongly associated with the GvL response (Dickinson et al. 2002; Fontaine et al. 2001). Targeting CTL to these antigens was thought to preferentially mediate GvL responses, as normal haematopoietic cells (derived from the donor) will express limited amount of antigens, while residual leukaemic cells will express high level of antigens and thus be targeted by these CTLs. HA-1 and HA-2 specific T cells have been generated *in-vitro* and have been proved, in some case, to be restricted to haematopoietic tissues and mediate leukaemic specific cytotoxicity (Brossart et al. 1999; Mutis et al. 2002; Warren et al. 1998a; Warren et al. 1998b). More recently the transfer of HA-1 and HA-2 specific T cells in CML patients who relapsed following HSCT have been demonstrated to induce complete cytogenetic remission (Marijt et al. 2003). However some of these HA specific T cells remain closely associated with GvHD responses and thus may not constitute the ideal targets for adoptive T cell immunotherapy (Mutis et al. 1999a).

1.9.3 Tumour specific antigens:

Another source of tumour antigen are those derived from proteins that are only expressed in cancer cells but not healthy cells, thus they are specific to the tumour. These tumour specific antigens are encoded by translocated or mutated proteins (p53, src or ras, (Gedde-Dahl et al. 1993; Melief and Kast 1991)), which arise as initiators or as part of the oncogenesis process.

Two specific chromosome translocations have been described as initiators of a malignant transformation, the PML/RARα t(15; 17) in acute promyelocytic leukaemia and the bcr/abl t(9;22) in CML (Chen et al. 1992; Kagan 1993; Shtivelman et al. 1986). The translocated genes translate into a fusion protein, which acquires altered function and acts as transcriptional repressor (PML/RARα) or activator (bcr/abl). The expression of these fusion proteins is restricted to the transformed cells and required for their malignant activity. Thus, they represent an ideal source of tumour specific antigen to target in immunotherapeutic strategies, and will be discussed in more detailed in the context of CML disease.

1.10: Tumour escape from Immune recognition:

Reactive T cells for various tumour antigens have been detected in some cancer patients, but failed in the majority of cases to eradicate the tumour. Thus, it appears that tumour cells have developed a number of mechanisms to evade the immune system.

1.10.1 Immune Evasion mechanisms:

The development from a normal cell to a cancerous cell is thought to be a multistep process of clonal evolution driven by a series of somatic mutations that progressively convert the cell from normal growth to a cancerous state with a very high intrinsic rate of growth. The generation of an efficient immune response for the eradication of the tumour cells may not actually occur fast enough to keep the tumour outgrowth under control.

The characteristic genomic instability of tumours cells has been described to induce the loss of the β-2 microglobulin or HLA heavy chain expression, as well as the loss or mutation of the tumour antigen in cancer cells (Bicknell et al. 1994; Cormier et al. 1998; de Vries et al. 1997; Maeurer et al. 1996; Paschen et al. 2003; Restifo et al. 1993). This so-called variant loss can lead to the selection of tumour cells, which are not recognized and therefore are resistant to immune cells. Such antigen loss has also been described to occur in response to immunotherapeutically induced anti-tumour immunity. The vaccination of melanoma patients with tumour peptides, or adoptively transferred antigen-specific T cells, has been shown to induce the loss of tumour antigen expression, especially in patients who relapsed following therapy (Ohnmacht et al. 2001; Yee et al. 2002). Many tumour cells have also been demonstrated to be deficient in the specific transporter of antigen proteins (TAP), which results in impaired tumour antigen presentation (Johnsen et al. 1999; Sanda et al. 1995). The down-regulation or lack of HLA/peptide complex presentation on the surface of tumour cells can result in their escape from T cell recognition, however it has been demonstrated to induce NK cell mediated lysis (Moretta et al. 1996; Schrier et al. 1991). Thus the modulation of HLA expression by tumour cells may not actually represent the major immune escape mechanism of tumour cells, especially in the context of haematological malignancies.

1.10.2 Immune Suppression mechanisms:

A wide range of tumour cell lines has been demonstrated to express negative signalling molecules and/or secrete immunosuppressive cytokines, which are believed to represent a major immune evasion mechanism. The expression of the cell-death signalling receptor FasL by tumour cells, including leukaemic cells has been shown to counter attack the attack by anti-tumour specific lymphocytes resulting in their escape from immune surveillance (Kamihira et al. 1997; O'Connell et al. 1998; Okada et al. 2000; Strand and Galle 1998). However this suppressive mechanism remains controversial as the engineering of tumour cells to express cell surface FasL have been shown to actually enhance *in-vivo* tumour regression (Arai et al. 1997). Additionally, the secretion of immunosuppressive cytokines, such as TGF-β and IL-10 have been characterised from the culture supernatant of tumour cell lines (Kim et al. 1995; Moretti et al. 1997). The increased level of expression of these cytokines has been demonstrated to correlate with disease progression and therefore support their role in tumour escape of immune surveillance (Conrad et al. 1999).

1.10.3 Immune Tolerance mechanisms:

The development of cancer from the initial molecular mutation can take several years and it has been therefore suggested that immunosurveillance may actually contribute to the selection of non-immunogenic tumour clones and induce the most profound immune tolerance. Besides the majority of tumour antigens are derived from self proteins, thus any reactive T cells would be expected to be negatively selected during T cell development in the thymus. As mentioned earlier, the high avidity self-antigen reactive T cells which have escaped this selection are believed to be deleted by peripheral tolerance mechanisms. Thus, the remaining anti-tumour specific T cells in many cases have been shown to recognize subdominant or cryptic tumour antigen determinants, which have very low affinity for binding to HLA molecules and are poorly immunogenic (Smyth et al. 2001).

In addition, since cancer cells initially arise from self-cells, these may fail in providing danger signals required for the generation of an effective T cell response. The uptake of tumour antigens by DCs, which have not received danger signals and are therefore immature, has been demonstrated to induce tumour specific T cell ignorance. Both the low duration of antigen presentation and the activation status of APCs have been suggested to contribute to the tumour escape from immune surveillance (den Boer et al. 2001; Spiotto et al. 2002). As previously mentioned, immature DCs can promote the generation of regulatory

T cells. In fact, a high frequency of $CD4^+CD25^+$ regulatory T cells has been detected from the peripheral blood and/or in tumour sites of cancer patients (Liyanage et al. 2002; Sakaguchi et al. 2001; Sasada et al. 2003; Wolf et al. 2003). Their increase has even been correlated with disease progression in some patients (Sasada et al. 2003). The presence of Treg in cancer patients is believed to be one of the major mechanisms for the induction of tumour-specific T cell tolerance as their removal in animal models was shown to restore antigen specific T cell immunity resulting in tumour rejection (Golgher et al. 2002; Shimizu et al. 1999; Tanaka et al. 2002a).

These observations have lead to the development of immunotherapeutic strategies aiming to increase or induce anti-tumour specific T cell responses. The majority of these strategies have been assessed in melanoma patients and will be mentioned as indications of available therapies and their outcome.

1.11: Immunotherapeutic strategies to enhance tumour immunity:

The tumour immunity of cancer patients can be enhanced by active or passive (also referred to as adoptive) immune-mediated therapies. Active therapies or vaccinations aim to stimulate endogenous responses targeted towards one or more antigens, which should result in the activation of T (and in some case B) lymphocytes. The progress in the molecular characterisation of tumour derived antigens has lead to the development of tumour-peptide vaccination trials. Although the use of antigenic peptides is restricted by the HLA type of each individual patient, their easy production to good manufacture practice, their stability and simple administration has proven to be very attractive for clinical use.

1.11.1 Active immunotherapies:

Peptide vaccination:

CML patients have been vaccinated with escalating doses of peptide pools derived from the BCR/ABL tumour specific antigen (Pinilla-Ibarz et al. 2000). Antigen-specific T cell proliferation was obtained with the highest dose of peptides and this then led to a phase II clinical trial (Cathcart et al. 2004). Although peptide dose was clearly demonstrated to correlate with an increase of *in-vivo* proliferative responses, no leukaemia-specific cytotoxic activity was observed. The lack of functional activity could be attributed to the large tumour burden of the patients enrolled in these trials. These patients were, in the majority of cases, in late stage disease where the induction of a tumour immune response is more likely to be tolerized *in-vivo*.

WT1 and hTERT peptide vaccinations have also been assessed in leukaemic or solid tumour patients (Oka et al. 2004; Parkhurst et al. 2004). An increased of antigen specific T cell frequency was observed in the majority of vaccinated patients and in the context of WT1, these were correlated with a clinical reduction of leukaemic blast cells or tumour sizes. In contrast, hTERT specific T cells detected from vaccinated patients failed to recognize endogenous tumour cells (Parkhurst et al. 2004).

Many small clinical trials have been performed, mainly in melanoma patients with metastatic tumour lesions using a wide range of antigenic peptides (Cormier et al. 1997; Coulie et al. 2002; Dutoit et al. 2002; Lee et al. 1999; Monsurro et al. 2002; Murray et al. 2002; Scheibenbogen et al. 2000). Although the frequency of tumour specific T cells detected in some patients following vaccination was also shown to increase proportionally

with the number of peptide injections, no correlation has been found with *in-vivo* tumour regression. Tumour specific T cells were shown to differentiate into memory and/or effector phenotype after vaccination, but the Monsurro group demonstrated that these cells were deficient in the perforin cytolytic protein, certainly accounting for their *in-vivo* unresponsiveness (Monsurro et al. 2002; Nielsen et al. 2000). Additionally, Murray et al. have shown that the *in-vitro* blockade of CTLA-4 signalling significantly increased the vaccine-induced tumour specific T cells anti-tumour activity (Murray et al. 2002). Thus, the lack of tumour specific responses following vaccination may be due to the presence of regulatory T cells (Tregs).

In an attempt to increase antigen specific T cell responses, a combination of peptide infusion with an adjuvant such as GM-CSF, QS-21 or incomplete Freund's adjuvant, was also assessed in some of these trials (Cathcart et al. 2004; Cormier et al. 1997; Dutoit et al. 2002; Murray et al. 2002; Pinilla-Ibarz et al. 2000; Scheibenbogen et al. 2000). These adjuvants were aiming to activate endogenous APCs especially DCs, to uptake the infused antigenic peptides and induce an immune response. Despite the increase of specific delayed type hypersensitivity (DTH) and $CD8^+$ T cell responses in these treated patients, the use of these adjuvants has not lead to the eradication of the tumours.

Modified or Heteroclitic peptide vaccination:

The immunogenicity of antigenic peptides can be substantially increased by the modification of a specific amino acid anchoring residue. The modification of a single amino acid residue in a wide range of tumour peptide sequences have been demonstrated to significantly increase the HLA/peptide affinity and subsequently its TCR interaction stability (Denkberg et al. 2002; Parkhurst et al. 1996; Scardino et al. 2002; Slansky et al. 2000; Tangri et al. 2001; Valmori et al. 1998; Valmori et al. 1999). These modified peptides, also referred to as heteroclitic peptides, have been demonstrated to be more immunogenic than the wild-type peptide, while retaining the primary tumour specificity. Melanoma patients were vaccinated with the HLA-A*0201 associated gp100 peptide, modified to contain a methionine at the second anchor position (Monsurro et al. 2001; Rosenberg 2001; Rosenberg et al. 1998a). Despite an apparent increase in the frequency of these peptide-specific T cells, no clinical responses were observed following vaccination with the modified peptide alone. In addition, the loss of native tumour cell recognition of these heteroclitic peptide specific T cells has been observed in some patients, suggesting that the anchor residue difference in the modified peptide may be recognized by T cells (Clay et al. 1999).

Heat-shock protein vaccination:

In an attempt to increase the antigenic peptides immunogenicity, the use of purified exosome or heat-shock proteins has been suggested as a source of tumour antigens for vaccination. Both exosomes and heat-shock proteins are released by stressed tumour cells and are associated with shared tumour antigens (Feng et al. 2001; Schartz et al. 2002; Trieb et al. 2000; Wolfers et al. 2001). Tumour derived exosomes or heat shock proteins have been demonstrated to elicit tumour specific immune responses *in-vitro* and to eradicate established tumour in mice models (Andre et al. 2004; Blachere et al. 1997; Yedavelli et al. 1999; Zitvogel et al. 1998). These specific tumour responses are believed to be mediated trough the transport of tumour antigens to APCs.

These proteins represent an ideal source of tumour antigens and do not require the knowledge of the specific antigenic peptide sequence. Clinical trials using autologous tumour derived exosomes are now under investigation (Chaput et al. 2003). The vaccination of melanoma and colon carcinoma patients with autologous tumour derived gp96 or heat shock protein 96, although only in a minority of patients, was demonstrated to induce potent anti-tumour specific T cell responses *in-vivo*, which were associated with an observed clinical response (Belli et al. 2002; Janetzki et al. 2000; Mazzaferro et al. 2003).

Recombinant viruses or DNA vaccination:

The advance in recombinant gene technology has also given rise to another route of tumour antigen delivery. Recombinant viruses or plasmid DNA encoding cancer antigens have been shown to generate potent anti-tumour responses in mice models (Nawrath et al. 1999; Tuting et al. 1999; Van Pel et al. 2001; Warnier et al. 1996). Viral vectors have been demonstrated to have the advantage of being able to mimick natural infection, leading to a strong cellular-mediated response and DNA vaccines have been shown to activate both the innate and adaptive immune system (Gurunathan et al. 2000). These strategies have not yet been assessed in leukaemic patients. Melanoma and prostate cancer patients have, however, been immunised with recombinant viral vectors or bacterial DNA plasmids targeted to different TAA (Eder et al. 2000; Lonchay et al. 2004; Rosenberg et al. 1998b; Tagawa et al. 2003; Wolchok et al. 2003). Despite boosting the immune responses with peptide vaccination in some trials, only weak anti-tumour specific T cell responses were observed in vaccinated patients. This was attributed to the presence of neutralizing antibodies against the viral envelope proteins or to the poor *in-vivo* DNA transfection efficiency.

All these different active vaccination approaches rely on the endogenous activation of APCs necessary for the generation of functional antigen-specific T cells. The relative lack of success of peptide vaccinations in cancer patients has lead to the development of other therapies aiming to increase the induction of tumour specific T cell expansion and activation. For this, the most potent APCs, the DCs have been suggested to represent the optimal cellular vaccination, which would directly present the antigenic peptide along with a controlled level of costimulatory molecules required for the priming of naïve T cells and the expansion of memory helper and cytotoxic T cells.

Dendritic cell vaccination:

The identification of specific DC markers and the development of culture protocols for the generation, maintenance and expansion of these cells have allowed the development of DC-based immunotherapies. A single clinical trial using autologous derived DCs, pulsed with the BCR/ABL peptides has been reported (Takahashi et al. 2003). Although DTH responses were observed in the three CML patients enrolled in this trial, no anti-leukaemic specific responses were generated. Again, these patients were in an accelerated phase, which may have accounted for the lack of tumour specific T cell responses.

A number of clinical trials using patient-derived DCs loaded with tumour antigens have been assessed in melanoma, prostate, gastric, breast or ovarian cancer patients (Brossart et al. 2000; Kono et al. 2002; Lodge et al. 2000; Nestle et al. 1998; Thurner et al. 1999; Vonderheide et al. 2004). In some cases, the responses to DC vaccination have been shown to induce a higher frequency of tumour specific CTLs activity than those generated with peptide vaccination (Thurner et al. 1999). In addition, the antigen specific T cell responses were correlated with clinical tumour metastasis reduction in some patients (Nestle et al. 1998). Thus, DC vaccination has demonstrated encouraging results in some patients. The protocols for optimal DC generation, the dose and the route of administration remained however still under investigation.

1.11.2 Passive (or adoptive) immunotherapies:

In contrast to active therapies, passive therapies consist of directly transferring the immune cells, ideally those with anti-tumour reactivity. Prior to the identification of tumour antigen recognized by T cells, tumour-infiltrated lymphocytes (TILs) were isolated from cancer patients and assessed for their function *in-vitro*. These TILs were demonstrated to be easily expanded with IL-2 and maintained their specific cytotoxic activity in tumour-bearing mice models (Rosenberg et al. 1986). The efficiency of autologous derived TILs and their adoptive transfer in melanoma patients has been assessed in many clinical trials (Arienti et al. 1993; Dudley et al. 2002; Dudley et al. 2003; Kradin et al. 1989; Rosenberg et al. 1988; Rosenberg et al. 1994; Topalian et al. 1988). Specific tumour recognition and destruction has been observed in about 35% of treated patients. Although these responses lead to tumour regression in some cases, these antigen specific T cell responses have been shown to be only transient (Dudley et al. 2002; Rosenberg et al. 1994).

As mentioned earlier, DLI induces GvL responses in haematological malignant patients who have relapsed following HSCT (Drobyski et al. 1993; Kolb et al. 1990; Mackinnon et al. 1995; Porter et al. 1994). The transplantation of donor derived immuno-competent T cells mediates potent anti-leukaemic responses. Some of these transferred T cells also recognize healthy tissues and induce graft-versus host disease (GvHD). Thus, more specific therapies need to be developed. Leukaemic reactive T cells, isolated from CML patients and expanded *in-vitro*, were transferred back into patients as a more specific DLI therapy and were reported to induce complete cytogenetic remission in some patients (Falkenburg et al. 1999).

The identification of HLA-associated peptides, in addition to the improvement of *in-vitro* culture techniques has allowed the development of further more specific T cell therapies, targeted to an individual antigenic epitope. Antigen specific T cell therapies have been performed for a number of viral (CMV, EBV, HIV) or tumour antigens (mHA, gp100, MART-1) (Brodie et al. 1999; Comoli et al. 2002; Dudley et al. 2001; Einsele et al. 2002; Marijt et al. 2003; Walter et al. 1995; Yee et al. 2000). In some cases, the transferred $CD8^+$ T cell clones have been demonstrated to expand *in-vivo*, localise to the tumour site (melanoma) and to generate antigen specific T cell responses, which lead to an observed clinical response. In other cases, the limited persistence of the transferred antigen specific T cells has been reported and associated with the lack of a long-term protective immunity (Dudley et al. 2001; Walter et al. 1995; Yee et al. 2000). The persistence of melanoma specific T cells has been improved by multiple T cell infusions along with IL-2 administration (Meidenbauer et al. 2003; Yee et al. 2002). However IL-2 has also been

shown to induce the loss of high avidity melanoma specific T cell clones, presumably by activation-induced cell death and this was associated with the lack of clinical response in vaccinated patients (Yee et al. 2002). The exhaustion of HIV-specific T cells following intensive expansion *in-vitro* has also been shown to limit their long-term *in-vivo* survival in treated patients (Brodie et al. 1999; Tan et al. 1999). The recent characterisation of T cell phenotypic markers has allowed monitoring the phenotypic status of the transferred T cell clones. Thus the optimisation of the conditions for the *ex-vivo* expansion of antigen specific T cell in order to maintain and transfer central memory along with effector memory T cell clones could improve their persistence *in-vivo*. In addition, the persistence of CMV specific $CD8^+$ T cell clones *in-vivo* has been demonstrated to rely on the endogenous reconstitution of CMV specific $CD4^+$ T cell immunity (Marzo et al. 2000; Walter et al. 1995). The characterisation and manipulation of antigen specific $CD4^+$ T cell has been impeded, certainly because of the limited availability of HLA class II specific T cells characterization tools. However their importance in viral and tumour immunity has been demonstrated and with the progress in the development of HLA class II tetramers, improvement of therapies in the future is promising.

More recently, a potential improvement of adoptive T cell therapy has been suggested by creating "more space" in the lymphocyte compartment for the transferred T cells. The higher persistence of the infused T cell clones has been demonstrated following immunosuppressive conditioning (Maine and Mule 2002). This has been attributed to the elimination of the unfavourable tolerance environment, potentially the reduction of regulatory $CD4CD25^+$ T cells (Shimizu et al. 1999). The efficiency of such therapy has been assessed in patients with metastatic melanoma. Patients received lymphodepletion-conditioning regimen prior to the transfer of melanoma specific T cell clones (Dudley et al. 2002). The transferred CTLs have been demonstrated to constitute more than 70% of circulating $CD8^+$ T cells and to persist longer in treated patients. Six out of thirteen vaccinated patients demonstrated tumour regression, which was associated with immunological findings. Thus, the preferential T cell repertoire reconstitution with anti-tumour or anti-viral immunity provides higher disease protection. These observations may account for the efficient anti-tumour reactivity of DLI in leukaemic patients. DLI has been in fact demonstrated to be an effective salvage therapy for 80% of CML patients who relapsed following HSCT (Dazzi et al. 2000; Drobyski et al. 1999). Unfortunately GvHD reactivity has also been reported and represents a major cause of morbidity and mortality in treated patients. Thus, the development of tumour specific T cell based therapy would provide a more specific and effective therapy for patients.

1.12: Basis for the development of CML immunotherapies targeting the BCR/ABL fusion protein:

The BCR/ABL protein is an attractive candidate antigen for the development of immunotherapeutic approaches because it exhibits selective expression on tumour cells, which limits the potential toxicity against normal tissues. In addition, BCR/ABL is central to the malignant phenotype, thus it is less likely for the tumour to evade immune recognition by loss of antigen expression. The BCR/ABL p210 (b3a2 and b2a2) and p190 (e1a2) protein are believed to be only expressed in leukaemic cells. In addition, the protein fusion creates a codon split, which results in a new amino acid, lysine instead of glutamic acid, in the b2a2 or e1a2 present at the exact fusion point in the b3a2 fusion protein. Therefore the unique amino acid sequences encompassing these BCR/ABL breakpoint proteins can be considered as truly tumour-specific antigens.

As the e1a2 BCR/ABL translocation has been shown to be mainly expressed in Ph positive ALL, the immunogenicity of this protein has been assessed for the development of immunotherapeutic strategies to treat ALL patients. Both HLA class I and HLA class II e1a2 specific CTLs have been generated *in-vitro* and demonstrated to recognize leukaemic cells (Tanaka et al. 2000; Tanaka et al. 2002b). However their efficiency to eliminate ALL cells *in-vivo* remains to be determined.

Although the BCR/ABL fusion protein has been the most studied over the past 10 years, recently the reciprocal ABL/BCR fusion protein has been assessed as a source of tumour specific antigens. The ABL/BCR protein is detected in about 80% of Ph positive CML patients and recently both HLA-class I and HLA-class II associated epitopes derived from the a1bb3 and a1bb4 fusion proteins have been described (Pawelec et al. 2001; Wagner et al. 2003). These peptides have been shown to elicit ABL/BCR specific T cells *in-vitro*, which recognized leukaemic cells.

Targeting the BCR/ABL (b3a2) tumour specific antigen in CML:

In the context of CML, the BCR/ABL p210 fusion protein have been assessed as a primary CTL target for the development of immunotherapeutic therapies, aiming to reproduce the clinical observed GvL effect. The b2a2 fusion protein is expressed in about 40% of CML patients. However no peptides derived from the breakpoint protein have been found to bind any HLA class I molecules with sufficient affinity (Bocchia et al. 1995; Greco et al. 1996). Some b2a2 derived peptides have been shown to bind and to elicit HLA-class II restricted T cell responses *in-vitro* (ten Bosch et al. 1999). Although some of these b2a2 specific CTLs were demonstrated to kill b2a2 transfected cells, none of these CTLs killed HLA-matched primary tumour cells.

In contrast, a large number of epitope peptides derived from the BCR/ABL b3a2 breakpoint protein have been demonstrated to bind with high or intermediate affinity to a wide range of both HLA-class I and HLA-class II molecules (Bocchia et al. 1996; Bocchia et al. 1995; Bosch et al. 1996; Buzyn et al. 1997; Greco et al. 1996; Mannering et al. 1997; Pawelec et al. 1996; ten Bosch et al. 1995; Yasukawa et al. 1998). Six b3a2 derived peptides were shown to bind to purified HLA-A*0301, -A*1101, -B*0801 and more recently HLA-A*0201 molecules (Bocchia et al. 1995; Buzyn et al. 1997; Yotnda et al. 1998). Interestingly some of these HLA types, HLA-A*0301 and/or HLA-B*0801, have been associated with a reduced incidence of CML (Posthuma et al. 1999). From these observations, a hypothesis was developed as to whether the potential processing and presentation of BCR/ABL b3a2 derived antigens on HLA-A*0301 and HLA-B*0801 results in the generation of a specific immune response, which in turn protects individuals bearing these HLA types from developing CML. In addition, the expression of the translocated bcr/abl fusion gene, especially the b3a2 gene, has been detected in 4 healthy donors (Biernaux et al. 1995; Bose et al. 1998). The expression of the b3a2 fusion gene without clear evidences of CML transformation could be argued as being evidence of a possible immune control of a low number of BCR/ABL positive clones. However the lack of confirmation of these experiments and the low detection (one out forty observable DNA amplification was considered positive) of this transcript questions the validity of these data.

Nevertheless, BCR/ABL b3a2 derived peptides have been demonstrated to elicit reactive T cells *in-vitro* that recognize peptide-pulsed target cells in an HLA-class I and HLA-class II manner (Bosch et al. 1996; Choudhury et al. 1997; Mannering et al. 1997; Nieda et al. 1998; Norbury et al. 2000; Osman et al. 1999; ten Bosch et al. 1995; Yasukawa et al. 1998;

Yotnda et al. 1998). The HLA restriction (Osman et al. 1999) and the efficiency of these specific T cells to lyse BCR/ABL b3a2 tumour cells have however not been clearly demonstrated in all of these culture systems (Nieda et al. 1998; Yotnda et al. 1998). These observations have subsequently questioned the natural processing and presentation of BCR/ABL derived antigens on the tumour cell surface. Indirect evidence of intracellular processing and presentation of these antigens has been provided by the generation of BCR/ABL b3a2 specific CTLs following *in-vitro* stimulation with peptide-unpulsed Ph positive DCs (Choudhury et al. 1997; Mannering et al. 1997; Yasukawa et al. 1998; Yasukawa et al. 2001).

Prior to the onset of my PhD study, our group had demonstrated and confirmed the natural processing and presentation of the previously described HLA-A*0301 b3a2 KQSSKALQR peptide on the cell surface of HLA-A*0301 transfected K562 leukaemic cell line and primary tumour cells derived from HLA-A*0301 CML patients (Clark et al. 2001). More interestingly, using peptide-specific HLA/tetramers, BCR/ABL specific T cells were detected from the peripheral blood of HLA-A*0301 CML patients. In addition, some of these patients mounted BCR/ABL specific cytotoxic T cells responses against autologous CML targets (Clark et al. 2001). These data provided the basis for the development of immunotherapeutic strategies for the treatment of CML patients, targeted to the specific BCR/ABL oncogenic protein.

Accordingly, preliminary clinical trials, including peptides and DC vaccination have been documented. As mention earlier, a phase I peptide vaccination trial has been performed in CML patients using escalating doses of BCR/ABL peptides (including the HLA-A*0301, -A*1101, -B*0801 described b3a2 epitopes and a 25-mer HLA class II potential epitope), and QS-21 molecule as an adjuvant. The peptides were found to be non-toxic, and *ex-vivo* peptide-specific T cell proliferation was found in three out of six patients vaccinated with the highest dose (Pinilla-Ibarz et al. 2000). However no antigen-specific cytotoxic T cells were found. Recently a phase II peptide vaccination trial was published by the same group (Cathcart et al. 2004). In this trial CML patients were vaccinated with the highest peptide dose, still in combination with the QS-21 adjuvant and including a new HLA-A*0201 associated b3a2 peptide (Yotnda et al. 1998). In this case, *ex-vivo* antigen-specific CD4 T cell proliferation was observed in eleven out of twelve vaccinated patients and antigen-induced INF-γ production was generated from six patients. Again, no leukaemia-specific cytotoxicity was found and was associated to the large tumour burden of these CML patients, the majority of whom were in accelerated phase of the disease. A small clinical trial using *ex-vivo* BCR/ABL (b3a2) pulsed DCs as a vaccine has also been attempted

(Takahashi et al. 2003). The antigen-specific T cell responses generated in three vaccinated CML patients was detected by DTH reactivity to the peptide antigen but no measurable leukaemia-specific cytotoxicity and clinical responses were observed.

In all these trials, patients were not selected for the expression of HLA types that have been associated with a potential BCR/ABL (b3a2) derived epitope, with the exception of the phase I peptide vaccination trial. Also, the majority of these patients were in accelerated phase, thus bearing a large tumour burden. These types of patients have been described to be refractory to many conventional therapies, including HSCT. It is therefore unlikely that these patients could easily mount a tumour specific T cells with such high tumour load and its associated tolerizing environment. For these reasons, our group in collaboration with Prof R. Clark from the Liverpool Royal Infirmary have initiated a phase I peptide vaccination trial. CML patients are selected for the expression of HLA-A*0201, -A*0301, -A*1101 and/or HLA-B*0801 molecules and for experiencing a partial haematological remission with stable but low levels of bcr/abl transcripts following Imatinib mesylate therapy. This would allow the assessment of a potential further reduction of bcr/abl levels upon generation of a specific vaccine-mediated immune response. In addition, a helper T cell epitope, PADRE has been included to promote $CD4^+$ T cell proliferation and cytokines secretion required for appropriate tumour specific $CD8^+$ T cell responses. This vaccination trial is still undergoing and no definitive conclusions are yet available.

As described previously, peptide vaccination may not provide the optimal stimulation necessary for the generation and/or expansion of tumour specific T cells *in-vivo* in cancer patients. These vaccinations rely totally on the endogenous activation of APCs for the subsequent activation of antigen-specific T cells. Thus, the development of *in-vitro* protocols for the efficient generation and/or expansion of BCR/ABL (b3a2) specific CTLs would provide a promising therapy for CML patients. These therapies could be applied for both post-HSCT CML patients who relapsed (as a specific DLI) and also for CML patients who do not have an available HLA-matched donor.

1.13: Aim of the thesis:

The success of haematopoietic stem cell transplantation (HSCT) and more specifically donor lymphocytes infusion (DLI) to eradicate leukaemia in Chronic Myelogenous Leukaemia (CML) patients have been attributed to the graft-versus leukaemia effect (GvL), mediated by the donor T cells infused with the graft (or with the blood infusion). Unfortunately the immuno-competent cells from the donor can also mount an immune response against the recipient cells causing graft versus host disease (GvHD). The characterisation of the antigen recognized by the T cells mediating the GvL response would provide a more specific T cell therapy for patients who relapse post-HSCT but may also be applied for patients who are not candidates for HSCT. In the context of CML, the BCR/ABL breakpoint fusion protein represents a good candidate antigen, as its expression is restricted to CML cells and involved in the disease pathogenesis.

Thus, the aim of this thesis is to develop immunotherapeutic strategies for the treatment of CML disease targeted to HLA associated epitopes derived from the BCR/ABL tumour specific protein.

Firstly the characterisation and confirmation of natural processing and presentation of BCR/ABL tumour specific epitopes, derived from the b3a2 breakpoint protein, will be assessed from the cell surface of leukaemic cells in the context of additional HLA class I molecules. This will allow the potential application of therapies to be extended to a wider number of patients.

The use of the peptide-specific HLA/tetramers to identify and quantify the frequency of BCR/ABL specific T cells circulating in the peripheral blood of CML would provide valuable information concerning the relevance of the BCR/ABL tumour antigen in the induction, or the lack of anti-tumour specific protection.

Subsequently, the feasibility of expanding or priming BCR/ABL specific T cells from both CML patients and healthy donors will be assessed *in-vitro* using established cellular stimulation protocols and developed acellular protocols. The resulting efficiency of these protocols would provide indications about the *in-vitro* immunogenicity of BCR/ABL derived peptides and consequently their potential relevance for the development of immunotherapeutic strategies to treat CML patients.

The findings from this PhD thesis demonstrate the feasibility of BCR/ABL based adoptive T cell immunotherapy, at least in the context of the HLA-A*0301 molecule for CML patients. In addition, the techniques presented here could be applied in the context of other antigens and developed in the context of other malignancies.

CHAPTER 2
Materials and Methods

The materials and methods described in this chapter were used to perform the research work mentioned throughout the chapters of this thesis.

Buffers and solutions

All buffers and solutions (except where specified) were purchased from Sigma (Surrey, UK) and BDH (Poole, UK).

2.1 Molecular Biology:

2.1.1 mRNA extraction:

mRNA was isolated from a minimum of 2 to 5 $\times 10^6$ fresh or thawed Peripheral Blood Mononuclear Cells (PBMCs). Cells were pelleted by centrifugation at 13000 rpm for 5 minutes and the supernatant was aspirated completely. Cell pellets could be stored at -70°C until required. The mRNA was extracted using the Rneasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. Cells were lysed in 350µl of denaturing guanidinium isothiocyanate buffer, which inactivates RNAses (RLT buffer). The cell pellet was disrupted by pipetting until a homogeneous cell lysate was obtained. Then 350µl of 70% ethanol was added and the samples were applied to a QIAamp spin column placed into a collection tube. The ethanol ensures appropriate binding conditions for the total RNA to the silica-gel membrane. The columns were centrifuged for 15 seconds at 10,000rpm (Biofuge, Heraeus Instruments, Germany) and the flow-through was discarded. Contaminants were removed by washing once with 700µl buffer RW1, and twice with 500µl ethanol-containing buffer RPE. Columns were spun after each wash at 10,000rpm for 15 seconds and the flow-through discarded. Finally, total RNA was eluted with 30-50µl of RNAse-free water and recovered in a sterile, RNAse-free microcentrifuge tube (provided in the kit) by centrifugation for 1 minute at 14,000rpm. The mRNA was used immediately as a template for complementary deoxyribunucleic acid (cDNA) synthesis or stored at -70°C until required for further use.

2.1.2 Quantification of total RNA:

The yield of total RNA was determined by UV spectrophotometry (Perkin Elmer Life, Zaventem, Belgium). RNA absorbs light at 260nm and one optical density (OD) unit is equal to 40μg/ml of single-stranded RNA.

2.1.3 Generation of complementary DNA:

Complementary DNA (cDNA) was generated from total RNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using the moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega, Southampton, UK). The M-MLV RT is an RNA-dependent DNA polymerase that synthesizes cDNA from RNA templates up to 5kb long. A volume of 10ml (or ~1mg) of purified mRNA was mixed with 0.5 mg of random primers (Promega, UK) in a sterile RNase free microcentrifuge tube. The random primers were annealed to the RNA by heating the tube to 70°C for 5 min in a hot block (Block Thermostat, Grant instruments Ltd, Cambridge, UK). The tubes were cooled down immediately on ice to prevent any partial secondary structure formation. The reagents listed in **Table 2.1** were then added to the tubes and incubated for 60 minutes at 37°C. Double-stranded cDNA was generated by incubating the tubes at 70°C for an additional 10 minutes. The samples were then briefly spun and stored at -70°C until required.

Reagents	*Volumes*
5X M-MLV reaction buffer (Promega, UK)	4μl
0.1M DTT (Sigma, St Louis, USA)	2μl
rRNasin® Ribonuclease Inhibitor, 20U/μl (Promega)	1μl
dNTPs (10mM stock, Bioline, UK)	1μl
M-MLV RT, 200U/μl (Promega)	1μl
Nuclease-Free Water to final volume	20μl

Table 2.1: Reagents for the synthesis of complementary DNA:

This table summarizes the components of the reverse transcriptase reaction performed from total RNA for the generation of cDNA.

2.1.4 Generation of double stranded DNA from M13 clone:

The DNA constructs coding for HLA class I molecules were provided by Dr Ann-Margaret Little (Anthony Nolan Histocompatibilty laboratory, London) and were maintained in M13 bacteriophage. M13 bacteriophages have circular, single stranded DNA. HLA encoding genes were inserted via 5'-Hind III and 3'-Sal I restriction enzyme sites. Prior to use as a template in PCR reactions, it was necessary to generate double-stranded DNA from each HLA coding sequence.

Competent bacteria expressing F' episome (sex pili necessary for infection by virus) were infected with the relevant M13 bacteriophage. The JM109 *E.coli* strain (F' positive, described in section 2.1.14) was grown at 37°C on a M9 minimal agar plate (**Table 2.2**) to increase the expression of F' episome necessary for permissive infection with the bacteriophage. The plating stock of JM109 was obtained by transferring a single colony into a flask (Pyrex, UK) containing 50ml of LB medium (medium preparation described in **Table 2.5**) and cultured for 5-8 hours at 37°C (G24 environmental incubator shaker, New Brunswick Scientific, Edison, N.J., USA), until the $O.D_{600nm}$ reached 0.8-0.9. Then 200µl of JM109 plating stock was aliquoted into a sterile 15ml tube (Bibby sterilin, UK) and infected with 10µl of a serial dilution of M13 bacteriophage containing the HLA coding region. After addition of 1ml of LB medium, the infected bacteria were grown overnight at 37°C. The culture was transferred into a sterile 1.5ml eppendorf tube (Elkay, UK) and spun at full speed in a microcentrifuge for 5 minutes. The viral particles were harvested from the supernatant and kept at -70°C.

M9 Salt 10X		**M9 Minimal Medium**	
Na_2HPO_4 (BDH, UK)	12g	M9 Salt 10X	100ml
KH_2PO_4 (BDH)	6g	$MgSO_4$ 1M (BDH)	1ml
NH_4Cl (BDH)	2g	$CaCl_2$ 100mM (BDH)	1ml
NaCl (BDH)	1g	Thiamine 1M (BDH)	1ml
Distilled H_2O	200ml	Glucose 20% (GibcoBRL, UK)	10ml
Autoclave at 120°C for 20 minutes, cool to 50°C.		Distilled H_2O	Up to 1L

Table 2.2: M9 medium composition:

First 10X M9 salts were prepared and autoclaved. $MgSO_4$ and $CaCl_2$ solutions were prepared in distilled water, autoclaved separately and kept at -20°C. Glucose was sterilised by filtration through a 0.45µm filter (Sartorius, Surrey, UK). M9 salts (100ml) were cooled down to 50°C and the other ingredients were added. For the preparation of solid agar medium, 15g of agar (Difco, UK) was added per litre prior to autoclaving.

The cell pellet contains the double stranded M13 DNA and was purified by DNA miniprep (described in section 2.1.17). A restriction enzyme digestion with Hind III and Sal I was performed and the product was analysed by agarose gel electrophoresis to confirm the correct DNA size. This double-stranded DNA encoding for HLA class I molecule was then used as a template in PCR reaction.

2.1.5 Polymerase Chain Reaction and Primer design:

The Polymerase Chain Reaction (PCR) allows the *de novo* synthesis of a DNA region flanked by 5' and 3' primers. PCR consists of three steps, a denaturing step which melts the DNA into single strands, an annealing step where the primers recognise and bind to their complementary sequence and an extension step where the *Thermus aquaticus* (Taq) DNA polymerase synthesizes the DNA flanked by the two primers. These heating and cooling steps can be repeated a number of times, resulting in the amplification of the DNA of interest. The reaction requires deoxynucleotides to provide both the energy and the nucleotides for the synthesis of DNA, a heat stable DNA polymerase, the 5' and 3' specific primers, the DNA template, and buffer containing magnesium. Magnesium is a mandatory cofactor for the activation and the fidelity of the Taq polymerase, however it also affects primer annealing and template denaturation and therefore its concentration needs to be optimised.

Primer design:

Two primers were designed for the amplification of the DNA sequence of interest. These primers are 15-30 nucleotides in length and are complementary to the DNA sequence at the 5' and 3' ends of the segment to be amplified. At the 5' end of each primer, a number of nucleotides were added for restricted enzyme recognition, in order to subsequently clone the amplified DNA into a vector of choice. Primer pairs that are complementary to each other or form internal hairpin structures are to be avoided. It is important that the primer pairs have an annealing temperature within 4°C of each other, and ideally that this annealing temperature is 4°C below the Melting temperature (Tm).

The Tm is the temperature at which half the DNA strands are single-stranded and half are double-stranded, it directly depends on the G-C content (as they share triple H bonds). Tm can be calculated for oligonucleotides with the following formula, where n is the number of A (adenine), T (thymidine), G (guanine) and C (cytosine) nucleotides:

$$Tm\ (°C) = 2\ (nA + nT) + 4\ (nG + nC)$$

The Tm of the primers used in this study was calculated using the following web site: http://www.gensetoligo.com/Calculation/calculation.html.
Finally, the specificity of the designed primers was confirmed by Blast search for DNA complementarity, using the following web page: http://www.ncbi.nlm.nih.gov/BLAST.

In this study, PCR reactions were performed to amplify HLA class I sequence for the subsequent production of HLA tetramers. In this case, the intracellular portion of the HLA class I molecule was deleted, as it was insoluble and not required for T cell receptor recognition. The positions of the HLA-pET3d forward and reverse primers in the HLA coding sequence are highlighted in yellow in **Figure 2.1**. The same primers were used for the amplification of HLA-A*0201, -A*0301 and –B*0801, and the nucleotides mismatches are shown in red. The primer sequences are summarized in **Table 2.3**, with the HLA matching sequence highlighted in yellow. The NcoI (forward) and BamHI (reverse) enzyme restriction sites incorporated for the subsequent subcloning into the pET3d vector are shown in purple.

The primers designed for the amplification of HLA class I sequences for transfection into eukaryotic cells are shown in green (**Figure 2.1**). In this case the whole HLA coding sequence was necessary and two sets of primers were designed: a set for HLA-A molecules (HLA-A*0201, -A*0301) and a set for HLA-B (HLA-B*0801). These primer sequences are summarized in **Table 2.3** and the HLA sequences are highlighted in green. HLA coding sequences were inserted into pBJ1Neo vector via 5'XhoI (forward) and 3'ClaI (reverse) enzyme restriction sites shown in purple.

Finally, CML patients were typed for their bcr/abl transcript type. The primer sequences for the amplification of the major bcr/abl breakpoint gene were described elsewhere and are summarized in **Table 2.3** (Devaraj, Foroni et al. 1995). The amplification of the housekeeping gene β-actin was carried out in parallel to control for the quality of the generated cDNA using the β-actin primers (**Table 2.3**).

PCR reactions were performed in PCR sterile tubes (ABgene®, Surrey, England) and run on a Peltier Thermal Cycler-200 (MJ Research, INC, Massachusetts, USA). The optimal annealing temperature used for PCR reactions was determined from the calculated Tm (described for each primer in **Table 2.3**). As the primers designed for the amplification of HLA molecules were not completely homologous to the template (addition of restriction enzyme sites), 5°C to 10°C was subtracted from the Tm to obtain the annealing temperature.

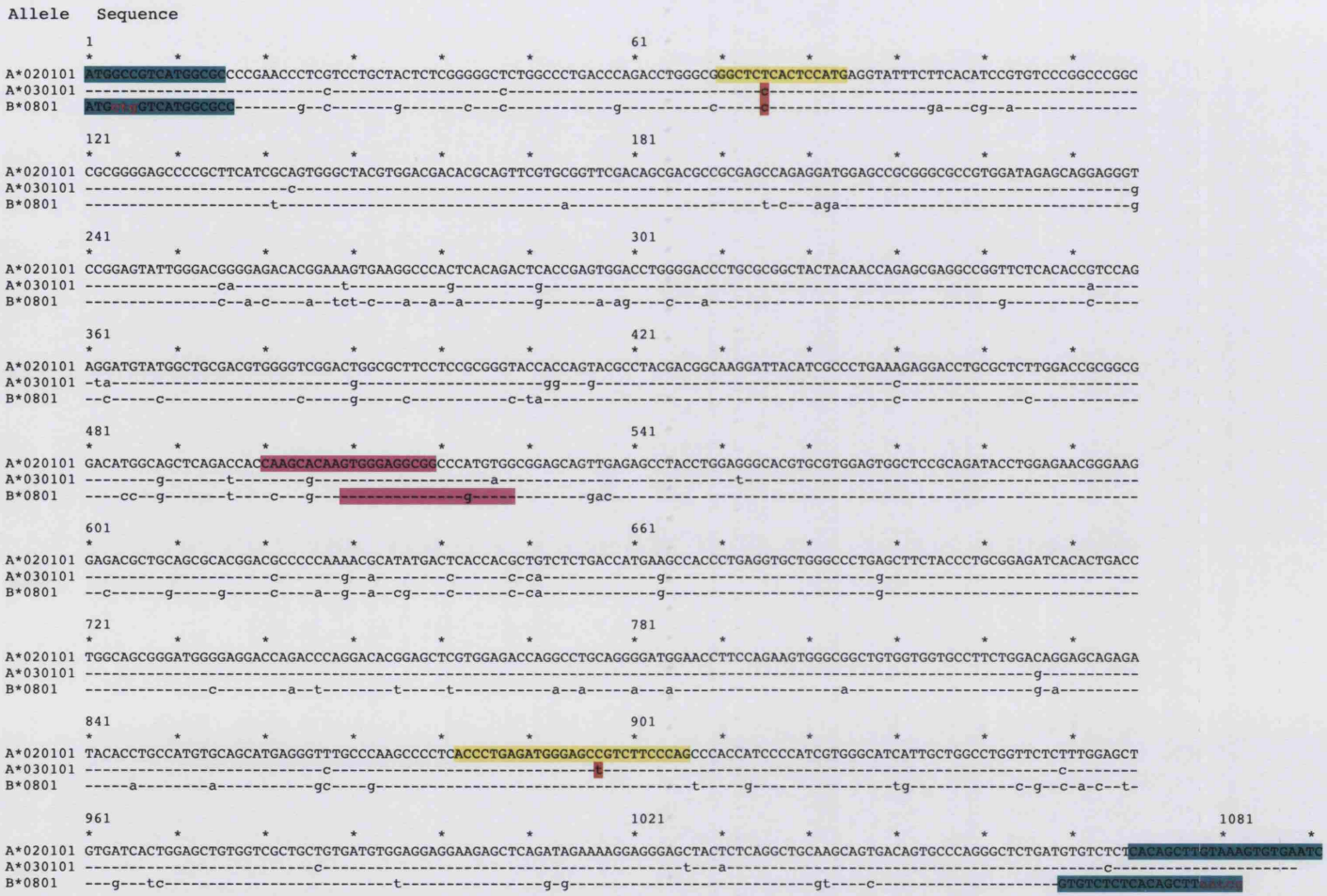


Figure 2.1: HLA class I alignement and primers:
HLA-A*0301 and HLA-B*0801 DNA coding sequences were aligned with HLA-A*0201 sequence using the IGMT/HLA database designed by James Robinson from the Anthony Nolan Bioinformatic group. Two sets of forward and reverse primers for DNA amplification of respectively HLA-A and HLA-B alleles and their subsequent subcloning into pBJ1Neo vector are shown ■. These primers are designed for the amplification of the whole HLA coding molecule. The primers for HLA class I DNA amplification and subcloning into pET3d vector are shown ■. These primers delete a 3' portion and the 5' intracellular portion of the molecule. The same primers were used for the amplification of HLA-A*0201, -A*0301 and B*0801, and the mismatched nucleotides are highlighted in red. In addition, HLA-A (upper) and HLA-B (lower) middle primers designed for the DNA sequencing of the generated clones are shown ■.

Name	*Sequences*	*Tm*
Primer for HLA DNA amplification and cloning into a protein expression vector		
HLA-pET3d forward	GCAC↓CATGGGCTCTCACTCCATG	64°C
HLA-pET3d reverse	CTGGGAAGACG↓GATCCCATCTCAGGGT	67°C
Primer for HLA DNA amplification and cloning into an eukaryotic expression vector		
HLA-A forward	C↓TCGAGATGGCCGTCATGGCGC	67°C
HLA-A reverse	GGCAT↓CGATTCACACTTTACAAGCTGTG	63°C
HLA-B forward	C↓TCGAGATGCTGGTCATGGCGCC	66°C
HLA-B reverse	GGCAT↓CGATTCAAGCTGTGAGAGACAC	63°C
Primers for amplification of housekeeping gene β-actin		
β-actin forward	TCATGAAGTGTGACGTTGACATCCGT	61°C
β-actin reverse	CTTAGAAGCATTTGCGGTGCACGATG	64°C
Primers for amplification of Major breakpoint of Bcr/abl gene		
Major forward	TTCAGAAGCTCTCCCTGACAT	50°C
ABL reverse	CGGCTCTCGGAGGAGACGTAGA	56°C

Table 2.3: Primer sequences for PCR amplification performed in this study:

This table lists the primer sequences and their Tm. Primers for the amplification of HLA class I molecules for the subsequent cloning into pET3d vector, HLA-pET3d Forward and Reverse are shown with the HLA sequence highlighted in yellow. Primers for the amplification of HLA-A*0201, HLA-A*0301 (HLA-A forward and reverse), and HLA-B*0801 (HLA-B forward and reverse) and the subsequent cloning into pBJ1Neo (necessary for eukaryotic cell transfection) are also shown, with the HLA coding sequences highlighted in green. The additional restriction enzyme recognition sites for the subcloning of these sequences are shown in purple. Finally, the sequence of the primers used for the amplification of the β-actin house-keeping gene and the bcr/abl breakpoint region are also summarized. The Tm of each of these primers is shown on the right column. All primers were purchased from Amersham Pharmacia Biotech, UK.

The different protocols used for PCR amplification are detailed on **Figure 2.2**. The protocol for amplification of the major bcr/abl breakpoint and β-actin was obtained from Dr. Letizia Foroni (Department of Haematology, Royal Free Hospital).

A. Protocol for amplification of HLA class I molecules

Reagents	*Volume*	*Temperature in °C*	*Time in minutes*	*Purpose*
cDNA template	1µl			
PfuTurbo DNA polymerase 10X buffer[a]	1µl	95	5 minutes	denaturation
Forward primer (10pM)	2.5µl	30 cycles: 94	1 minute	denaturation
Reverse Primer (10pM)	2.5 µl	30 cycles: 55	30 seconds	annealing
$MgCl_2$ (50mM)[a]	1.5µl	30 cycles: 72	1 minute	extension
dNTPs (10mMeach)[b]	1µl	72	10 minutes	extension
Pfu Turbo DNA polymerase[a]	0.5µl	4	forever	storage
RNAse free water[c]	Up to 50µl			

B. Protocol for amplification of bcr/abl breakpoint gene

Reagents	*Volume*	*Temperature in °C*	*Time in minutes*	*Purpose*
cDNA template	1µl			
Taq DNA polymerase 10X buffer[b]	2.5 µl	94	5 minutes	denaturation
Forward primer (10pM)	1 µl	35 cycles: 94	30 seconds	denaturation
Reverse Primer (10pM)	1 µl	35 cycles: 60	30 seconds	annealing
$MgCl_2$ (50mM)[b]	1 µl	35 cycles: 72	1 minute	extension
dNTPs (10mM each)[b]	1µl	72	10 minutes	extension
Taq DNA polymerase[b]	0.2µl	4	forever	storage
RNAse free water[c]	Up to 25µl			

C. Protocol for amplification of β-actin gene

Reagents	*Volume*	*Temperature in °C*	*Time in minutes*	*Purpose*
cDNA template	1µl			
Taq DNA polymerase 10X buffer[b]	2.5 µl	94	2 minutes	denaturation
Forward primer (10pM)	1 µl	40 cycles: 94	30 seconds	denaturation
Reverse Primer (10pM)	1 µl	40 cycles: 65	40 seconds	annealing
$MgCl_2$ (50mM)[b]	1 µl	40 cycles: 68	40 seconds	extension
dNTPs (10mM each)[b]	1µl	68	7 minutes	extension
Taq DNA polymerase[b]	0.2µl	4	forever	storage
RNAse free water[c]	Up to 25µl			

Figure 2.2: PCR protocols:

The protocols for the amplification of HLA molecules (**A**), bcr/abl breakpoint (**B**) and β-actin (**C**) are summarized. The reagents required for PCR amplification are described and were purchased from Stratagene (La Jolla, USA)[a], Bioline (London, UK)[b], or B.Brain (Melsungen, Germany)[c]. PCR programs are described for each DNA amplification.

2.1.6 DNA electrophoresis:

Agarose gel electrophoresis was performed to verify the successful amplification of the target gene. DNA is negatively charged and migrates through the agarose matrix towards the anode in an electric field at a speed relative to its size. Therefore, electrophoresis separates DNA by size. The agarose gel was prepared by dissolving 0.8% electrophoresis grade agarose (Invitrogen, Paisley, UK) in 100 ml of Tris-boric acid-EDTA buffer (TBE: 0.1M Tris, 0.09M boric acid, 1mM EDTA, Biowhittaker, Walkersville, Maryland, USA). Ethidium bromide (1μg/ml final concentration, Sigma, UK) was added to visualise the DNA. The gel was poured into a sealed gel tray (Biorad, Hercules, California USA), combs were placed to create the loading wells, and the gel was left to solidify. TBE buffer was overlayed on top of the gel and the combs were removed before loading the samples. Then 5μl of the PCR reaction, or approximately 1 μg of DNA, mixed with 1μl of Orange G loading buffer (0.1 mg final concentration, Pel Freez, Merseyside, UK), in parallel with 0.5μg of 1kDa DNA ladder (GibcoBRL, Paisley, UK) were loaded into the wells. DNA separation was achieved after a 40 minutes run at 120 volts. The DNA bands were visualized under UV light and a photograph was taken using the molecular analyst® software (Biorad, Hercules, California USA). The DNA agarose gel of the HLA class I coding sequences PCR amplification is shown in **Figure 2.3**.

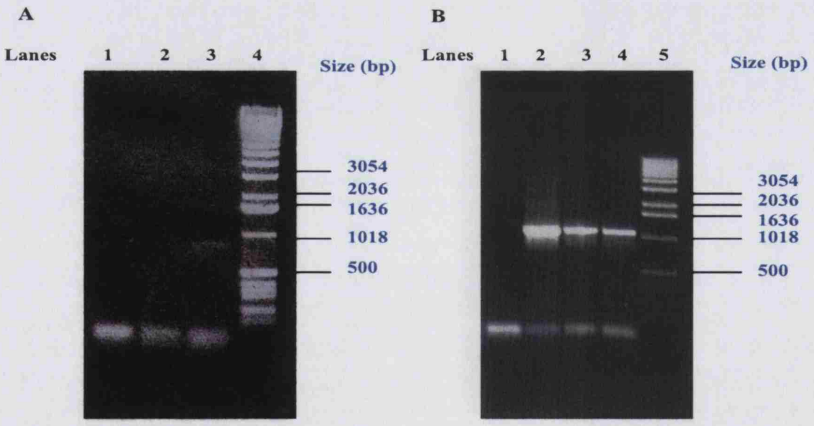


Figure 2.3: PCR amplification of HLA cDNA sequences:

PCR products of **A**) HLA-B*0801 cDNA amplification for the subsequent production of HLA tetramers and **B**) HLA class I cDNA amplification for subsequent cloning into pBJ1Neo vector and cells transfection were separated on a 0.8% DNA agarose gel. **A**) 10μl of: lane 1: no DNA, PCR negative control; lane 2: product of HLA-B*0801 cDNA amplification, 1:100; lane 3: product of HLA-B*0801 amplification, 1:10; lane 4: 1Kb DNA ladder were loaded. A DNA band of approximately 878 base pairs (bp) is visible in lane 3. **B**) 10 μl of; lane 1: no DNA, PCR negative control; lane 2: HLA-B*0801 cDNA amplification, non diluted; lane 3: HLA-B*0801 cDNA amplification, 1:10; lane 4: HLA-A*0301 cDNA amplification, 1:10; lane 5: 1Kb DNA ladder were loaded. Sharps bands of 1100bp (whole HLA class I coding sequence) are visible in lane 2, 3 and 4. These DNA bands were purified from the gel using a GFX column (section 2.1.11) for subsequent subcloning into the corresponding vector.

2.1.7 Plasmid vectors and cloning procedure:

The PCR primers used in this study contain very short 5' and 3' overhangs, which do not allow restricted enzyme digestion for the subsequent ligation into the vector of interest. Therefore, the PCR products were initially cloned into pCR®2.1 vector (TA cloning kit, Invitrogen, UK, Appendix 2.1). This linear vector is designed for the rapid cloning and further maintenance of the DNA of interest. It contains poly-thymidine overhangs that allow ligation of poly-adenosine PCR products (poly-A tail added by the Taq DNA polymerase during PCR reaction). The first 146 amino acids coding for the β-galactosidase (Lac Z) are encoded within the poly-cloning site, which with the carboxy-terminal portion encoded by the host cells (Ω portion, XL1-Blue strain of *E.coli*) can complement and produce an active form of the enzyme (β-galactosidase). The addition of isopropylthio-β-D-galactosidase (IPTG, Alexis® Biochemicals, Nottingham, UK) in the medium induces the T7 promoter to express the β-galactosidase. In the absence of the insert, the β-galactosidase will be functional and metabolise the 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, the β-galactosidase substrate, Alexis® Biochemicals, UK) into a blue colour. This α-complementation allowed the rapid white (presence of the insert)/blue (absence of the insert) screening of positive clones. The pCR®2.1 vector poly–T overhangs are flanked by two EcoRI restriction sites that also permit a rapid screening for the presence of the insert, by a single DNA endonuclease digestion.

Addition of 3' overhandings to the PCR products:

The Pfu DNA polymerase used for the amplification of the HLA class I gene has a 3' to 5' exonuclease proofreading activity and this confers a high fidelity with the DNA template but also removes the 3' overhangs necessary for the TA cloning. Therefore, PCR products were further incubated for 8 minutes at 72°C with 0.7 unit of Taq polymerase (Bioline, London, UK) for the addition of 3' overhangs. The DNA was immediately extracted with an equal volume of phenol-chloroform and kept on ice. Then 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol were added to precipitate the DNA. The tubes were centrifuged at 1600rpm for 2 minutes and the DNA pellet was air-dried. The DNA was recovered with 20-30μl sterile distilled water containing 10μg/ml of RNAse A solution (Promega, Southampton, UK), and was directly ligated into the pCR®2.1 vector.

2.1.8 Protein expression using the pET cloning system:

The HLA class I heavy chains were expressed using a modified pET3d vector (4.637Kb, Novagen, Merck Biosciences Ltd, Nottingham, UK; Appendix 2.2). The targeted gene is cloned downstream of the T7 promoter. The high efficiency of this system relies on the expression of cloned genes under the tight control of the bacteriophage T7 RNA polymerase expressed by the host cell, which itself is under the control of the lac UV5 operon. This allows the complete diversion of the host protein synthesis machinery towards the selective expression of the target gene by the addition of the Lac operon substrate, IPTG (isopropylthio-β-D-galactosidase), which induces the synthesis of T7 RNA polymerase. Usually foreign proteins are very toxic for the bacterial host and affect their growth. This system allows the control and timing of protein expression, by supplementing IPTG in the bacterial broth at the exponential phase of growth. The *E.coli* host used for target protein expression was the BL21(DE3)pLysS (Novagen). This specific strain is deficient in lon and ompT protease, contains the Lac operon and T7 encoding gene, chloramphenicol resistance, and has an additional plasmid encoding for the T7 lysozyme for more stringent control of protein expression. However, the plasmid containing the insert was maintained in a host lacking T7 RNA polymerase to avoid plasmid instability (XL1-Blue *E.coli*, Novagen).

Modification of the pET-3d vector:

The pET-3d vector has been modified by Chrissy Zamoyska (Research Scientist, ANRI) to contain a 3' biotinylation site for fusion to the cloned gene. A peptide tag sequence containing a biotinylation site was chosen from Schatz *et al.* work (Schatz 1993) and was synthesised as four oligos (or linker) to avoid errors. The linkers A, B, C and D are represented in **Figure 2.4** and the lysine (K) residue necessary for the biotinylation is highlighted. This NcoI-BglII cassette was inserted into the poly-cloning site of the pET3d vector at its NcoI (546)-BamHI (510) site. Cutting the BamHI site in the pET3d vector leaves a BglII overhang and the modified pET3d-bsp2 vector has a new poly-cloning site: Bpu-**BglI-bsp2-BamHI-NcoI**-XbaI-BglII. The HLA class I encoding genes were inserted via NcoI, giving rise to a codon start ATG, and BamHI, flanking the bsp2 site at the 3' of the gene, necessary for directional biotinylation of the protein product.

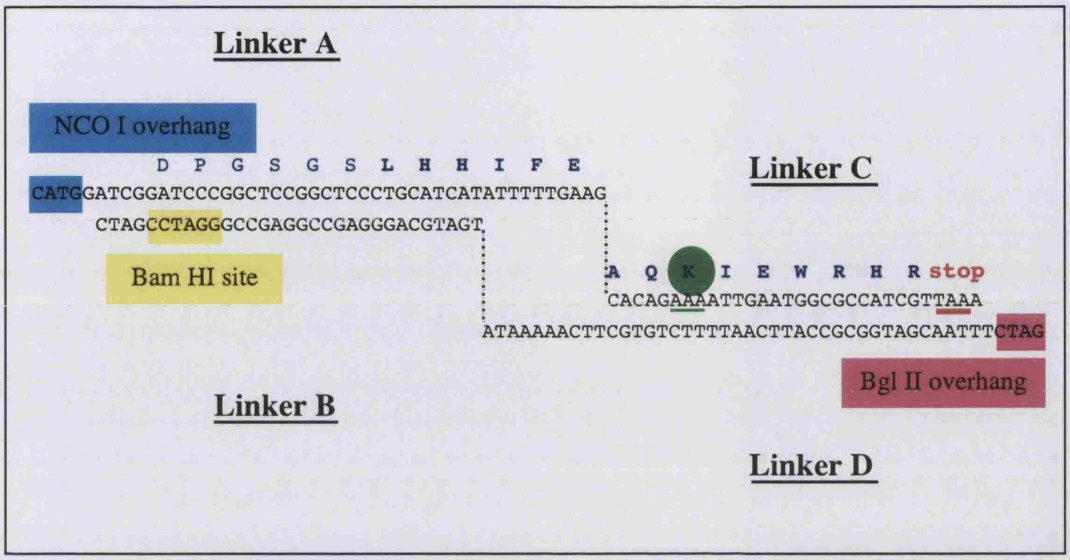


Figure 2.4: Peptide tag sequence for biotinylation:

The four different linker sequences (A, B, C and D) are described. The lysine biotinylation residue is highlighted in green. This sequence contains a 5' NcoI and a 3' BglII overhang, which will ligate to the pET3d vector in frame and form NcoI and BglII restriction enzyme sites. A BamHI restriction site is present in the linker B. HLA coding genes are inserted into pET3d vector via NcoI and BamHI restriction sites. This creates a codon start ATG (NcoI) and allows having the biotinylation site on the C-terminal of the protein. The figure is a kind gift from Dr Geraldine Aubert.

2.1.9 Cloning into pBJ1Neo vector for transfection of eukaryotic cells:

The pBJ1Neo vector (8Kb) was created from the pcDL-SRα296 vector (Takebe, Seiki et al. 1988). A poly-linker containing the restriction sites: 5' XhoI-XbaI-SfiI-NotI-EcoRI-EcoRV-HudIII-ClaI-3' was inserted and placed between the SRα (human T cell lymphotropic virus I) promoter and the SV40 poly-adenylation site. This vector contained an initial ampicillin resistance gene for the selective maintenance and propagation of the vector in a bacterial host. The neomycin resistance coding gene (derived from PHβ-Apr1 vector, (Gunning, Leavitt et al. 1987)) was inserted between the ampicillin resistance gene and the SRα promoter to allow selection of transfected mammalian cells with the G418 antibiotic. The map of the modified vector is described in Appendix 2.3. The HLA class I encoding genes were inserted at 5' Xho I and 3' Cla I sites in the pBJ1Neo vector.

2.1.10 DNA endonuclease restriction enzyme digestion:

All the DNA endonucleases and their corresponding buffers were purchased from Gibco BRL®, Paisley, UK. The specific DNA sequences recognised and digested by the different restriction enzymes are represented in **Table 2.4**. Briefly, 1 to 5 μl of DNA (maximum of 5μg) was mixed in a sterile microcentrifuge tube (Elkay, UK) with 1μl of reaction buffer and 1μl of restriction enzyme (when two different enzymes were used, 0.5μl of each were added) in a final volume of 10μl (completed with sterile double distilled water). The digestion was carried out for 1 to 2 hours in a 37°C water bath. The digested DNA was visualised on a 0.8% DNA electrophoresis gel. The screening of HLA class I recombinant clones for the subsequent cloning into transfection vector (**Figure 2.5**) and for the production of HLA tetramer (**Figure 2.6**) are represented.

Name	*Recognition site*	*Reaction conditions*
EcoRI	5'G↓AATT C-3' 3'-C TTAA↑G-5'	REACT® buffer 3 50mM Tris-HCl (pH8) 10 mMMgCl2 100mM NACl
NcoI	5'-C↓CATG G-3' 3'-G GTAC↑C-5'	
BamHI	5'-G↓GATC C-3' 3'-C CTAG↑G-5'	
ClaI	5'-AT↓CG AT-3' 3'-TA GC↑TA-5'	REACT® buffer 2 50mM Tris-HCl (pH8) 10 mM MgCl2 50mM NaCl
XhoI	5'-C↓TCGA G-3' 3'-G AGCT↑C-5'	

Table 2.4: Restriction endonucleases and their optimum buffers:

The specific DNA sequences recognized and digested by the restriction enzymes used in this study are summarised in this table. The endonuclease digestion was performed in the enzymes' optimal reaction buffer as listed here. The optimal buffer for Cla I is REACT® buffer 1, but this enzyme also has 100% activity in the optimal buffer for XhoI, REACT® buffer 2. All restriction endonucleases and their buffers were purchased from GibcoBRL, Paisley, UK.

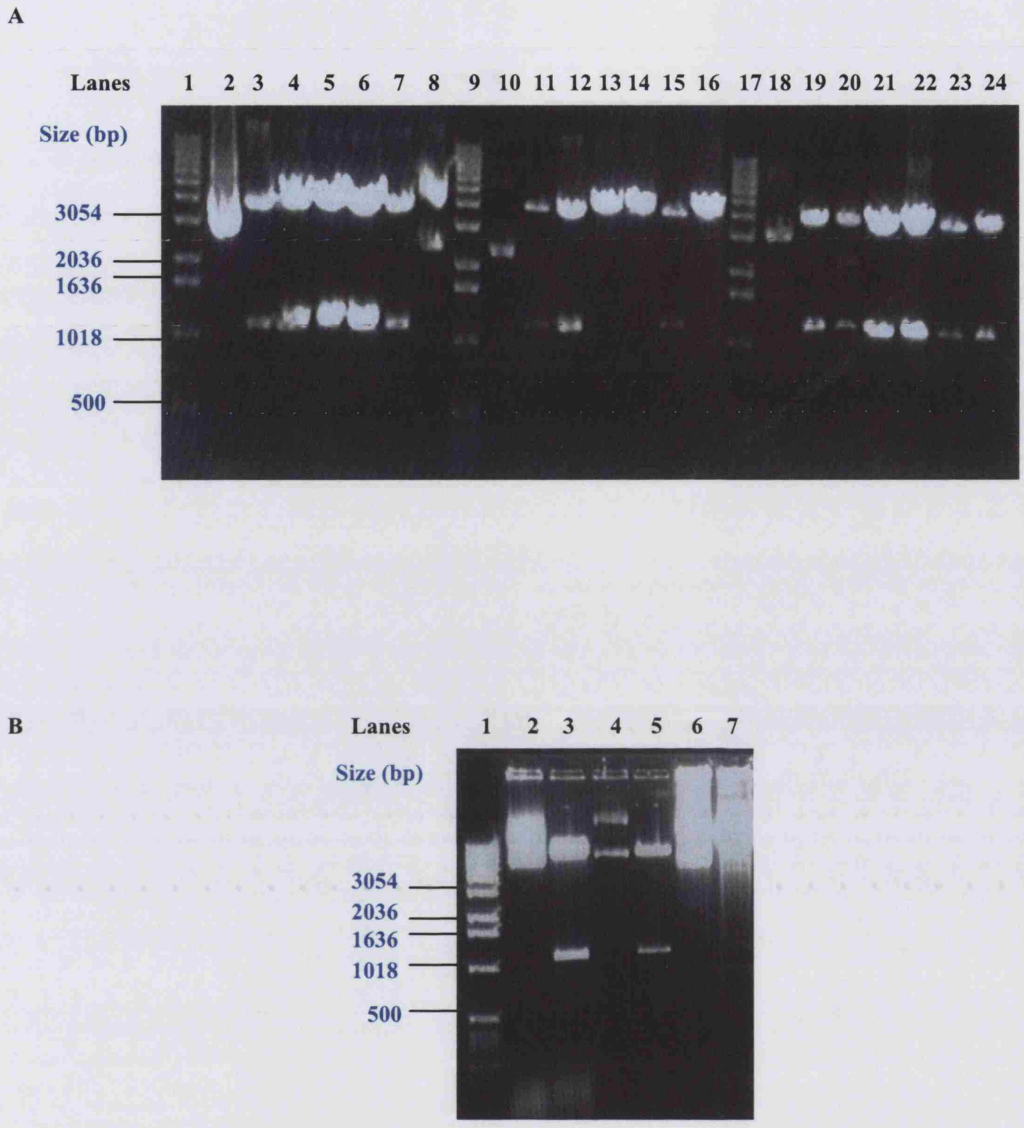


Figure 2.5: Screening HLA class I recombinant bacterial clones for transfection:

DNA mini-preparation products were digested with the corresponding restriction enzyme and were loaded on a 0.8% DNA agarose gel. A 1Kb DNA ladder was loaded in lane 1, 9 and 17 (Panel **A**) and lane 1 (panel **B**). Panel **A**: Uncut DNA clones were loaded in lane 2, 10 and 18. HLA-A*0201 (lane 2 to 8), HLA-A*0301 (lane 10 to 16) and HLA-B*0801 (lane 18 to 24) recombinant DNA clones in pCR2.1 vector were digested with EcoRI. DNA bands of 1.1Kb were obtained and clone 4 (HLA-A*0201), clone 12 (HLA-A*0301) and clone 22 (HLA-B*0801) were selected for the subcloning into pBJ1Neo vector. Panel **B**: HLA-B*0801 (lane 2 and 3), HLA-A*0301 (lane 4 and 5) and no insert (lane 6 and 7) recombinant DNA clones in pBJ1Neo vector were screened for the presence of the insert. Undigested (lane 2, 4 and 6) and ClaI/XhoI digested (lane 3, 5 and 7) DNA clones are shown. These clones were selected and DNA sequenced (section 2.1.19) prior to their use for the transfection of eukaryotic cell lines.

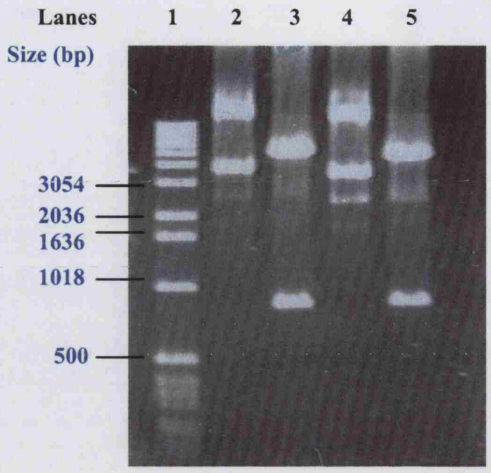


Figure 2.6: Screening HLA-B*0801 recombinant bacterial clones for HLA tetramer production:

HLA-B*0801 recombinant DNA clones in pET3d/bsp vector were screened for the presence of the insert. On a 0.8% DNA agarose gel a 1Kb DNA ladder was loaded in lane 1. Undigested (lane 2 and 4) and NcoI/BamHI digested (lane 3 and 5) of two DNA mini-preparation products were then loaded. DNA bands of 878bp are shown in lane 3 and 5. These clones were selected and tested for optimal protein expression (section 2.2.2).

2.1.11 DNA GFX column purification:

For subsequent cloning and/or sequencing, DNA was purified using a GFX PCR DNA purification kit (Amersham Pharmacia Biotech, Piscataway, USA) following the manufacturer's instructions. The purification was performed from digested DNA fragments purified from DNA electrophoresis gel bands (for cloning steps) or directly from PCR reaction tubes (for sequencing). For the purification of DNA from agarose gels, the agarose slice containing the DNA of interest was cut out using a scalpel blade and a maximum gel slice weight of 300mg was placed into a 1.5ml sterile microcentrifuge tube. Then 10% v/w capture buffer was added and after vigorous vortexing, the agarose was left to dissolve for 10 to 15 minutes at 60°C. The dissolved agarose containing the DNA was then transferred into a GFX column and incubated for 1 minute. When purifying fragments from PCR reactions, 500µl of capture buffer was added on top of the GFX column and 25µl of PCR

product was transferred to the column and mixed by pipetting. The chaotropic agent present in the capture buffer denatures proteins and promotes DNA binding to the glass fiber matrix. GFX columns were microcentrifuged (Biofuge 13, Heraeus instrument, Germany) at full speed for 30 seconds and the flow through was discarded. Contaminants were extracted by the addition of 500 µl of wash buffer (ethanol buffer) and the column was microcentrifuged for 30 seconds at full speed. The GFX column was then transferred to a sterile microcentrifuge tube and the bound DNA was eluted by the addition of 25-50µl of sterile water and incubated for 1 minute at room temperature. DNA was recovered by microcentrifugation at full speed for 1 minute. The recovered DNA was kept at -20°C until required for further use.

2.1.12 Determination of DNA concentration:

DNA concentration was determined by UV spectrophotometry (Perkin Elmer Life, Zaventem, Belgium) as described for mRNA. One O.D unit at 260 nm corresponds to 50µg/ml of double-stranded DNA.

2.1.13 DNA Ligation:

The ligation of two DNA fragments relies on the catalysis of phosphodiester bonds between the 3' hydroxyl and the 5' phosphate termini of double stranded DNAs by the T4 DNA ligase in the presence of ATP. Ligation of PCR products into the pCR2.1® vector was performed using the TA cloning kit (Invitrogen, Paisley, UK). Briefly, 10 to 15ng (depending on the size of the fragment to ligate) of amplified DNA product was ligated to 50ng of pCR2.1® vector (2µl of 25ng/µl) with 1µl of T4 DNA Ligase (4.0 Weiss units) in 1µl of 10X Ligation buffer (all reagents were provided in the kit). The volume was made up to 10µl with sterile double distilled water. Ligation reactions were carried out overnight in a 14°C water bath.

The subsequent subcloning into pET3d or pBJ1Neo was performed as above. Both the DNA fragments and the vectors were first digested with the relevant restriction enzymes and separated on a DNA electrophoresis gel. Linearized DNA fragments were purified from agarose bands using GFX columns (described in section 2.1.11). DNA concentration was then determined to maintain an adequate insert to vector ratio calculated as follows:
Xng of insert × size of vector in bp= Yng of vector × size of insert.

The pET3d vector is 4.6kb and the HLA class I is 878bp (without the intracellular portion). The pBJ1Neo vector is 8kb and the whole HLA coding gene is 1.1kb long. Therefore an approximate insert to vector ratio of 1: 6 was used for the ligation of HLA class I molecules into the modified pET3d vector and a ratio of 1:7 for the ligation of HLA class I molecules into pBJ1Neo vector.

The DNA insert and vector (1-3µl volume) were incubated with 2µl of 5X ligation buffer (GibcoBRL, 250mM Tris-HCl (pH7.6), 50mM $MgCl_2$, 5mM ATP, 5mM DTT, 25% (w/v) polyethylene glycol), 1µl of T4 DNA Ligase (GibcoBRL, 5U/µl) and complete up to 10 µl with double distilled water. The ligations were incubated overnight at 14°C and kept at -20°C until required.

2.1.14 Bacterial culture and transformations:

Different bacterial strains were used in this study, for the maintenance and propagation of plasmids (XL1-Blue, DH5α, JM109), for the selection of clones transformed with positive ligation products by β-galactosidase α-complementation (XL1-B), for the generation of double stranded DNA from M13 and its super-infection via F' episome (JM109) or for the expression of targeted proteins (BL21[DE3]pLysS). These strains were purchased from Novagen (Merck Biosciences Ltd, Nottingham, UK), maintained in 10% v/v glycerol stock and kept at -70°C.

Bacteria were grown in sterile, liquid or solid, TYM, LB or 2 XYT mediums, described in **Table 2.5**. Culture media were prepared in 100ml glass bottles (Schott, Duran, UK) and autoclaved prior to use. Solid media were cooled down to 50°C before the addition of selective antibiotics (when required) and poured into sterile Petri dishes (Bibby sterilin, UK). Plates were left to solidify and dry before plating bacteria, and placed up side down in the incubator (Heraeus Instrument, Germany). For liquid medium culture, single colonies were inoculated into 15ml sterile tubes (Falcon®, Becton Dickinson, London, UK) or Flasks (Pyrex, UK) and incubated at 37°C (G24 environmental incubator shaker, New Brunswick Scientific, Edison, N.J., USA).

Components	*2XYT*	*LB*	*TYM*
Bacto-tryptone (GibcoBRL®, UK)	16g	10g	20g
Bacto-Yeast extracts (Oxoid, UK)	10g	5g	5g
NaCl (BDH, UK)	5g	10g	5.8g
Bacto-Agar (only for solid medium, Difco, UK)	15g		$MgCl_2$ 2g
Reagents were dissolved in 1L distilled water and autoclaved After cooling down, antibiotics were added when required			

Table 2.5: Composition of bacterial growth medium:

This table summarizes the composition of the three different bacterial media used in this study.

2.1.15 Generation of competent cells:

Competent bacteria were generated using a calcium chloride membrane permeabilisation protocol. The optimum medium for the generation of competent cells is TYM medium because it contains magnesium chloride ($MgCl_2$). The treatment of bacteria with ice cold $MgCl_2$ followed by heat-shock (see transformation section) induces a transient "competent" state of bacteria, which allows the up-take of DNA plasmids by bacterial cells.

Competent bacterial cells were generated from the strain of interest. For this, a single bacterial colony was picked with a pipette from an agar plate, or from a glycerol stock and pre-grown overnight in 2ml TYM medium (see **Table 2.5**) at 37°C. Then 1ml of confluent growth was transferred into a flask containing 100ml of TYM and left at 37°C until the OD_{550nm} reached 0.5-0.6. Bacterial cells were transferred into a sterile 50ml Falcon tube (BD, UK), and pelleted by centrifugation at 3000rpm for 20 minutes at 4°C. The supernatant was discarded and the bacterial pellet was completely resuspended in 10ml (per 50ml culture) of TfI solution (30mM KOAc, 50mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$, 15%w/v Glycerol, pH5.8) and left for 30 minutes on ice. Bacterial cells were then centrifuged at 1000rpm for 20 minutes at 4°C and resuspended in 2ml (per 50 ml culture) of TfII solution (10mM MOPS, 75mM $CaCl_2$, 10mM KCl, 15% w/v glycerol, pH 7). Cells were mixed very gently and 200µl were aliquoted in sterile 1.5ml centrifuge tubes (Elkay, UK). These competent bacterial cells were kept at –70°C until needed for further use. The efficiency of transformation was verified by performing a transformation using a closed vector (no insert) and plating on selective medium.

2.1.16 Transformation and selection of bacteria:

The transformation of bacteria with the recombinant plasmid vector was performed by heat-shock treatment of competent *E.coli* strains.

5µl of ligation product was pipetted in a thawed 200µl vial of competent bacteria, mixed and kept on ice for 30 minutes. The vial was transferred into a 42°C water bath for 3 minutes, and immediately placed back on ice for 2 minutes. The transformed bacteria were grown for 2 hours in 1ml of 2XYT medium at 37°C. No selective antibiotic was added at this point, to allow both the recovery of cells and the expression of resistant protein derived from the newly acquired plasmid. 20µl and 200µl of the transformed bacterial cells were spread on selective agar medium and grown overnight at 37°C (Heraeus Instrument, Germany).

The successfully transformed bacteria were selected in 2XYT medium supplemented with the appropriate antibiotic as listed in **Table 2.6**. Only clones containing the vector and subsequently expressing the resistant protein were capable of growing. Clones were then tested for the presence of the insert by purification of the plasmid DNA (by DNA miniprep, see following section) and restriction enzyme digestion of the insert.

Vector	*Bacterial strain*	*Selection*
PCR2.1	XL1-Blue	Ampicillin (Sigma, 50µg/ml) (IPTG and X-Gal can be added for α-complementation)
PET3d-bsp2	XL1-Blue (maintenance)	Ampicillin (50µg/ml)
PET3d-bsp2	BL21(DE3)pLysS (protein expression)	Ampicillin (50µg/ml) Chloramphenicol (34µg/ml for pLysS plasmid selection)
PBJ1Neo	JM109 or DH5α	Ampicillin (50µg/ml)

Table 2.6: Antibiotic selection of transformed bacteria:

This table summarizes the antibiotic required for the selection of transformed bacteria strain with the different plasmids used in this study. All antibiotics were purchased from Sigma (Surrey, UK). 100X stock solutions were prepared in distilled water (ampicillin) or ethanol (chloramphenicol) and kept at -20°C. Chloramphenicol was required to select for the pLysS plasmid encoding for the T7 lysozyme.

2.1.17 DNA miniprep:

12 to 16 clones were tested for the presence of the recombinant vector. These clones were selected with a sterile pipette tip and transferred into a 15ml sterile tube (Bibby sterilin, UK) containing 3ml 2XYT supplemented with the selective antibiotic. The bacteria were cultured overnight at 37°C in a shaking incubator (G25 model, incubator shaker, New Brunswick Scientific Inc., USA). Confluent cultures were transferred into sterile 1.5ml microcentrifuge tubes and bacterial cells were harvested by centrifugation for 10 minutes at 1300rpm. Supernatants were completely aspirated using a Pasteur pipette (John Poulten Ltd, Essex, UK) linked to a vacuum pump and cells were lysed in 100µl ice-cold hypotonic glucose solution (Solution I: 50mM glucose, 25mM Tris.Cl [pH 8], 10mM EDTA [pH8], autoclaved and kept at 4°C) vortexed vigorously and set on ice for 5 minutes. Bacterial proteins and DNA were lysed by the addition of 200µl detergent solution II (0.2M NaOH, 1% SDS). Cells were mixed by inverting the tubes a few times. This SDS-alkali treatment denatures both the linear genomic DNA and the circular recombinant plasmid. The rapid neutralisation with 150µl of solution III (KOAc: 60ml 5M potassium acetate, 11.5ml glacial acetic acid AcOH, 28.5ml dH2O: final concentration 3M potassium, 5M acetate) causes the linear genomic DNA to aggregate, but the recombinant plasmid is left intact as the two strands of this circular plasmid are tightly intertwined together. The tubes were vigorously vortexed and set on ice for 5 minutes. Proteins and denatured genomic DNA were separated from recombinant plasmid by centrifugation at 1300rpm for 5 minutes. Supernatants were transferred into fresh 1.5ml tubes containing 450µl of phenol:chloroform:isoamyl alcohol (25:24:1, BDH, Poole UK), mixed and centrifuged at 1300rpm for 5 minutes. The supernatant was recovered into a fresh 1.5ml tube and plasmid DNA was precipitated by the addition of 1ml of 100% ethanol. Tubes were mixed and incubated for 5 minutes at room temperature. DNA was pelleted at 1300rpm for 5 minutes. The supernatant was discarded and DNA was washed with 1ml 70% ethanol. After centrifugation at 1300rpm for 5 minutes, the DNA pellet was air-dried and then dissolved in 50µl of distilled water supplemented with DNAse free RNAse solution A (10µg/ml, Promega, Southampton, UK). Purified recombinant plasmid DNA was kept at -20°C until required.

2.1.18 DNA midi/maxi preparations:

DNA midi and maxi preps were performed following the protocol for DNA miniprep (section 2.1.17) scaled up to 50ml (midi) or 500ml (maxi) bacterial cultures. This was

performed to obtain a higher yield of DNA (0.25mg for midi, 2.5 mg for maxi) necessary for DNA transfection of eukaryotic cells.

2.1.19 DNA sequencing:

The positive clones for HLA-A*0301, -B*0801 in pET3d and HLA-A*0201, -A*0301, -B*0801 in pBJ1Neo were sequenced to verify their authenticity. The HLA class I genes cloned into pET3d were sequenced using primers designed for the pET3d vector and the HLA class I genes cloned in pBJ1neo were sequenced using the original primers (HLA-A or –B forward and reverse, described in **Table 2.3**). Using the original primers results in the loss of the first 30-50bp of sequence after the primers, therefore additional primers were designed to cover the entire HLA coding region. Two different sets of primers were designed, one for HLA-A alleles and one for HLA-B alleles. The position of these primers is shown in **Figure 2.1** (highlighted in pink). The sequencing primers are described in **Table 2.7**.

Primers for sequencing cloned HLA molecules in pET3d vector	
pET3d forward	GGAGACCACAACGGTTTCCC
pET3d reverse	TGCTAGTTATTGCTCAGCGG
Primers for sequencing cloned HLA molecules in pBJ1Neo	
HLA-A Middle forward	CAAGCACAAGTGGGAGGCGG
HLA-A Middle reverse	CCGCCTCCCACTTGTGCTTG
HLA-B Middle forward	GTGGGAGGCGGCCCGTGTGG
HLA-B Middle reverse	CCACACGGGCCGCCTCCCAC

Table 2.7: Description of primers used for sequencing:

This table describes the sequences of the different primers designed for the sequencing of the recombinant plasmids. All the primers were purchased from Amersham Biosciences, Bucks, UK.

Recombinant plasmids were amplified using the sequencing primers, following the original PCR program described in **Figure 2.2**. The PCR products were run on 0.8% DNA agarose gels, to verify the successful amplification of the insert as shown in **Figure 2.7**. The DNA was purified from primers and salt contaminants directly from the PCR tubes using GFX columns (described in section 2.1.11). DNA was recovered in 150µl of deionised water and sequenced using the ABI-Prism big dye terminator reactions.

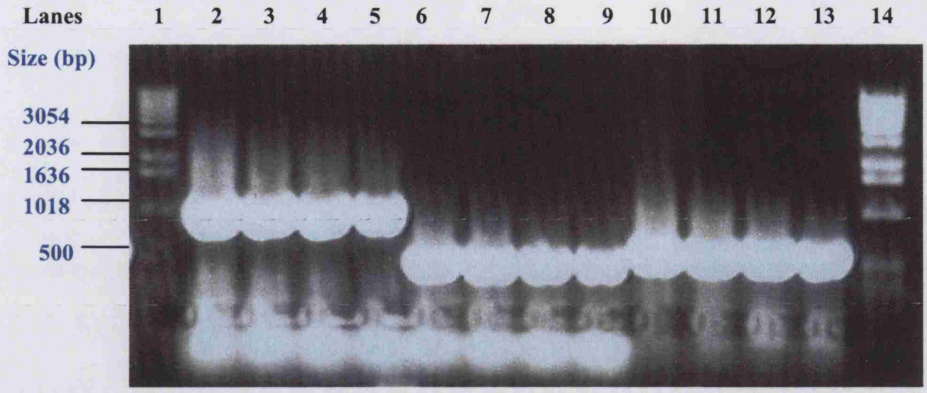


Figure 2.7: PCR amplification of HLA-B*0801/pBJ1Neo recombinant clones for DNA sequencing:

PCR products of four different HLA-B*0801/pBJ1Neo clones were loaded on a 0.8% agarose gel. A 1Kb DNA ladder was loaded in lanes 1 and 14. The different HLA-B*0801/pBJ1Neo clones were amplified with: HLA-B forward/HLA-B reverse primers (lanes 2 to 5), HLA-B forward/HLA-B middle reverse primers (lanes 6 to 9) and HLA-B Middle Forward/HLA-B Reverse primers (lanes 10 to 13). Strong, single DNA bands of: 1100bp (lanes 2 to 5), 501bp (lanes 6 to 9) and 600bp (lanes 10 to 13) are shown. The PCR products were purified from the PCR tubes using GFX columns and were sequenced.

The big dye terminator reaction relies on the presence of dideoxynucleotide analogs that are incorporated during the terminator cycle in the growing polynucleotide. This dideoxynucleotide analog contains a 3'-OH group, which prevents other nucleotides from incorporating, thus terminating the DNA chain. This results in the random generation of truncated polynucleotide chains, using a single primer (forward or reverse) per reaction (two reactions per DNA sample to sequence are required). The four dideoxynucleotides (A, T, C and G provided in the kit) are fluorescently labelled with a different dye and are detected at different wavelengths. The sequencing gel electrophoresis separates the DNA chains according to their length and the detection of the fluorescently labelled dideoxynucleotide incorporated from the 3' end, identifies the nucleotide at that position in the sequence. Analysing all the PCR fragments in the reaction results in the determination of the whole nucleotide sequence.

The Big Dye Terminator cycle was performed from GFX purified DNA as described in **Table 2.8**. The DNA product was harvested by the addition of 60μl of isopropanol (BDH, Poole, UK). DNA was precipitated 15 minutes and pelleted by centrifugation at 3200rpm for 30 minutes. Immediately after the centrifugation, PCR tubes were opened, inverted on tissue

paper and centrifuged at 1500rpm for 1 minute. Loading buffer was prepared by diluting Blue Dextran/EDTA (ABI, Applied Biosystems, Warrington, UK) with formamide at a ratio of 1:4 (v/v). The DNA pellet was recovered in 1.5µl of loading buffer and denatured for 2 minutes at 95°C in a thermal cycler (MJ Research, INC, Massachusetts, USA) and immediately placed on ice prior to loading.

Reagents	*Volume*	*Temperature in °C*	*Time*
DNA template	2µl		
Reaction buffer	1 µl	96	10 seconds
Sequencing mix	2 µl	25 cycles { 50	5 seconds
Forward or Reverse primer (1.6 pM)	1 µl	60	4 minutes
Deionised water	Up to 10µl	4	Forever

Table 2.8: Big Dye terminator Reaction components:

This table summarizes the components mixed for the Big Dye terminator reaction and the cycle program. Reaction buffer and sequencing mix were provided in the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK).

The sequencing gel was prepared as described in **Table 2.9**. The gel was filtered (to remove the resin) and degassed for 5 minutes. For this, the filtration apparatus (150 filter Unit, 0.2µm, Vivascience, Germany) was set up and attached to a vacuum pump. 5ml of 10X TBE buffer (Biowhittaker, Walkersville, Maryland) was first filtered and then the gel was poured. The sequencing plates were assembled with spacers and combs following the manufacturer's instructions. The gel was polymerised with 250µl of 10% ammonium persulphate (APS, Amersham Pharmacia Biotech, Bucks, UK) and 25µl TEMED (BDH, Poole, UK), gently mixed by swirling and immediately taken up into a 50ml syringe avoiding air bubbles. The gel was cast, the comb immediately inserted and the gel was left to polymerize for 2 hours. After removing the comb and gently washing off the excess of polymerised gel from the plates, the sequencing gel was then placed on the automated ABI 377 DNA sequencer according to the manufacturer's instructions. A disposable paper sharks teeth comb (36-wells, ABI Prism377, PE Biosystems) was placed on top of the gel to create the loading wells and 1X TBE buffer was poured into the top and bottom tanks. A plate-check run was first performed to verify any excess of polymerised gel on the plate, resulting in excess fluorescence detection.

Prior to loading the samples, the sequencing gel was conditioned at the appropriate temperature, salt concentration and electric field during the 30 minute pre-run. 1.5µl of denatured sample was loaded into alternate wells and run for 2 minutes before loading the remaining samples. The gel was run for 7 hours at 50°C using the ABI PRISM™377 data collection software and the sequences were analysed using the ABI PRISM™377 Sequencing analysis v.3.4.1 (Applied biosystems, Warrington, UK).

Reagents	*Amount*
Urea (Biorad, Hercules, USA)	18g
Amberlite resin (PE Applied Biosystem, Foster City, USA)	0.5g
Long Ranger solution (FMC Bioproducts, Rockland, USA)	5ml
Distilled water	27ml

Table 2.9: Sequencing gel composition:

The sequencing gel was prepared in a clean 200ml beaker (Pyrex, UK). A magnetic bar was added and the gel stirred for 10 minutes at room temperature.

2.2 Biochemistry:

2.2.1 Synthetic peptides:

The synthetic peptides used in this study are described in **Table 2.10**. All peptides were produced on an ABI synthesiser using F moc chemistry and purified by high performance liquid chromatography (HPLC) by Alta Bioscience, The University of Birmingham, UK. Their purity of more than 90% was confirmed by mass spectrometry. The peptides were dissolved in DMSO at 10mg/ml and stored at -20°C.

Peptide sequence	*Protein*	*HLA RESTRICTION*
ATGFKQSSKALQRPVAS	BCR/ABL	Multiple HLA
KQSSKALQR	BCR/ABL	HLA-A*0301
GFKQSSKAL	BCR/ABL	HLA-B*0801
SSKALQRPV	BCR/ABL	HLA-A*0201
SLKALQRPV	Modified BCR/ABL	HLA-A*0201
KALQRPVAS	BCR/ABL	HLA-A*0201/-B*0801
ILAKFLHWL	Human telomerase s/u,hTERT	HLA-A*0201
RMFPNAPYL	Wilms' tumour antigen, WT1	HLA-A*0201
VLQELNVTV	Proteinase 3 antigen, PR1	HLA-A*0201
NLVPMVATV	Cytomegalovirus, pp65	HLA-A*0201
IGDQYVKVY	Cytomegalovirus, pp150	HLA-A*0301
DANDIYRIF	Cytomegalovirus, pp65	HLA-B*0801
GILGFVFTL	Influenza Virus	HLA-A*0201

Table 2.10: Antigenic peptide sequences:

This table summarises the antigenic peptide sequences used in this project and their HLA restriction.

2.2.2 HLA heavy chains and β-2 microglobulin expression:

HLA-A*0201 (cloned by Dr Geraldine Aubert, ANRI), HLA-A*0301 and HLA-B*0801 were cloned into the modified pET3d vector (section 2.1.9) and expressed in BL21(DE3)pLysS *E.coli*. The β2m was provided by Dr Don Wiley, cloned into the pHN1 plasmid and expressed in XA90 *E.coli*. The HLA heavy chains and β2m were produced as insoluble proteins, following the same protocol.

A single colony or a scraping from a glycerol stock was inoculated into a 15ml sterile tube (Elkay, UK) containing 5ml 2XYT medium supplemented with the relevant antibiotic and grown overnight at 37°C (G25 environmental incubator, New Brunswick Scientific, Edison, N.J., USA). The bacterial culture was then transferred into 1L of autoclaved 2XYT media supplemented with the selective antibiotic and cultured at 37°C until the mid-log phase of exponential growth was reached ($O.D_{550}$ of 0.6-0.9). The recombinant protein expression was induced by the addition of 1mg of Isopropyl β-D-thiogalactopyranoside (IPTG, Alexis Corporation Ltd, Nottingham, UK) and the culture was incubated for an additional 4 hours.

Bacterial cells were transferred into a 1L centrifuge bottle and harvested by centrifugation at 4000g (or 6000rpm) for 20 minutes at 4°C (Beckman, JB-6, Buckinghamshire, UK). The supernatant was disinfected and discarded. The bacterial cell pellet was lysed with 10ml Bug lysis buffer (25% (w/v) sucrose, 50mM Tris-HCl, 1mM EDTA, pH 8.0, filtered), then transferred into a 50ml Falcon tube (BD, London, UK) and centrifuged for 10 minutes at 4000g (6000rpm)/4°C (Jouan KR221 high speed centrifuge, France). From this stage, all steps were performed on ice. The pellet was resuspended in 10ml B-Per® Bacterial Protein Extraction Reagent (Pierce, Cheshire, UK) supplemented with 25mg lysozyme (Sigma, Surrey, UK) and the proteins obtained as insoluble inclusion bodies were isolated from the bacterial cell pellet by five 15 second pulses of sonication performed on ice at 45 seconds intervals (50% duty, Jencons Scientific Ltd, Vibra Cell™, Danbury, CT, USA).

The insoluble inclusion bodies were purified and washed once with 10ml detergent buffer (20mM Tris-HCl, 0.2M NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P40, 2mM EDTA (GibcoBRL) pH7.5) and three times with 10ml Triton buffer (50mM Tris-HCl, 100mM NaCl, 1mM EDTA, 0.5% (v/v) Triton X100, pH 8,0). After each wash, the tubes were spun at 10,000g (or 9440rpm)/4°C for 20 minutes and the supernatant discarded. The inclusion bodies were kept as a pellet at -70°C or solubilised in urea buffer.

Inclusion bodies were dissolved in 15-25ml Urea buffer I (8M urea, 0.1mM EDTA, 0.1mM DTT , 0.01M Tris-HCl, 0.1M NaH_2PO_9, pH8) for HLA-B*0801and β2m, or Urea buffer II (8M Urea, 0.1mM EDTA [GibcoBRL], 0.1mM DTT, 50mM MES, pH6.5) for HLA-A*0201, -A*0301, depending on the overall ionic charges of the protein. Urea buffers were first deionised overnight by the addition of 10g of the ion exchanger Amberlite (Merck, Germany) and stirred at 4°C. Inclusion bodies were solubilised overnight by rolling at 4°C. Proteins were kept for a short time at -70°C.

2.2.3 Measurement of protein concentration:

Protein concentration was determined using the micro BCA™ Protein Assay Reagent Kit (Pierce Ltd, Cheshire, UK). This assay is based on protein quantification by the Lowry method. The peptide bond reduces Cu^{2+} in an alkaline environment, and the resulting cuprous ion (Cu^{1+}) chelates two molecules of Bicinchoninic acid (BCA) and forms a purple complex, which absorbs at 562nm. The quantity of purple complex formed is proportional to the quantity of peptide bonds present in the sample.

Briefly, a serial dilution of protein sample was prepared in 500µl of distilled water and aliquoted in 1.5ml eppendorf tubes. In parallel, a serial dilution of BSA protein standard (from 0.5 to 10µg/ml) was prepared. BCA reagent (25 parts BCA reagent MA, 24 parts BCA reagent MB, 1 part BCA reagent MC) was freshly prepared and 500µl was aliquoted in each tube. The samples were incubated for 1 hour at 60°C and the U.V absorbances were read at 562nm against a distilled water blank. The concentration of protein in the samples was determined from the BSA standard curve.

2.2.4 Electrophoretic separation of Proteins:

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE):

SDS-PAGE allows the separation of proteins according to their size. Under reducing conditions and heat, proteins are dissociated into polypeptides that are negatively charged by binding to SDS and migrate through an electric current according to their size. Polyacrylamide gels are composed of a stacking gel (high porosity) that deposit the proteins on the surface of the resolving gel. The sample protein size was estimated by loading protein markers of known molecular weight.

Lower gel buffer (1.5M Tris, 0.4% SDS, pH8.8) and upper gel buffer (0.5M Tris, 0.4% SDS, pH6.8) were filtered and kept at 4°C. The SDS-PAGE apparatus was assembled (Biorad, Hercules, California, USA) and gels were prepared in 50ml Falcon tubes as described in **Table 2.11**. Ammonium persulphate (Amersham Pharmacia Biotech, Bucks, UK) and TEMED (BDH, Poole, UK) cross-link and polymerise the gel and therefore were added immediately before pouring the gel between the two glass plates. First, the resolving gel was poured and overlaid with 1ml of isopropanol to create an even surface. Once polymerised, the stacking gel was prepared and poured on top of it after removing the isopropanol. A well comb was inserted and the gel left to polymerize.

Reagents	*Stacking gel (upper gel)*	*Resolving gel (lower gel)*
30% Acrylamide, Biorad	1.5ml	15ml
Upper/lower gel buffer	2.5ml	7.5ml
Water	6ml	7.5ml
10% ammonium persulphate, Pharmacia Biotech	50µl	100µl
TEMED, BDH	10µl	10µl

Table 2.11: SDS-Polyacrylamide gel composition:

This table summarises the reagents necessary to cast two 10% acrylamide SDS-PAGE gels. The stacking and resolving gels were prepared separately in 50ml Falcon tubes (BD). Ammonium persulphate and TEMED were added just prior pouring the gels between the two glass plates (Biorad apparatus).

Samples containing at least 5µg of protein were mixed in small eppendorf tubes with 10-15µl of SDS loading buffer (50mM Tris.HCl pH6.8, 2% (w/v) SDS, 0.1% (w/v) Bromophenol Blue, 10% (v/v) glycerol, 100mM Dithiotreitol, Sigma) and heated for 3 minutes at 100°C (hot block). Once the gel polymerised, SDS electrophoresis buffer (25mM Tris, 250mM Glycine, 0.1% (w/v) SDS) was overlayed and the combs removed. Samples were loaded in the wells in parallel with a low and a high molecular weight standard (Biorad). The gels were run at 150 Volts, 400 mA (Biorad powerpac 300) until the bromophenol blue dye reached the bottom of the gel (approximately 1 hour). Gels were stained with Coomassie Blue and dried for analysis (section 2.2.5). The SDS-PAGE separation of HLA class I and β2-microglobulin inclusion body preparations is shown in **Figure 2.8**.

Figure 2.8: SDS-PAGE of large scale protein expression:

Inclusion bodies of HLA-A*0201 (lane 1), human β-2 microglobulin (lane 2) and HLA-B*0801 (lane 5) were separated on a 12.5% acrylamide SDS-PAGE gel. High range (lane 3) and low range (lane 4) molecular weight markers (MW expressed in Dalton, Da) were loaded in parallel (Biorad prestained SDS-PAGE standards). Predominant protein bands of 35,000Da (HLA heavy chain without the intracellular portion) are observed in lane 1 and 5; and a protein band of 12,000Da (β-2 microglobulin) is observed in lane 2. Several protein bands are seen in the inclusion body preparation as a result of bacterial protein contamination.

Native gel shift assay:

The biotinylation efficiency of recombinant proteins (section 2.2.8) was estimated in a native gel shift assay. Native gel electrophoresis under non-reducing conditions separates proteins according to their size, charges and tertiary structure. Biotinylated proteins in the presence of extravidin form a tetrameric complex and migrate more slowly than non-biotinylated protein. In the presence of an excess of extravidin, the shift obtained between the migration distance between HLA monomers (with no extravidin) and extravidin-biotinylated HLA monomer complexes was proportionally related to the percentage of biotinylation. Native gels were prepared similarly to SDS-PAGE gels. The native resolving and stacking gel composition is described in **Table 2.12**. After polymerisation, gels were pre-run in the native gel running buffer (24.8mM Tris/192mM Glycine) for 30 minutes at 150 volts to remove any excess salts that might affect the protein migration.

A minimum of 2μg of non-biotinylated and biotinylated HLA monomers were aliquoted in small eppendorf tubes and mixed with 0μg, 3μg, 6μg or 9μg of extravidin (Sigma). Protein complex formation was obtained after incubation for one hour at room temperature. The

samples were then mixed with 5µl of native gel loading buffer (50mM Tris-HCl pH 6.8, 0.1% (w/v) Bromophenol blue, 10% (v/v) Glycerol) and loaded in the wells. Gels were run for approximately 1h30 mins at 150 volts, stained with Coomassie Blue and dried for analysis (section 2.2.5). A sample native gel shift assay is represented in **Figure 2.9**.

	Resolving gel	*Stacking gel*
Acrylamide 30% (Biorad)	8ml	1.7ml
Double distilled water	14.5ml	6.8ml
Tris, Hcl (BDH)	7.5ml of 1.5M stock, pH8.8	1.25ml of 1M stock, pH6.8
Added immediately before pouring the gels:		
Ammonium persulphate 10%	100µl	
TEMED (BDH)	10µl	

Table 2.12: Native polyacrylamide gel s composition:

This table summarises the reagents used to cast two Native gels. The stacking and resolving gels were prepared separately in 50ml Falcon tubes. Ammonium persulphate was purchased from Amersham Pharmacia Biotech, Bucks, UK.

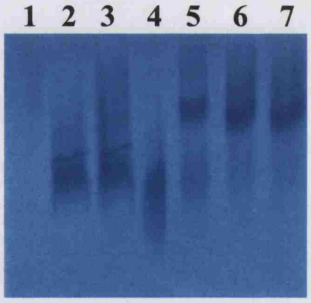


Figure 2.9: Native gel shift assay:

HLA-B*0801/CML monomers pre and post-biotinylation were incubated with extravidin for 1 hour at 37°C and separated on a 10% acrylamide native gel. The non-biotinylated monomers in the absence of extravidin (lane 2) or in the presence of 3µg of extravidin (lane 3) demonstrate a similar migration distance. The post-biotinylated monomers without extravidin are shown in lane 4; and in the presence of 3µg, 6µg or 9µg of extravidin in lanes 5, 6 and 7 respectively. The biotinylated monomers form a tetrameric complex with the extravidin, modifying the overall protein structure and charge, which results in a different distance of migration. This is observed by a "shift" of the protein band in the presence of a saturating concentration of extravidin (from lane 4 to lane 5, 6 and 7). 3µg of extravidin was loaded in parallel and is shown in lane 1.

2.2.5 Protein detection by coomassie blue staining:

SDS-PAGE and Native gels were carefully transferred into a plastic container, and immersed in Coomassie blue staining solution (10% (v/v) glacial acetic acid, 0.25% (w/v) Coomassie brillant blue G250, 50% (v/v) methanol, in distilled water). Gels were stained for at least 30 minutes at room temperature with gentle shaking. The coomassie blue solution was recovered for future use. Gels were destained with Coomassie destain solution (165ml methanol, 50ml acetic acid, 785ml water) overnight, changing the solution 4 to 5 times. Once proteins were clearly visible, the gel was placed on a Whatman paper (3MM, Whatman®), covered with saran wrap and dried for 2 hours (Gel Dryer, model 583, Biorad).

2.2.6 HLA/peptide complexes refolding:

Each HLA class I heavy chain was refolded with the appropriate peptide and β2m as follows. A refolding buffer (400mM arginine (Sigma), 100mM Tris, 5mM reduced glutathione (Sigma), 0.5mM oxidised glutathione (Sigma), 2mM EDTA (GibcoBRL), pH8) was prepared in a 400ml glass beaker (Pyrex, UK) and pre-cooled at 4°C. For a 1X scale preparation, 4.8mg of β2-microglobulin, 2 mg of peptide and 9.6mg of HLA class I heavy chain were added respectively drop-wise into the refolding buffer and incubated for 72 hours at 4°C with continuous stirring. The refolding mixture was transferred into 50ml Falcon tubes and centrifuged at 10000rpm/4°C for 15 minutes to exclude any aggregates. The supernatant was transferred into an ultrafiltration stirred cell (Amicon® ultrafiltration cell, model 8400, Millipore, Bedford, USA) and concentrated down to 25ml through a 10,000 molecular weight cut off (MWCO) membrane (YM 10, 10,000 MWCO, Millipore, Bedford, USA) under nitrogen gas pressure. The refolded HLA monomers were transferred into a 20ml Vivaspin concentrator (10,000 MWCO, Vivascience, Epsom, UK) and further concentrated down to 1-2 ml. The concentrated product was kept at 4°C for no longer than 24 hours before purification by Fast Protein Liquid Chromatography (FPLC) gel filtration.

2.2.7 Purification of HLA class I monomers by FPLC gel filtration:

HLA class I monomers were purified from aggregates, free β2m and peptide by FPLC gel filtration on an Akta purifier (Amersham Pharmacia Biotech, Bucks, UK). The gel filtration was performed on superdex™ 75 matrix (separation range of 3000-70000 MW protein, Amersham Pharmacia Biotech) packed onto a XK16/70 column. The matrix consists of dextran covalently bound to cross-linked agarose beads, which results in the separation of protein by size exclusion. The gel filtration run was acquired on Unicorn 3.10 software. A maximum pressure of 0.3 Mpa was set, the buffer was run at a speed of 1ml/min and eluted fractions were collected in a volume of 3ml per tube. Protein elution was monitored by UV absorbance at 280nm. Prior to protein purification, the column efficiency was tested with 20mg/ml acetone and a minimum of 13 $000m^{-1}$ theoretical plates was expected (Amersham Pharmacia Biotech). The column resolution was estimated by running protein standards of known molecular weight (Gel filtration molecular weight markers, Sigma). This allowed us to determine at which fraction a particular protein was expected to be eluted.

The column was first equilibrated with one and a half column volumes of the appropriate buffer. The concentrated HLA refolded mixture (<2ml) was filtered through 0.2μm filter (Low protein binding, Acrodisc®13, Gelman Sciences, Portsmouth, UK) in a sterile 2ml eppendorf tube before loading the sample onto the FPLC. HLA refolded monomers were purified with a very low salt buffer (10mM Tris-HCl, 5mM NaCl, pH8, filtered and deionised) to obtain the optimum salt concentration for the enzymatic biotinylation reaction. Post biotinylated HLA monomers were purified from free biotin following the same protocol using a physiological salt buffer (20mM Tris-HCl, 150mM NaCl, pH8, filtered and deionised). The purification of HLA-B*0801 monomers is shown in **Figure 2.10**.

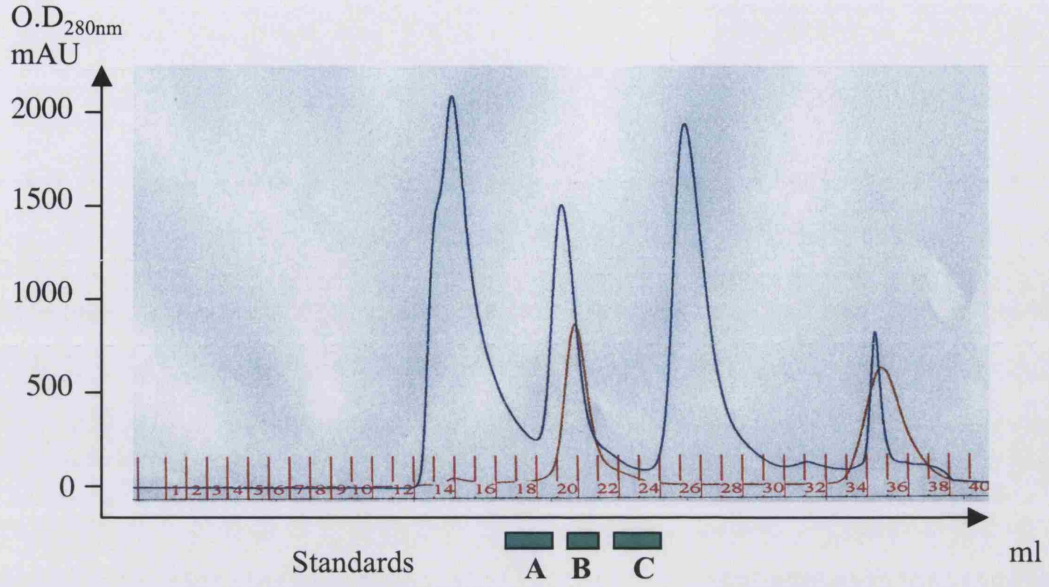


Figure 2.10: FPLC purification trace of HLA class I refolded complexes:

The FPLC profile of HLA-B*0801/CML monomers purification pre- (blue curve) and post-biotinylation (red curve) are represented. Protein elution was monitored by UV absorbance at 280nm (Y-axis, expressed in mAU). Eluted protein composition was determined by a calibration curve obtained by separating proteins of known molecular weights (Standards, Sigma): **A**, Albumin, 66,000Da; **B**, Ovalbumin, 45,000Da; and **C**, Carbonic anhydrase, 29,000Da. Refolded HLA monomers purification profile (blue curve) separated **p1**, the aggregates; **p2**, the refolded HLA monomers; **p3**, free β2-microglobulin; and **p4**, arginine (contained in the refolding buffer) and free peptide. The **p2** fraction was collected, biotinylated and purified (red curve, **p1**) from the free biotin (**p2**).

2.2.8 Biotinylation of refolded HLA proteins:

Biotinylation of HLA class I monomers was performed using a biotinylation enzyme BirA produced "in house". This enzyme catalyses the formation of biotinyl-5'-adenylate from biotin and adenosine tri-phosphate (ATP), which is transferred to the specific lysine (K) residue on the biotin substrate peptide tag sequence expressed at the C-terminus of the HLA class I heavy chain molecule.

The purified HLA monomer fractions were transferred into a 5ml Vivaspin concentrator (Vivascience, Epsom, UK) and concentrated down to 200µl by centrifugation at 3000rpm, 4°C. This was transferred into a sterile 500µl eppendorf tube and 25µl of Biomix A (0.5M Bicine, pH 8.3), 25µl of Biomix B (100mM ATP, 100mM MgOAc, 400µm Biotin); protease

inhibitors (2.5µl of leupeptin at 5mg/ml, 2.5µl of pepstatin at 2.5mg/ml, Sigma); 2.5µl of D-biotin (100mM stock in Tris-HCl, pH8, Sigma) and 20µl of Bir A enzyme (1mg/ml stock) were added. The biotinylation reaction was carried out overnight at room temperature. Biotinylated HLA monomers were purified from excess of biotin by FPLC (section 2.2.7 and **Figure 2.10**).

2.2.9 Generation of HLA-tetrameric complexes and storage:

The tetramerization of HLA class I molecules was necessary to obtain sufficient avidity for the T cell receptor ligand to form a stable, relatively long lasting complex. The use of fluorescently conjugated streptavidin allows the visualisation by flow cytometry of specific T cells that recognize and bind to HLA class I molecule/peptide complexes (tetramers). Avidin or a modified version, streptavidin has the capacity to form four strong non-covalent bonds with biotin, thus forming a tetramer complex.

Purified biotinylated HLA class I monomers were concentrated down to 100-200µl using Vivaspin concentrators (Vivascience) and the protein concentration was measured by BCA assay (described in section 2.2.3). The correct refolding and the biotinylation efficiency of HLA class I molecules were verified by an Enzyme Linked Immuno-Sorbent Assay (ELISA) and native gel shift assay respectively, prior to tetramerization (these methods are described in the following sections).

Biotinylated refolded HLA class I monomers were tetramerized with phycoerythrin (PE) or allophycocyanin (APC) conjugated streptavidin (Biogenesis, Poole, UK) at a molecular weight ratio of four to one respectively. Biotinylated HLA monomers were placed on a shaker at 4°C, and 0.375µg of conjugated streptavidin was slowly added per 1µg of HLA monomers. The amount of conjugated streptavidin necessary to tetramerize four molecules of HLA class I monomers was calculated according to their molecular weight (MW). Very small volumes of streptavidin were added over a few hours time, favouring the formation of tetrameric complexes. Finally 10% (v/v) sterile glycerol and 0.1% (w/v) sodium azide were added to maintain the integrity and sterility of the tetramer complex. HLA-tetrameric complexes were kept aliquoted at -70°C for long-term storage, -20°C for up to 6 months, or at 4°C for up to 1 month.

2.2.10 Generation of soluble Antigen-presenting complexes: Modified HLA/tetrameric complexes:

Antigen presenting cells (APCs) prime and/or activate antigen specific T cells by the presentation of HLA/peptide complexes and secondary costimulatory signals. Soluble artificial antigen-presenting complexes (sAPCs) were thus generated by cross-linking HLA/peptide monomers with a costimulatory antibody. Goat-anti human biotinylated IgG antibodies were purchased from R&D (Minneapolis, USA) and are listed in **Table 2.13**.

Name	*Function*
CD27	Early and late T cell activation signal
CD28	Early T cell activation signal (signal 2)
CD40L	Late T cell activation signal (T helper)

Table 2.13: Biotinylated T cell costimulatory antibodies:

This table summarises the different costimulatory antibodies used for the generation of soluble artificial Antigen-Presenting Complexes.

Biotinylated HLA/peptide monomers and biotinylated antibodies were cross-linked with phycoerythrin (PE)-streptavidin at the theoretical ratio of 3 to 1. The amount of HLA/peptide monomers to mix with costimulatory antibody was calculated according to their molecular weight (MW): 49,000Da for HLA/peptide complex and 150,000Da for IgG antibody. As the molecular weight of IgG antibody and in order to make sure that three HLA/peptide complexes would be cross-linked in these sAPCs, a little excess of HLA/peptide monomers were added. Thus, the theoretical calculation was performed at a ratio of 3.5 to 1.

For a ratio of 3.5:1: 171,500Da (3.5×49,000):150,000

1μg of HLA/peptide monomer was cross-linked with 0.8μg of costimulatory antibody.
Biotinylated costimulatory antibodies were resuspended in the same buffer as the HLA/peptide monomers (20mM Tris.Hcl, 150mM NaCl, pH8) and were incubated with the HLA/peptide monomers at 4°C with shaking for 1 hour. The cross-linking was performed by

slowly adding 0.186μg of PE-extravidin per μg of complex (or 0.372μg/μg of HLA monomer) over two hours. The final concentration of these sAPCs was 0.53μg of HLA/peptide and 0.43μg of costimulatory antibody per μl of complex. These modified HLA complexes, or soluble artificial antigen-presenting complexes were kept at -20°C for long-term storage or 4°C for short-term use. The cross-linking of both HLA/peptide monomers and costimulatory antibody in the complex was verified in a modified Enzyme Linked Immuno-Sorbent Assay (ELISA described in section 2.2.13).

2.2.11 Dot Blot:

The refolding and biotinylation of HLA class I monomers was verified in a Dot Blot immuno-assay. A nitrocellulose membrane (Hybond ™ ECL, Amersham Life Science) was placed into a vacuum dot-blotter (Hybridot Manifold, Besthesda Research laboratories) and pre-wetted with transfer buffer (25mM Tris, 192mM Glycine, 0.05% (w/v) SDS, 20% (v/v) methanol). A minimum of 5μg protein sample was loaded into the wells in duplicate and fixed to the membrane under vacuum pressure. Each well was then washed twice with 200μl of PBS. The membrane was transferred into a 50ml Falcon tube containing 20ml of blocking buffer (PBS, 1% (w/v) BSA, 0.05% (v/v) Tween) and incubated overnight, rolling at 4°C. The membrane was subjected to three 15 minute washes with PBS-T buffer (PBS, 0.05% (v/v) Tween), and cut into two identical strips for testing against different antibodies. The strips were incubated either with 5μg/ml W6/32 (HLA class I specific antibody) or with Streptavidin-Horse radish peroxidase conjugated (biotin specific, used at 1:1000 dilution, Sigma) diluted in 15ml PBS-T buffer and incubated for 1 hour, rolling at room temperature. After three 15 minute washes in PBS-T buffer, the strip tested against W6/32 antibody was incubated for one hour with the second layer goat-anti mouse peroxidase-conjugated antibody (1:2500 dilution, Sigma) diluted in 15ml PBS-T buffer. The strips were finally washed three times with PBS-T buffer and the presence of peroxidase was revealed by incubating the membranes in ECL chemi-luminescent reagent for 1 minute (ECL™ reagent kit, Amersham Pharmacia Biotech). The nitrocellulose membranes were then wrapped in saran wrap and exposed to X-Ray photographic film (MXB film, GRI autoradiography blue sensitive, Kodak) for different periods of time (from 30 seconds to 30 minutes). An example of a Dot blot assay for the analysis of HLA class I monomers biotinylation is represented in **Figure 2.11**.

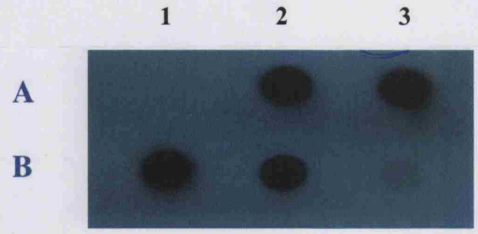


Figure 2.11: DOT BLOT analysis for biotinylation:

Proteins were blotted onto a nitrocellulose membrane and probed with Streptavidin-Peroxidase. 5μg of commercial non-biotinylated (**A 1**) and biotinylated (**A 2** and **3**) proteins are shown. HLA-B*0801 monomers pre-biotinylation (5 μg, **B 3**) and post-biotinylation (2.5 μg **B 2** and 5 μg **B 1**) were blotted in parallel. A visible dark spot is observed for positive biotinylated proteins.

2.2.12 Monoclonal antibody affinity chromatography purification:

W6/32 (HLA class I specific), BB7.2 (HLA-A*0201 specific), GAP-A3 (HLA-A*0301 specific) and L243 (HLA class II specific) antibodies were produced from hybridoma cell line culture (described in section 2.3.9). Antibodies were harvested from the culture supernatants by centrifugation at 3000rpm for 15 minutes. The supernatants were transferred into an ultrafiltration stirred cell (Amicon® ultrafiltration cell, model 8400, Millipore) and concentrated down to 20ml through a 10,000 Molecular Weight Cut Off (MWCO) membrane (YM 10, 10,000 MWCO, Millipore) under nitrogen gas pressure.

Crude concentrated antibodies were used for indirect cell surface detection of HLA molecules by flow cytometry. A titration of the quantity of antibody necessary for the staining was performed on HLA positive and negative cell lines (described in section 2.4.5 and shown in **Figure 2.17**). The antibodies were kept in 0.05% sodium azide at -20°C for long-term storage, or 4°C for short-term storage.

A portion of these concentrated antibodies were purified by affinity chromatography using the Affi-Gel® Protein A MAPS® II Kit (Biorad) for their subsequent use in immuno-assays such as Dot Blot or Elisa. The affinity chromatography matrix consists of protein A chemically bound to agarose beads. Protein A is derived from Staphylococcus aureus and has a high affinity for immunoglobulin Fc regions. Antibodies specifically bind to the Protein A agarose beads and contaminants are washed off. The antibodies are then eluted by

a low pH elution buffer and immediately recovered in pH9 Tris/HCl buffer to neutralise the acidity. The matrix, the column and the buffers are provided in the kit.

5ml of agarose beads were packed and left to settle in a 10 cm Econo-column. The column was manually connected to a peristaltic pump (Pharmacia Fine Chemicals, Gilson, France) and set at a flow rate of 1ml/min. The column was equilibrated with 5 volumes (25ml) of binding buffer before loading a maximum of 30mg of antibody diluted 1:1 in the binding buffer. Protein contaminants were washed off with 15 bed volumes (75ml) of binding buffer. The antibodies were then eluted with 5 bed volumes (25ml) of elution buffer (pH3) and were collected in two sterile 50ml falcon tubes containing 8ml of neutralising buffer (1M Tris HCl, pH9, filtered). An additional 5 bed volumes of elution buffer was applied on the column to ensure total removal of antibodies. The column was regenerated with 5 bed volumes of regeneration buffer and kept in PBS + 0.05% sodium azide at 4°C. The purification efficiency was estimated by separating the various fractions (flow-through, washes and elutions) on an SDS-PAGE gel, including non-purified antibody and pure antibody controls as shown in **Figure 2.12**.

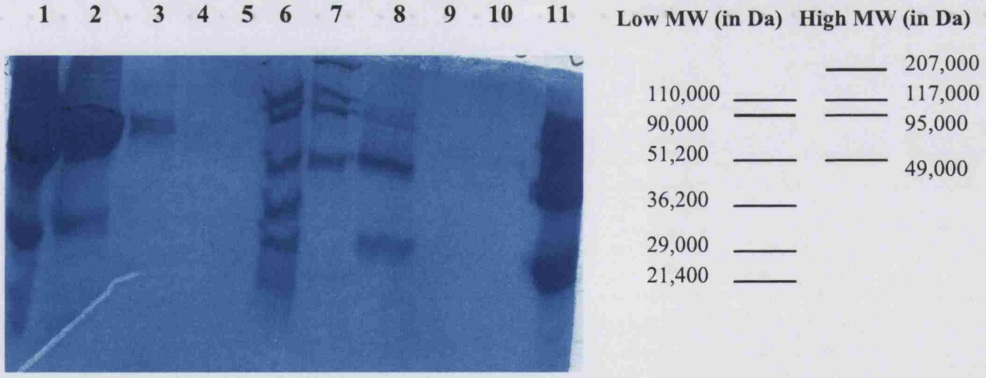


Figure 2.12: W6/32 antibody purification:

W6/32 antibody was purified by affinity chromatography (Affi-Gel®II Biorad Kit). The different fractions obtained were separated on a 10% SDS-PAGE to estimate the purification efficiency. 10µl of the flow-through (lane 2), wash 1 (lane 3), wash 2 (lane 4), wash 3 (lane 5), eluate 1 (lane 8), eluate 2 (lane 9) and eluate 3 (lane 10) were loaded. An aliquot of W6/32 pre-purification (lane 1) and a pure IgG Antibody (BB7.2 Ab, lane 11) were loaded in parallel. The pre-stained low range (lane 6) and high range (lane 7) molecular weight standards are shown (size expressed in Da). IgG immunoglobulins in denaturing conditions, are separated in two bands of 50,000Da (IgG heavy chain) and 25,000Da (IgG light chain). The Antibody was recovered in the elution 1 fraction (lane 8), and purified from the culture medium protein contaminants observed in lane 1 (pre-purification). A significant amount of Antibody is observed in the flow-through fraction (lane 2), which corresponds to unbound antibody (due to the maximum capacity of the column to bind 30mg of Antibody). This fraction was saved and re-purified.

The purified antibodies were precipitated by the addition of 0.314g of ammonium sulphate granules per ml of eluted antibody and the mixture was left for 1 hour, rolling at 4°C. Antibodies were pelleted by centrifugation at 10,000rpm for 30 minutes/4°C (Jouan KR221 high speed centrifuge, France) and solubilised in 1ml of PBS. Ammonium persulphate ions were removed by buffer exchange dialysis, using a 6000-8000 MW cut off dialysis tubing (Spectrum®, UK). The dialysis tubing (15 cm) was sealed at one extremity, pre-wetted in PBS for 10 minutes before carefully transferring the 1ml antibody sample into the tubing and sealing the other extremity. Dialysis was performed in 1L PBS stirring at 4°C for 48 hours, exchanging the PBS buffer 4 times per day.

The antibodies were recovered from the tubing in sterile eppendorf tubes, with the addition of 0.05% of sodium azide and kept at -20°C for long-term storage, or 4°C for short-term. Antibody concentration was determined by BCA assay (section 2.2.3).

2.2.13 Enzyme Linked Immunosorbant Assay (ELISA):

ELISA immunoassay was performed to verify the correct refolding of the generated HLA class I monomers and the cross-linking of costimulatory molecules with HLA class I monomers. The ELISA sandwich reaction is described in **Figure 2.13**.

ELISA for testing correct HLA class I refolding:

Maxisorb immuno-plates (Nunc® Nalge Nunc International, Roskilde, Denmark) were coated with 0.5μg of W6/32 antibody per well and incubated for 3 hours at 37°C (a control lane without antibody was also included). The plates were blocked with 1% BSA in PBS overnight at 4°C to avoid non-specific binding. After 6 washes with PBS, 5 μg of protein sample (from the FPLC fractions) were aliquoted in the first well of each column (except for the control) in a final volume of 200μl. In parallel, controls including HLA heavy chain, β-2 microglobulin and correctly refolded controls were loaded. Serial dilutions were performed by transferring 100μl from the first well into the following wells and the plates were left for 1 hour at RT. After 6 washes with PBS, 100 μl of rabbit anti-human β2m (DAKO, UK) diluted 1:5000 in PBS was added to each well and incubated for 20 minutes at RT. After a further 6 washes with PBS, 100μl of alkaline phosphatase goat-anti rabbit Ig G(Sigma) diluted 1:5000 in PBS was added to each well and incubated for 20 minutes at RT. Finally, after 6 washes with PBS, 100μl of the alkaline phosphatase substrate was added to the wells

(Sigma Fast™ p-Nitrophenyl phosphate tablet sets, Sigma) and the plates were left at room temperature until a yellow colour was visible, 10 to 45 minutes. The reaction was stopped by the addition of 50µl of 0.1M EDTA, pH8.0 and the plate was read in an ELISA plate reader (Titertek Multiskan® MCC/340, Labsystems, Finland) at a wavelength of 405nm. The $O.D_{405nm}$ values obtained for the protein samples were plotted against the $O.D_{405nm}$ values obtained for the control (no antibody). A representative ELISA for the evaluation of the correct refolding of HLA/peptide monomers is shown for the HLA-B*0801/BCR-ABL complexes in **Figure 2.14**.

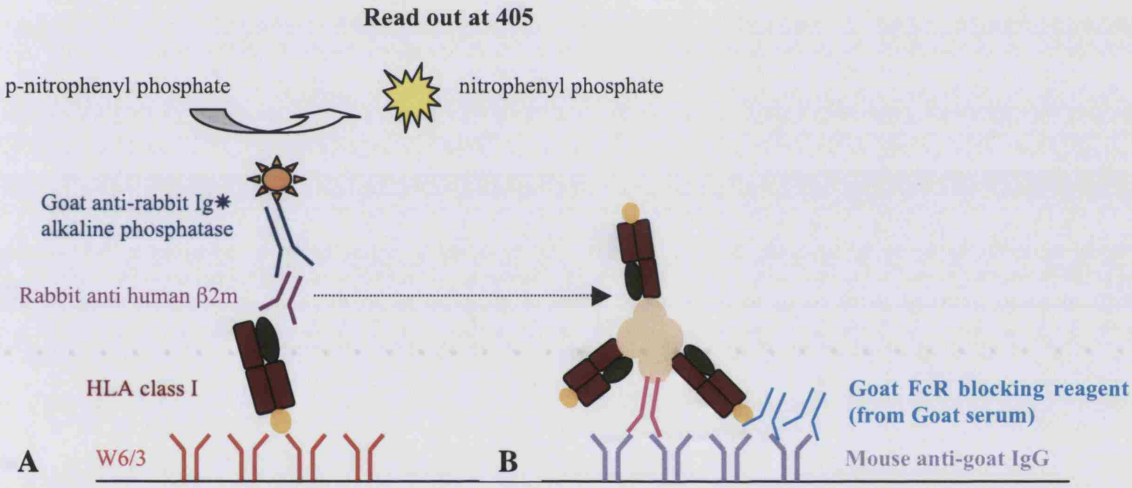


Figure 2.13: ELISA sandwich:

ELISA immunoassay was performed to assess the refolding of HLA class I monomers (A) and the presence of both costimulatory molecules and HLA class I monomers on the modified HLA class I tetramers (B, artificial sAPCs). The coating antibody to capture HLA class I molecules was W6/32 (conformationally-dependant HLA class I specific antibody) and to detect costimulatory molecules, the coating antibody was mouse anti-goat IgG (all the costimulatory IgG antibodies were produced in goat). The plates were blocked two hours at room temperature (or overnight at 4°C) with 1% BSA in PBS (100µl/well). After washing the plates 6 times with 200µl of PBS, 5µg of protein samples were loaded in the first well of each column. Serial dilutions were performed by transferring 100µl from the first lane to the following lanes. Refolded HLA molecules were then detected with a rabbit anti-human β2-microglobulin antibody. The second layer was a goat anti-rabbit IgG alkaline phosphatase conjugated antibody. Therefore, the plates coated with mouse anti-goat IgG (panel B) were blocked with goat Fc Receptor blocking reagent to avoid non-specific binding of the second layer detection antibody with the capture antibody. The specific binding of the tested molecules to the capture antibody was finally revealed by the alkaline phosphatase substrate (Sigma Fast™p-Nitrophenyl phosphate tablet sets, Sigma) and detected at a wavelength of 405 nm.

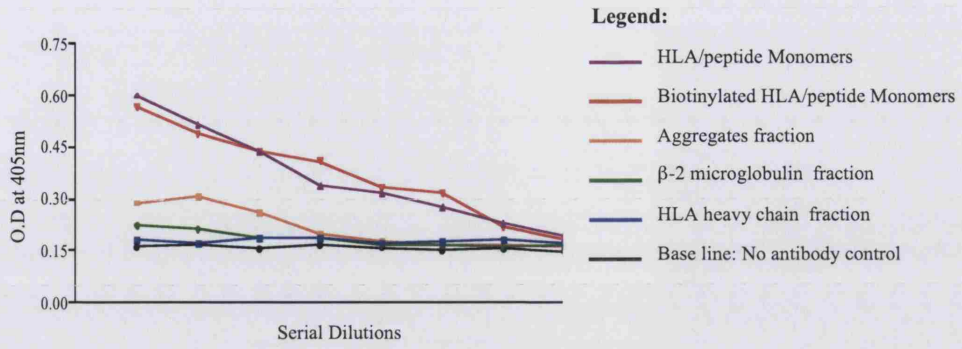


Figure 2.14: Representative assessment of HLA/peptide monomers refolding by ELISA:

The structural integrity of HLA-B*0801/BCR-ABL monomers was verified in an ELISA assay as described in section 2.2.13. The HLA-B*0801/BCR-ABL monomers pre-biotinylation (purple line) and post-biotinylation (red line) were assessed in parallel to the Aggregate fraction (orange line), the β-2 microglobulin fraction (green line), and the HLA heavy chain fraction (blue line). A negative control was also performed (No antibody, black line). The samples were serially diluted (1:2) from 5 μg to 0.039μg. The optical density (O.D) was read at 405nm.

ELISA for testing the modified tetramers:

The presence of both costimulatory antibody and correctly refolded HLA class I molecules in the generated modified tetramers was tested following the ELISA method described above using a different capture antibody. All the costimulatory molecules were produced in goat, thus the ELISA plates were coated for 3 hours at 37°C with anti-goat IgG antibody (1:3000 dilution in PBS, Sigma). After capturing the modified HLA tetramers, the plates were blocked with goat serum (1:100 dilution in PBS, sigma) for an additional two hours at room temperature. This was necessary as the second antibody layer, the anti-rabbit alkaline phosphatase conjugated antibody, is produce in goat and could directly bind to the capture antibody. The subsequent steps were identical to the ELISA assay described previously for testing the correct refolding of HLA class I monomers (**Figure 2.13**). Controls including no antibody, tetramer and soluble costimulatory antibodies were assessed in parallel (discussed in chapter 6).

2.3 Cell Separation and Cell culture:

2.3.1 Patient recruitment:

In this study, CML patients were recruited from six different centres with ethics committee approval from the local institutional boards (detailed in Appendix 1): Royal Free Hospital, London; Kings College Hospital, London; BARTS hospital, London, Hammersmith hospital, London, University College Hospital, London and Liverpool Royal Infirmary, Liverpool. Patients were approached by clinicians and informed about the study. They received a detailed information sheet describing the objectives of the study and participating patients signed a consent form (Appendix 1). Patients were undergoing different treatments, including INFα, Hydroxyurea, autologous or allogeneic stem cell transplantation, and/or Imatinib mesylate therapy. HLA typing for HLA-A, HLA-B, HLA-C and in some cases HLA-DR, HLA-DQ and HLA-DP alleles was performed at the allelic level by molecular techniques at the histocompatibility laboratories of the ANBMT. The screening for CML patients' bcr/abl transcript type is not a routine test in all the hospital centres and if not available, it was performed as described in section 2.1.5.

2.3.2 Blood collections:

Blood samples were taken at the same time as routine hospital checks, and did not require additional vene-punctures. Approximately 20ml of blood was obtained in tubes provided containing 20μl of heparin (monoparin 1000U/ml, CP pharmaceuticals, UK) to prevent blood clotting. Samples received from London hospital centres were processed on the same day. With regards to the samples received from Liverpool Royal Infirmary, 50ml falcon tubes containing 20ml of sterile transport medium (RPMI 1640, 3.3% w/v trisodium citrate, 5μM mercaptoethanol, filtered) were sent to our collaborator, in which blood samples were diluted 1:1 to preserve cell viability. These samples were then sent to us by mail and processed as soon as they were received.

2.3.3 Healthy volunteer recruitment:

Volunteers from the Anthony Nolan Research Institute laboratory and Royal Free Hospital personnel were also recruited. Participants were informed of the study and consented to participate. Donors were HLA typed at the Anthony Nolan Trust and selected for the presence or absence of HLA-A*0201, -A*0301, and/or –B*0801.

2.3.4 Peripheral blood mononuclear cell separation:

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation. Whole blood was layered over an equal volume of lympholyte®-H reagent (Cedarlane® laboratories Ltd, Canada) in a sterile 20ml universal tube (Bibby sterilin, UK) with as little disturbance of the interface as possible. The tubes were centrifuged at 2400rpm for 20 minutes, without braking to prevent disturbance of the lymphocyte layer at the interface. Lymphocytes were collected with a Pasteur pipette, transferred into a 50ml Falcon tube and washed with RPMI 1640 medium (with L-Glutamine, BioWhittaker, Wokingham, UK). The cells were centrifuged at 1700rpm for 15 minutes. This washing step was repeated to remove any contaminants from the lympholyte reagent, and the cell pellet was resuspended in 10ml RPMI 1640 medium. Lymphocytes were enumerated and used immediately or cryopreserved for future experiments.

2.3.5 Cell counting and viability:

Cells were counted using the Trypan Blue exclusion method. A 10µl aliquot of cell suspension was mixed with 10µl of 0.4% Trypan Blue solution (Sigma) and transferred into a cell counting chamber (0.1mm depth, Weber Scientific International, West Sussex, UK). Cells were visualised under a phase contrast microscope (DMLB Leica, Germany, ×200-×400) and enumerated, excluding dead cells (blue) from viable cells (translucent).

2.3.6 Cell cryopreservation and thawing:

Cells were cryopreserved in a freezing solution containing 90% Foetal Calf Serum (FCS, BioWhittaker) and 10% Dimethylsulphoxide (DMSO, BDH). FCS was heated inactivated at 55°C for 20 minutes and filtered through a 0.2µm filter (Sartorius, Surrey, UK) prior to use. Cells were washed in RPMI 1640 medium and centrifuged at 1700rpm for 10 minutes. The pellet was resuspended in freezing solution at a concentration of 10×10^6 cells/ml and transferred into 1.5ml cryovials (Cryotube Vials, Nunc®, Denmark). Aliquots were placed in a sealed polystyrene box at -80°C for 24 hours and then placed into liquid nitrogen for long-term storage. When cells were required, one aliquot was thawed at 37°C and rapidly diluted into 20ml of RPMI 1640 medium. Cells were centrifuged at 1700rpm for 10 minutes and washed with 50ml of medium to remove any DMSO contaminants. Prior to setting-up any assay, cells were allowed to recover at 37°C for 24 hours.

2.3.7 Cell line culture: Medium and conditions:

Cell lines were cultured in RMPI 1640 medium (with L-Glutamine, BioWhittaker) supplemented with 1U/ml penicillin and 1μg/ml streptomycin (BioWhittaker) and 10% heat-inactivated FCS (BioWhittaker). This will be referred to as complete medium. Sterile conditions were maintained by working in a class II microflow cabinet. Cells were grown in sterile 25, 75 or 175 cm^2 culture Flasks (Falcon™, BD) depending on cell density, at 37°C in a humidified 5% CO_2 incubator (IG 150, Jouan, France). Cells were grown at a density of $0.5\text{-}0.9\times10^6$ cells/ml and medium was changed every three days, or when the pH indicator colour changed to yellow (acidic). Cells were transferred to a 50ml Falcon tube, centrifuged at 1600rpm for 10 minutes and the cell pellet was resuspended in two volumes of fresh medium. The remaining cells were frozen to replace the liquid nitrogen stock, or discarded.
In some experiments, G418 sulphate antibiotic was required for the growth selection of neomycin resistant transfected cells (section 2.3.10). The G418 sulphate antibiotic (GibcoBRL) was dissolved in PBS, filtered through 0.45μm filter (Sartorius) and used at a final concentration of 1mg of active antibiotic per ml of medium.

2.3.8 Cell lines:

K562:

K562 is an immortal human erythroleukemic cell line derived from a CML patient in terminal blast crisis (Lozzio and Lozzio 1975). This cell line contains the Philadelphia chromosome and expresses the b3a2 type of the bcr/abl fusion protein. This cell line also lacks HLA molecule expression on the cell surface (erythropoietic cell lineage).

T2:

The T2 cell line is a hybrid between the mutant EBV transformed 721.174 cell line and a T lymphoblastoid cell line (Zweerink, Gammon et al. 1993; Young, Mulder et al. 1998). This immortal cell line are HLA-A*0201 positive and TAP deficient, resulting in a low level of HLA expression on the cell surface unless an exogenous source of peptide antigen is provided. T2 is a standard cell line for HLA-A*0201 peptide binding assays and also a target for cytotoxic killing assays.

EM-3:

The EM-3 cell line was established from an HLA-A*0301 CML patient in blast crisis after a second relapse (Keating 1987), with Philadelphia chromosome positive, b3a2 transcript

type. This cell line was used as a positive control for testing the HLA-A*0301 specificity of GAP-A3 antibody.

KCL-22:

The KCL-22 cell line was established from a CML patient in blast crisis expressing the translocation t(9;22) b2a2 fusion gene (Kubonishi and Miyoshi 1983). This cell line served as a control for the b2a2 transcript type.

2.3.9 Hybridoma cell lines:

W6/32, BB7.2, GAP-A3 and L243 hybridoma clones were grown for the production of the respective monoclonal antibodies. Hybridoma cell cultures were grown up to 2L in volume and antibodies were harvested from the culture supernatant by centrifugation at 1700rpm for 10 minutes. The purification of these antibodies is described in section 2.2.12.

2.3.10 Transfection of cell lines:

The HLA negative K562 cell line was transfected with a DNA plasmid encoding for either the HLA-A*0201, -A*0301 or HLA-B*0801 molecule by electroporation. Briefly, 10×10^6 cells with a minimum of 80% viability were resuspended in 800µl of RPMI 1640 medium and transferred into a sterile 0.4cm width electroporation cuvette (Biorad). Cells were transfected with 10-40µg of plasmid DNA (filtered through a 0.2µm filter) using a gene pulser® (Biorad) set at 250 volts, 960µF capacity for 12msec. Cells were immediately resuspended in 5ml of RPMI 1640 medium supplemented with 20% of FCS and transferred into a $25cm^2$ tissue culture flask. After 48 to 72hours, cells were resuspended at 1×10^6 cells/ml and cultured in selective medium (complete RPMI supplemented with 1mg/ml G418 antibiotic [GibcoBRL]).

2.3.11 Transfected cells cloning:

Transfected K562 cells were cloned following a standard dilution protocol. G418 Neomycin antibiotic resistant cells were harvested and plated in 96-U bottom tissue culture plates (BD) at a density of 0.3, 3 and 30 cells per 100µl complete RPMI medium +1mg/ml G418. Growing clones were tested for the level and specificity of HLA-class I molecule expression on their cell surface by flow cytometry (section 2.4.5). Highly expressing clones were

selected and transferred first into 25cm^2, then into 75cm^2 flasks for cell expansion. HLA expressing clones were used immediately or cryopreserved in liquid nitrogen for future use.

2.3.12 Peptide elution from transfectants and Mass spectrometry analysis:

HLA-A*0201 and HLA-B*0801 transfected K562 clones were cultured in 125cm^2 Flasks (see medium and conditions). Half medium exchange was performed every three days and cells were expanded to a confluence of 0.5-1×10^9 viable cells. Transfected cells were harvested in 50mls Falcon tubes and washed twice in serum-free RPMI medium.

All the plastic and glass bottles were first treated with Sigmacote (Sigma) to prevent the absorption of eluted peptides, washed with distilled water and air-dried. Cells were centrifuged at 1500rpm at 4°C for 5 minutes and cells pellet were resuspended in 5ml of citrate phosphate buffer (66mM sodium phosphate Na_2HPO_4, 130mM citric acid, pH 3.3 adjusted with 1M NaOH, prepared and filtered immediately before use). The buffer was rolled over the cells for 5 minutes and recovered by centrifugation (1500rpm, 4°C, 5 minutes). The supernatant, containing peptide eluates, was filtered through a 0.2μm filter and transferred into a silanised glass bottle. The eluted peptides were stored at -80°C and sent to Nottingham University for Mass spectrometry analysis. The Mass spectrometry analysis was performed following the protocol outlined in (Clark, Dodi et al. 2001).

2.3.13 Human T cell culture: Medium and conditions:

PBMCs were cultured in RPMI 1640 supplemented with 1U/ml penicillin, 1μg/ml streptomycin and 10% AB serum (BioWhittaker). X-Vivo 10 medium (BioWhittaker) was used for the generation of dendritic cells and the subsequent culture of responding lymphocytes. Medium was filtered through a 0.45μm filter (sartorius) and sterile conditions were maintained by working in a class II microflow cabinet. Lymphocytes were cultured at 37°C in a humidified 5% CO_2 incubator (IG 150, Jouan). T cell priming cultures were carried out in sterile 6, 12, 24-wells or 96U-bottom tissue culture plates (Falcon™, BD).

2.3.14 Cell selection:

The enrichment of a particular cell population was performed by magnetic sorting using antibody-conjugated microbeads (Miltenyi Biotec, Auburn CA, USA). Cells were magnetically labelled with the relevant antibody-conjugated microbeads and were separated from unlabelled cells in a MS^+ column placed in a magnetic field (MACS separator). Labelled cells are retained in the column and the unlabelled cells (negative fraction) are washed through. The labelled cells (positive fraction) are then eluted by removing the column from the magnetic field.

In this study, the positive selection of monocytes ($CD14^+$) and plasmocytoid dendritic cells ($BDCA\text{-}4^+$) was carried out according to the manufacturer's protocols.
A minimum of 10×10^6 cells were washed and resuspended in 80μl of MACS buffer (PBS, 0.5% w/v BSA, 2mM EDTA, sterile filtered). For the selection of very rare $BDCA\text{-}4^+$ plasmocytoid DCs, 50×10^6 PBMCs were washed and resuspended in 300μl of MACS buffer. Then 100μl of Fc receptor blocking reagent (Miltenyi Biotec) was added to avoid the non-specific binding of the BDCA-4 Microbeads to cells expressing Fc receptor (such as monocytes or dendritic cells).

Cells were incubated with 20μl (or 100μl) of magnetically labelled anti-CD14 MicroBeads, or anti-BDCA-4 Microbeads respectively (Miltenyi Biotec) for 15 minutes at 6°-12°C (fridge). Cells were washed with 10-20 volumes of MACS buffer and centrifuged at 1600rpm for 10 minutes. The positive MS^+ selection column was placed in the MACS magnetic field and equilibrated with 500μl of MACS buffer. Cell pellets were resuspended in 500μl of MACS buffer and applied to the column. The column was washed with 3x500μl of MACS buffer and the unlabelled cells were collected in sterile 15ml Falcon tubes. The

column was then removed from the magnetic field and placed in a sterile 15ml Falcon tube. The positive fraction was recovered with 1ml of MACS buffer that was flushed out using a provided plunger.

Positive cell fractions were stained with the relevant fluorescently labelled antibody and analysed by flow cytometry to assess the magnetic purification efficiency. Cells were then enumerated and used immediately or cryopreserved.

2.3.15 Generation of T-cell blasts:

T cell blasts were generated by incubating lymphocytes with the polyclonal mitogen Phytohemagglutinin (PHA, Abbot Murex, Berkshire, UK). 2×10^6 PBMCs were plated in a 24-well plate at a density of 2×10^6/ml of complete RPMI medium containing 2 μg/ml of PHA. Cells were grown for three days at 37°C, and half the medium was exchanged thereafter every 3-4 days with complete RPMI medium plus 10UI/ml of recombinant IL-2 cytokine (R&D, Minneapolis, USA). T cell blasts were used as a source of antigen presenting cells (APC), or as a positive control in proliferation assays. Cells were used immediately or cryopreserved.

2.3.16 Generation of monocyte-derived Dendritic cells (mDCs):

Monocytes were purified from PBMCs by the adherence method or positive selection of $CD14^+$ cells using MACs microbeads (section 2.3.14). Monocytes have the property of adhering to plastic support and can therefore be enriched from non-adherent lymphocytes. This method was used for the generation of mDCs from healthy donors and CML patients in remission. It is believed that CML cells have a defect in their adhesion property to marrow stroma, thus it was difficult to obtain monocytes by the adherence method from CML patients with active disease. Therefore the generation of mDCs from these patient samples was performed by positive selection of $CD14^+$ cells (section 2.3.14).

For the adherence method, a minimum of 10×10^6 cells per well (in 12-well plate) to up to 30×10^6 cells per well (in 6-well plate) were incubated in complete X-VIVO 10 medium and left for 4 to 12 hours at 37°C. The non-adherent cells were removed by gently resuspending and washing the wells with medium and cryopreserved until required.

Functional mDCs were produced in two steps as illustrated in **Figure 2.15**. First, immature mDCs were generated from monocytes with Granulocyte Macrophage Colony Stimulator

Factor (GMCSF, R&D) and IL-4 (R&D). Then mDCs were matured with Tumour Necrosis Factor α (TNFα, R&D), polyinosinic-polycytidylic acid (poly I:C Sigma), soluble recombinant CD40L/TRAP (PeproTech Inc, Peterborough, UK), and the antigenic protein or peptide.

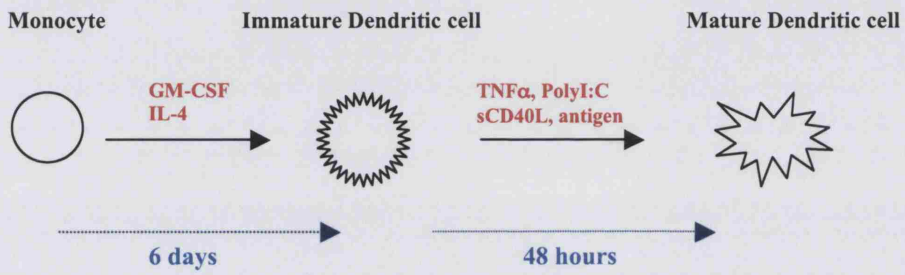


Figure 2.15: Protocol for the generation of monocyte-derived dendritic cells.

The adherent cells, or monocytes (in 6-well plate) were incubated in 2ml of complete X-Vivo 10 medium supplemented with 200ng/ml GMCSF (stimulates growth and differentiation of monocytes) and 100ng/ml IL-4 (stops macrophage growth) at 37°C. On day 2, 2ml of medium supplemented with 400ng/ml GMCSF and 200ng/ml IL-4 was added in each well. On day 6, half of the medium was exchanged with fresh medium containing 400ng/ml GMCSF and 200ng/ml IL-4. Monocytes will have differentiated into immature mDCs, with characteristic extensive dendrides visible under a phase contrast microscope as shown in **Figure 2.16**, panel **A** and **B**. These were matured or cryopreserved for use in future experiments. Frozen immature mDCs were thawed and incubated for at least 48 hours with 2ml complete medium plus 200ng/ml GMCSF and 100ng/ml IL-4 prior to maturation.

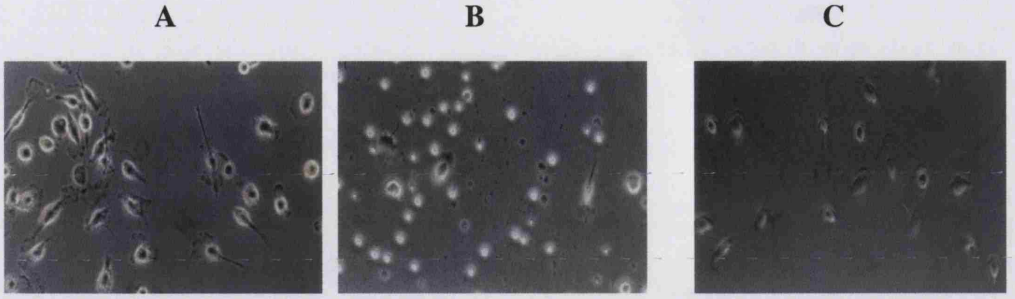


Figure 2.16: Generation of monocytes-derived and plasmacytoid derived dendritic cells:

Photographs of monocytes-derived DCs generated from a healthy donor (panel **A**) and CML patient (panel **B**) and plasmacytoid DCs generated from a healthy donor (panel **C**) are shown. Monocytes derived DCs were cultured with GM-CSF and IL-4 for 6 days (as described in section 2.3.16) and pictured on photographic microscope. Fewer visible dendrites are observed at this stage from the CML patient than from the healthy donor (panel **B** and **A** respectively). Thus prior maturation, patients' derived dendritic cells were cultured longer with these cytokines to allow the development of visible dendrites. Plasmacytoid DCs were cultured in IL-3 for 48 hours and photographed (panel **C**). These cells demonstrate a typical lymphocyte-like morphology with some dendrites. Photographs are shown for a magnification of ×20.

mDCs maturation was carried out on day 6, by exchanging 1 ml of medium with fresh medium containing 10ng/ml TNFα, 12.5μg/ml poly I:C and 1μg/ml sCD40L. Antigenic peptides that were required to be up-taken and processed by mDCs (ie 17-mers antigenic peptides) were added at 20μg/ml on the same day of maturation. However short 9-mers peptides were pulsed, also at 20μg/ml on day 8, prior to their use as APC. Cell surface expression of costimulatory molecules (CD80, CD83, CD86, HLA-DR) was analysed by flow cytometry to ensure that the mDCs acquired a mature phenotype prior to use as APCs in different assays.

2.3.17 Generation of plasmacytoid Dendritic cells (pDC):

Plasmacytoid DCs were purified from peripheral blood samples by positive selection of BDCA-4$^+$ cells using a MACS kit (Miltenyi Biotec, described in section 2.3.14). Purified pDCs were resuspended in 1ml of X-VIVO 10 medium supplemented with 10ng/ml of IL-3 (required for pDCs survival, R&D), plated in a 48-well plate and incubated at 37°C. On day 2, half the medium was exchanged with fresh medium containing 20ng/ml of IL-3. Plasmocytoid DCs were matured by exchanging 0.5ml of medium with fresh medium containing 20ng/ml of IL-3 (R&D), 2μg/ml of sCD40L (PeproTech Inc) and 20μg/ml of antigenic peptide, and incubated at 37°C for 24-48 hours. Cells were immediately used as APCs. As shown in **Figure 2.16**, panel **C**, pDCs have a lymphoid cell-like morphology.

2.3.18 Stimulation of antigen specific T cells with conventional methods:

Antigen specific T cells were generated from both CML patients and healthy volunteers using various stimulation protocols. The non-adherent cell fraction from DC separation or PBMCs were thawed, washed in RPMI medium and cultured overnight at 37°C in complete X-Vivo medium supplemented with 10ng/ml of IL-7 (T cell survival cytokine, R&D). Lymphocytes were plated at a ratio of 1×10^5/100μl in 96-well plate, or $1\text{-}3\times10^6$/ml in 24-well plate. These cells were primed with autologous monocyte-derived DCs or pDC (ratio of T cells:DC of 10:1), with autologous irradiated T cell blasts or PBMCs (ratio of T cells:APC of 5:1) or with modified HLA/tetramer (described in the following section). The APCs were pulsed with 20μg/ml antigenic peptide for at least 3 hours at 37°C. APCs were then washed in X-Vivo medium and resuspended in 100μl (for experiments carried out in 96-well plate) or 0.5 ml (24-well plate) complete medium plus 10ng/ml IL-7. APCs were irradiated at 30Gy (DCs and PBMCs) or at 96Gy (T cell blasts). It was not necessary to irradiate pDCs because of their short survival in culture once matured.

T cell cultures were restimulated every 7 to 10 days with autologous irradiated (or not) peptide-pulsed APCs aliquoted in complete medium plus IL-7 (10ng/ml), for up to 4 weeks. Three days after each stimulation, half of the medium was exchanged with fresh medium supplemented with IL-2 (T cell proliferation, 10-50 UI/ml final concentration, R&D) and IL-15 (also a T cell growth factor, 10ng/ml final concentration, R&D).

2.3.19 Stimulation of antigen specific T cells with sAPCs:

For the stimulation with the sAPCs, PBMCs were resuspended in complete X-Vivo medium supplemented with 10ng/ml of IL-7 and plated at a density of 3×10^6 cells in 24-well plate. Cells were stimulated with 1.5µl of sAPC complexes per ml of culture medium (0.8µg HLA/peptide-0.64µg costimulatory antibody) every 5 or 6 days, for up to 3 weeks. For the control stimulation conditions, 0.8µg of HLA/peptide tetramers and 0.64µg of soluble costimulatory antibodies (R&D) were aliquoted per ml of culture medium. Initially, IL-7 (10ng/ml), IL-15 (10ng/ml) and IL-2 (10UI/ml) were added to the cultures three days following each stimulation. After optimisation of this protocol (shown in Chapter 6), cells were washed from the remaining sAPCs three days after each stimulation and resuspended in complete X-Vivo medium supplemented with IL-7, IL-15 and IL-2 (aliquoted at the same concentration).

T cell expansion was observed under a light microscope and confluent cells were split into two. T cells were assessed for antigen specificity and functionality as described in the following section.

2.4 IMMUNOASSAYS:

2.4.1 Flow Cytometry:

Flow cytometric analysis of cell surface markers was performed using a Fluorescent Activated Cells sorter (FACs) Calibur system (BD). FACs is used to measure several parameters, including size and granularity, as well as up to four different fluorescent labelled specific markers (antibodies) on a single cell subset. The instrument contains an argon-ion laser (blue laser with emission peak of 488nm, collecting FSC, SSC, FITC, PE, PerCP signals) and a second red-diode laser (red laser with emission peak of 635nm, collecting APC signal). Cells pass through the red laser and microseconds later through the blue laser. To ensure that all the parameters for a single cell are collected at the same time, FL4 laser is electronically delayed. The range of wavelengths at which the different fluorescent molecules conjugated to the antibodies are collected is shown in **Table 2.14**. The acquisition of stained cell populations was performed using the CellQuest™ software version 3.3 (BD) and their subsequent analysis was performed using the FlowJo software (Tree Star).

Name	*Complete name*	*Emission peaks (in nm)*
FSC	Forward Scatter	488
SSC	Side Scatter	488
FITC	Fluorescin isothiocyanate	530
PE	Phycoerythrin	585
PerCP	Peridinin chlorophyll protein	670+
APC	Allophycocyanin	661

Table 2.14: FACs calibur optical components:

This table summarises the emission peaks (in nm) of the FSC, SSC signals and the fluorescent components conjugated to monoclonal antibodies. The emission peaks are obtained after excitation of the argon-ion laser or the red-ion laser (for APC).

2.4.2 Cell staining and cell fixation:

A minimum of 1×10^5 cells were transferred into a 96-V bottom plate (Nunc). Cells were stained and washed with staining medium (RPMI 1640 supplemented with 10% heated inactivated FCS and 0.1% sodium azide which prevents antibody/receptor internalisation). After staining with the different antibodies (see following sections), cells were washed with 200µl of staining medium and centrifuged for 3 minutes at 1600rpm/4°C. Cells were resuspended in 200µl of fixing solution, to prevent the dissociation of the antibodies from their ligands. Fixing solution was composed of 1% w/v of paraformaldehyde dissolved in PBS or RPMI 1640 medium. Fixed cells were kept at 4°C in the dark. Samples were analysed by flow cytometry within 48 hours when stained with HLA tetramers, or otherwise within 7 days.

2.4.3 HLA/Tetramer staining:

For HLA class I tetramer staining, 1×10^6 cells were resuspended in 50µl of staining medium containing 1µg of the relevant HLA tetramer and incubated for 30 minutes at 37°C. Plates were centrifuged at 1600rpm for 3 minutes at 4°C. Supernatant was removed and cells were washed with 200µl of staining medium. After centrifugation, the cell pellet was resuspended in 50µl of staining medium containing fluorescently conjugated antibodies specific for the relevant cell surface markers.

2.4.4 Characterisation of cell surface markers with fluorescently conjugated antibodies:

All the fluorescent conjugated antibodies were purchased from BD Pharmingen, San Diego, USA. They were used at a concentration of 2.5-5µl per 50µl volume up to a maximum cell number of 1×10^6 according to the manufacturer's recommendations. Cells were stained for 15-30 minutes on ice and in the dark. The cell surface antibodies used in this study are summarised in **Table 2.15**.

Name	*Description*
T cell / NK cell markers	
CD3	T cell receptor
CD8	T cell co-receptor
CD25	IL-2 receptor α chain
CD28	Positive receptor of T cell activation for CD80 (B7.1) and CD86 (B7.2)
CD69	Early activation marker for T, B and NK cells
Dendritic cell markers	
CD11c	Myeloid cell marker
BDCA-2	Plasmacytoid DCs marker
BDCA-3	Blood DCs subset marker
BDCA-4	Plasmacytoid DCs marker
CD123	IL-3 receptor
Costimulatory molecules markers	
W6/32	Pan-HLA class I cell surface molecule
HLA-DR	HLA class II cell surface molecule
CD80	B7.1 ligand, binds to CD28 (activation) or CD152 (anergy) receptors
CD83	Costimulatory ligand
CD86	B7.2 ligand, binds to CD28 (activation) or CD152 (anergy) receptors
T cell receptors, phenotype markers	
CD27	Thymocyte receptor
CD45RO	Leukocyte common antigen, RO isoform

Table 2.15: Antibodies to Immune cell surface molecules:

This table summarises the different mouse anti-human cell surface antibodies used to characterise immune cell subsets by flow cytometry. The different markers are regrouped for T cell/NK cell markers, B cell/DC markers, APC Costimulatory markers and T cell phenotypic markers. These markers are fluorescently conjugated (except for W6/32 antibody), which allow visualising the specific cellular subset by FACs analysis. All antibodies were purchased from Becton Dickinson Pharmingen, San Diego, USA.

2.4.5 Indirect staining:

HLA class I and class II specific antibodies were produced in-house from hybridomas and were not labelled with fluorescent molecules. Therefore, the staining for HLA cell surface expression was performed in a two-step procedure. Cells were stained with a primary antibody (W6/32, BB7.2, GAP-A3, or L243, **Table 2.16**) for 30 minutes on ice.

Name	*Specificity*
W6/32	HLA class I
BB7.2	HLA-A*0201
Gap-A3	HLA-A*0301
L243	HLA class II

Table 2.16: HLA specificity of generated antibodies:

This table summarises the HLA specificity of the monoclonal antibodies generated and purified from hybridoma cell cultures. These antibodies were used to characterise the HLA specificity of transfected cell lines and to evaluate the level of cell surface HLA expression on APCs or in peptide binding assays.

The antibodies were produced in the culture supernatant (detailed in section 2.3.9) and a titration of the amount necessary for the detection of these HLA surface molecules was performed. A positive HLA expressing cell line (for example HLA-A*0201 K562 for BB7.2 antibody) and a negative HLA expressing cell line (K562) were stained with a series of antibody concentrations (supernatant volumes) to determine the optimum volume to use. The excess of primary antibody was removed by washing the plates with 200µl of staining medium. Plates were centrifuged for 3 minutes at 1600rpm/4°C and resuspended in 50µl of staining medium. Cells were then stained with the secondary FITC conjugated anti-mouse IgG antibody (used at a dilution 1:20, Sigma) for 30 minutes on ice, in the dark. A control was performed to ensure antibody specificity by staining an aliquot of cells with the secondary antibody alone. The titration of BB7.2 antibody is described in **Figure 2.17**.

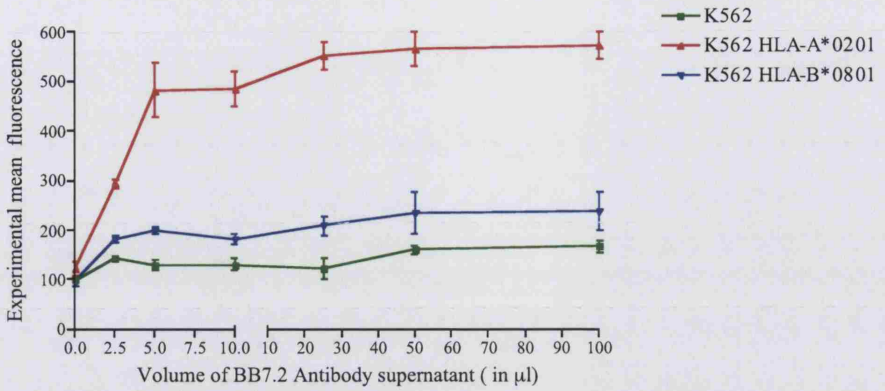


Figure 2.17:HLA class I antibody titration:

The titration of culture BB7.2 antibody volume required for the specific detection of HLA-A*0201 molecules is shown. K562 (Green line), K562 HLA-B*0801 (Blue line) and K562 HLA-A*0201 (Red line) were stained with different amount of primary BB7.2 antibody (from 2.5µl to 100 µl). HLA-A*0201 molecules were detected with the anti-mouse IgG FITC conjugated secondary antibody and analysed by flow cytometry. Cells were also stained with secondary antibody alone (0µl of BB7.2 antibody). The expression of HLA-A*0201 molecules is represented as experimental mean fluorescence. The error bars represent the standard deviation between duplicate samples. The optimal amount of BB7.2 antibody required for the specific detection of HLA-A*0201 molecules was 5µl.

2.4.6 Peptide binding Assay:

HLA-A*0201 positive T2 cells are deficient in transporter associated with antigen processing (TAP) protein. HLA class I molecules are therefore retained in the endoplasmic reticulum unless an exogenous source of antigenic peptides is provided. These cells make an ideal tool for studying the affinity of antigenic peptides for binding to HLA-class I molecules, particularly HLA-A*0201, by measuring the level of HLA class I expression on the cell surface after peptide pulsing. Cell surface up-regulation of HLA class I expression was measured by flow cytometry.

1×10^6 T2 cells resuspended in complete RPMI medium were incubated in a 24-well plate at 37°C. The various antigenic peptides to be tested were aliquoted in duplicate at 10µg/ml. A negative control (no peptide) and a positive control (high affinity peptide binder) were tested in parallel. The relative affinity of 9-mer peptides for the HLA-A*0201 molecule were defined through a time course experiment. For this, replicates were performed and harvested after 1, 2, 8 and 24 hours of incubation. Cells were washed in RPMI 1640 medium and stained for HLA-A*0201 cell surface expression with W6/32 and/or BB7.2 antibodies (Section 2.4.5). Samples were analysed by flow cytometry.

2.4.7 Proliferation assay:

CFSE labelling:

5-(and-6)- carboxyfluorescein diacetate succinyl ester (CFSE, Molecular Probes, Eugene, Oregon, USA) is an amine reactive fluorescein probe that diffuses passively into cells and couples with the amine group chains of intracellular and cell surface proteins. CFSE is equally distributed between two dividing cells, resulting in a halving of cellular fluorescence as cells proliferate. Cell proliferation was followed by flow cytometry as CFSE emits at 525nm.

CFSE was prepared in PBS as a 10mM stock, filtered and kept at -20°C. PBMCs were washed with RPMI 1640 and centrifuged at 1600rpm for 3 minutes. Cells were resuspended at a density of 10×10^6cells/ml and stained with 1μM CFSE for 10 minutes at 37°C. Cells were recovered by two washes with pre-warmed RPMI 1640 supplemented with 1% FCS (favouring cell recovery). CFSE labelled lymphocytes were incubated with antigenic stimuli in accordance with the priming protocol. In parallel, an aliquot of cells was incubated with medium only (negative control) and PHA polyclonal mitogen (positive control). Cell proliferation was assessed at 24 hour, 3 days and 7 days post stimulation. Lymphocytes were stained with the specific HLA tetramer and monoclonal antibody for CD3 and CD8 and analysed by flow cytometry. Thus this method allowed assessing the proliferation of the different cell subsets.

Thymidine incorporation assay:

Thymidine is a nucleic acid base component of DNA. As cells divide, DNA replicates and is equally distributed between daughter cells. Radioactive labelled thymidine (^{3}H-Thymidine, Amersham, Buckinghamshire, UK) was exogenously added to antigen-stimulated lymphocytes and the incorporation of ^{3}H-Thymidine into harvested cell DNA was measured.

Lymphocytes were plated in a 96-U bottom plate at 1×10^5 cells/well in complete RPMI supplemented with 10% AB serum. Antigenic peptides were added in triplicate at a final concentration of 10μg/ml and the cells were incubated at 37°C for 24 hour, 3 days and 5 days. ^{3}H-Thymidine was aliquoted at 1μCi/well and plates were left at 37°C for an additional 16 hours. A negative control (cells with medium only) and a positive control (cells with PHA) were set-up in parallel. Cells were harvested with an automatic cell

harvester (Harvester 96®, Tomtec, Orange, USA). The cellular debris and DNA were collected onto a glass fibre filter (Printed Filtermat A, Wallac, Turku, Finland). After three washes with distilled water, membranes were dried in a microwave and inserted into a sample bag (Sample Bag, Perkin Elmer life Science, Wallac). The edges of the bag were heat-sealed (Heat Sealer, Wallac), leaving a hole for the addition of approximately 5ml of scintillant (Optiphase "Supermix", Wallac, Turku, Finland), which solubilises ^{3}H-Thymidine. The bag was heat-sealed and placed into a cassette holder. ^{3}H-Thymidine incorporation was counted by a 1450 Microbeta Plus liquid scintillation counter (Wallac).

2.4.8 Cytotoxic killing assay: Chromium release assay:

The functionality of antigen primed T cells was verified in cytotoxic assays. Activated T cells recognize and kill cells that present their cognate specific antigenic peptide/HLA complex on the cell surface. CTL activity was tested in a chromium release assay. Target cells were labelled with radioactive sodium chromate ($Na_2{}^{51}CrO_4$, Amersham, Buckinghamshire, UK). Upon specific killing, radioactive chromium is released into the culture supernatant, which is harvested and counted in a 1450 Microbeta Plus liquid scintillation counter (Wallac).

In this study, peptide-pulsed T2 cells and allogeneic CML leukapheuresis samples were used as targets. Target cells required at least 75% viability to decrease the spontaneous chromium release background. If the viability was lower, which was often the case for leukapheuresis samples, a small lymphoprep was performed to remove the dead cells (see section 2.3.4). A minimum of 5×10^3 target cells per assay was required. The total number of target cells was defined by the number of CTLs to be tested and the ratio of targets to CTLs used. Approximately $1\text{-}2\times10^6$ target cells were resuspended in complete RPMI supplemented with 10%FCS and plated in a 24-well plate. Cells were labelled with 150μCi of $Na_2{}^{51}CrO_4$ and incubated for 2-3 hours (for peptide-pulsed PBMCs and leukapheuresis samples) or 24 hours (for peptide-pulsed T2 cells) at 37°C in a designated incubator. Antigen primed T cells were enumerated and diluted in the priming medium at a concentration defined by the ratio of CTLs to target cells used. For example, if using a 1:10 ratio, 5×10^4 CTLs/well was required. CTLs were prepared at a final concentration of 5×10^5T cells/ml, and 100μl was aliquoted in duplicate into a 96-U bottom plate. Radioactively labelled target cells were resuspended, transferred to a 15ml Falcon tube and washed at least twice with complete RPMI medium plus 10% FCS and plated with the CTLs (5×10^3 targets/well/100μl). Each target was plated in triplicate with medium alone (spontaneous

chromium release) and with 5% triton X-100 (BDH) in PBS (total chromium release). CTLs and target cells were incubated at 37°C for 3 to 4 hours. Plates were centrifuged at 1600rpm for 4 minutes, and 25µl of supernatant was harvested and transfered to a 96-well sample plate (Wallac, Turku, Finland). The addition of 100µl of optiphase scintillation fluid (Optiphase "supermix", Wallac) to each well was required to solubilise the chromium. Plates were sealed (Plate sealer, Wallac) and homogenized on a shaking platform (Luckham) for 5 minutes. Chromium release was measured using a 1450 Microbeta Plus liquid scintillation counter (Wallac).

The percentage of specific lysis was calculated as follows:
= (experimental release –spontaneous release)/ (total release –spontaneous release)× 100

2.4.9 Statistical analysis:

The statistical analyses of the data presented in this study were performed using the Prism® 4 Software (GraphPad, San Diego, USA).

CHAPTER 3

Definition of BCR/ABL derived epitopes associated with HLA class I molecules

INTRODUCTION

The development of an epitope-based immunotherapeutic treatment for CML patients, targeted to the BCR/ABL tumour specific antigen requires 1) the definition and confirmation that BCR/ABL antigenic peptides are presented on HLA molecules, 2) the identification and confirmation of T cells specific for those HLA/peptide complexes circulating in the peripheral blood of patients and finally 3) the demonstration that functional BCR/ABL specific T cells can be primed and/or expanded *in-vitro*.

Bocchia *et al.* and Buzyn *et al.* have both screened the BCR/ABL protein for potential peptide epitopes associated with HLA class I molecules (Bocchia et al. 1995; Buzyn et al. 1997). They selected and analysed over 150 peptides spanning the BCR/ABL b2a2 and b3a2 regions for 8 to 11-mers peptides predicted to bind to HLA class I molecules following the Rammensee prediction algorithm (Syfpeithi database, described in chapter 1). In 2000, Berke *et al.* also extended the study to the ABL/BCR reciprocal translocation protein and selected peptides from both translocated proteins predicted to bind HLA class I molecules using the Bimas prediction database (Berke et al. 2000). In total, 21 peptides derived from the BCR/ABL protein (or 14 with an anchor motif for common HLA molecules) were predicted to bind HLA class I molecules by the Syfpeithi algorithm, compared with 9 BCR/ABL peptides predicted by the Bimas algorithm. A few b3a2 peptides were shown to bind with intermediate or high affinity to HLA-A*0301, -A*1101, -B*0701 or -B*0801 (Berke et al. 2000; Bocchia et al. 1995; Buzyn et al. 1997). All the groups consistently characterised four potential BCR/ABL b3a2 derived epitopes associated with HLA-A*0301 (ATGFKQSSK and KQSSKALQR), HLA-A*1101 (ATGFKQSSK) and HLA-B*0801 (GFKQSSKAL) molecules.

The characterization of antigenic peptides associated with HLA class I molecules using the described prediction algorithms however has the limitation of being theoretical. Another method to characterize and more essentially, confirm the processing and presentation of antigenic proteins consists of eluting and purifying HLA-associated antigenic peptides from the cells surface. These antigenic peptides are then sequenced by mass spectrometry (MS) and compared with the target protein sequence of interest. The sensitivity of MS renders the technique difficult to apply for blind screening of HLA-associated antigenic peptides. One major obstacle of this method is that the low frequency of tumour specific peptides may be masked by the greater frequency of other antigenic peptides, including house-keeping peptides, presented on other HLA molecules. In the context of CML, the use of the K562 cell line has a number of advantages. This erythroleukemic cell line is HLA negative and expresses the b3a2 bcr/abl mRNA (Lozzio and Lozzio 1975). Thus, the transfection of K562 cells with the HLA class I molecule of interest allows the characterisation of b3a2 derived epitopes presented in the context of a single HLA allele and helps to reduce the peptide background obtained with heterozygous cells, expressing up to six different HLA class I molecules. Prior to the work described in this thesis the ANRI group, in collaboration with Prof R. Clark from the University of Liverpool and Prof R. Rees from the University of Nottingham, have demonstrated that the KQSSKALQR BCR/ABL derived peptide is presented in the context of HLA-A*0301 on transfected K562 cells and on primary CML cells (Clark et al. 2001).
In this study, the analysis was extended to other HLA class I molecules, including the HLA-A*0201 and HLA-B*0801 molecules.

Once identified, the antigenic peptides are assessed for their capacity to be recognized by T cells and to generate functional antigen specific T cell responses. *In-vitro* assays were established to characterize and in some cases quantify, antigen recognition by lymphocytes. The capacity of antigenic peptides to induce T cell proliferation can be assessed by measuring ^{3}H-thymidine incorporation into DNA. The successful refolding of HLA class I/peptide complexes has made the direct visualisation of antigen specific T cells possible (Garboczi et al. 1992). TCRs were however demonstrated to bind MHC/peptide monomers with low affinity and to have fast dissociation off-rates (Matsui et al. 1994). The cross-linking of four biotinylated MHC/peptide monomers on a streptavidin core, to form a so-called HLA tetramer, allowed a sufficient increase in the T cell avidity for a stable and relatively long lasting interaction (Altman et al. 1996). HLA/peptide tetramers have since become an invaluable tool for the detection, quantification and purification of antigen-specific T cells *ex-vivo*.

The aims of this chapter were to screen the BCR/ABL (b3a2) tumour specific protein for potential HLA class I binding peptides. A number of HLA-A*0201, -A*0301 and –B*0801 associated BCR/ABL derived peptides were selected using epitope and proteosomal cleavage prediction algorithms. In the context of the HLA-A*0201 molecule, the selected BCR/ABL peptides were then assessed experimentally for their HLA binding affinities. Additionally, the natural processing and presentation of b3a2 BCR/ABL derived peptides on the cell surface of CML cells was further evaluated in the context of HLA-A*0201 and HLA-B*0801 molecules. In order to achieve this, HLA-bound peptides were eluted from b3a2 HLA-A*0201 or HLA-B*0801 positive cells and analysed by tandem mass spectrometry. Subsequently the presence of BCR/ABL specific T cells circulating in the peripheral blood of CML patients and healthy individuals was characterised by measuring the *ex-vivo* lymphocyte proliferative responses upon BCR/ABL peptide stimulation and by measuring the frequency of BCR/ABL specific $CD8^+$ T cells using HLA/peptide tetramers.

RESULTS

3.1 Prediction of HLA class I associated BCR/ABL peptide epitopes:

3.1.1 BCR/ABL peptides binding prediction:

The BCR/ABL b3a2 fusion protein sequence was screened for potential epitopes binding to the HLA-A*0201, HLA-A*0301 and HLA-B*0801 molecules using the Syfpeithi and the Bimas epitope prediction algorithms. For comparative purposes, known HLA-associated peptide ligands were also included in this study. These were the described cytomegalovirus protein pp65 derived epitopes associated with HLA-A*0301 (IGDQYVKVY, selected from an in-house CMV epitope mapping study, unpublished data) and with HLA-B*0801 molecules (DANDIYRIF) (Wills et al. 1996). The high affinity HLA-A*0201 binding peptide from influenza virus A matrix protein (GILGFVFTL), referred to as "Flu antigen", as well as a set of described HLA-A*0201 tumour associated epitopes derived from Wilms' tumour antigen (WT1), Proteinase 3 (PR1) and telomerase catalytic subunit (hTERT) proteins were also included (Molldrem et al. 1996; Oka et al. 2000; Vonderheide et al. 1999). These three HLA-A*0201 tumour associated antigens (WT1, PR1 and hTERT) were chosen as control antigens and used throughout this study. The binding affinity of these peptides for HLA class I molecules predicted by Syfpeithi and Bimas are shown in **Table 3.1**. The score values obtained by the two algorithms cannot be directly compared since the epitope prediction for binding to HLA molecules are calculated using different formula (as described in chapter 1). However the score ratings of the different BCR/ABL epitopes were assessed and compared with the known immunogenic CMV, Flu or TAA peptides associated with the same HLA molecules (underlined peptides, **Table 3.1**).

In accordance with previous studies, two of the BCR/ABL derived peptides associated with the HLA-A*0301 molecules (ATGFKQSSK and KQSSKALQR) obtained a high or moderate scoring values by both prediction databases (**Table 3.1**, top panel) (Berke et al. 2000; Bocchia et al. 1995; Buzyn et al. 1997). Additionally, the GFKQSSKAL BCR/ABL derived peptide was predicted to bind the HLA-B*0801 molecule with high affinity, at least by the Syfpeithi database (**Table 3.1**, middle panel). These peptides obtained higher score values from both prediction databases compared to the described CMV pp65 epitope. The score ratings obtained from the two prediction databases were however quite different. For example, the top scoring BCR/ABL derived peptide predicted to bind to the HLA-B*0801 molecule by the Bimas database was the SSKALQRPV peptide (score of 0.6, **Table 3.1**), yet the Syfpeithi database predicted the GFKQSSKAL peptide as the highest binder (score

of 23). Likewise the KALQRPVAS BCR/ABL derived peptide was the third highest scoring epitope predicted by the Syfpeithi database to bind to the HLA-A*0301 molecule (score of 13), however this same peptide was given a very low score by the Bimas database (score of 0.001).

Peptides Sequences	Peptides Binding Values	
	Bimas[a]	*Syfpeithi*[b]
	HLA-A*0301	
IGDQYVKVY	0.018	12
ATGFKQSSK	1.000	19
KQSSKALQR	0.720	17
SATGFKQSS	0.002	5
KALQRPVAS	0.001	13
SKALQRPVA	0.000	8
	HLA-B*0801	
DANDIYRIF	0.060	10
SSKALQRPV	0.600	12
GFKQSSKAL	0.400	23
KALQRPVAS	0.200	14
SATGFKQSS	0.020	8
ATGFKQSSK	0.020	10
KQSSKALQR	0.003	10
	HLA-A*0201	
GILGFVFTL	550.9	30
ILAKFLHWL	1745.7	30
VLQELNVTV	484.78	28
RMFPNAPYL	313.97	22
SSKALQRPV	0.003	12
SLKALQRPV*	0.487	22
KALQRPVAS	0.013	10
GFKQSSKAL	0.001	10
ATGFKQSSK	0.000	8

Table 3.1: Predicted binding of BCR/ABL derived peptides to HLA class I molecules using two peptide-binding algorithms:

Peptides spanning the b3a2 BCR/ABL protein were screened for potential epitopes showing binding to HLA-A*0301, -B*0801 and –A*0201 molecules using two epitope algorithm databases: [a] Bimas: www.bimas.dcrt.nih.gov/molbio/hla_bind and [b] Syfpeithi: www.syfpeithi.de. The CMV pp65 derived epitopes associated with HLA-A*0301 and HLA-B*0801 molecules as well as the HLA-A*0201 Flu epitope were included as positive controls and are underlined. In addition the three described HLA-A*0201 Tumour associated antigens hTERT, PR1 and WT1 respectively were included and are shown in italic. * The high score BCR/ABL peptide predicted to bind HLA-A*0201 was modified to contain a leucine dominant anchor residue at position two and was included in the study (The Leucine insertion is in bold).

In the context of the HLA-A*0201 molecule, the scores obtained for the potential BCR/ABL epitopes were very low compared to the HLA-A*0201 high binding Flu derived peptide or the different tumour associated peptides, especially using the Bimas database (from 0 to 0.013, **Table 3.1**, lower panel). One of the BCR/ABL derived peptides, SSKALQRPV was predicted by the Syfpeithi algorithm to bind to the HLA-A*0201 molecule with moderate affinity and given a score value of 12. This peptide was modified to contain a HLA-A*0201 dominant anchor residue at position 2 (Leucine instead of Serine), which significantly increased the score values obtained from both the Bimas database (from 0.003 to 0.487 value) and the Syfpeithi database (from 12 to 22 value, SLKALQRPV peptide, **Table 3.1**). The Syfpeithi HLA binding value of this peptide is identical or similar to the values predicted for the known HLA-A*0201 tumour associated peptides WT1, PR1 and hTERT. The Bimas score value of this modified BCR/ABL peptide remained however low, especially compared to these control peptides.

Although a discrepancy between the epitope prediction scores was observed between the two different algorithms, some BCR/ABL derived peptides were predicted by the Syfpeithi database to bind to HLA-B*0801 (GFKQSSKAL), HLA-A*0301 (ATGFKQSSK, KQSSKALQR) and HLA-A*0201 molecules (SSKALQRPV) with high, intermediate and moderate affinity respectively. The Bimas epitope prediction algorithms predicted different BCR/ABL derived peptides to bind to HLA-B*0801 (SSKALQRPV and also GFKQSSKAL) and did not predict SSKALQRPV to bind to HLA-A*0201. Unlike Syfpeithi, the Bimas prediction database is based on experimental data (β-2 microglobulin dissociation), which may explain the differing scoring values observed between the two databases.

3.1.2 BCR/ABL proteosomal cleavages prediction:

BCR/ABL peptides were next assessed for proteosomal cleavage using Paproc, Netchop and Fragpredict prediction algorithms and are shown in **Table 3.2**. The values obtained for the control peptides both for Flu and for tumour associated antigens, are shown in the upper panel. The predicted proteosomal cleavage values of the potential BCR/ABL derived 9-mer peptides are shown in the lower panel.

Peptide	*Protein*	*Paproc*[a]	*Netchop*[b]	*Fragpredict*[c]
GILGFVFTL	Influenza virus	Wt (I) 390.41	0.999	0.500
ILAKFLHWL	Human telomerase	NP	0.035	<0.100
VLQELNVTV	Proteinase 3	NP	0.813	0.978
RMFPNAPYL	Wilms'tumour Ag	Wt (I) 182.42	0.995	0.622
SATGFKQSS		Wt (I) 106.38	0.356	0.499
ATGFKQSSK		NP	0.577	0.499
GFKQSSKAL	BCR/ABL	Wt (III) 151.17	0.746	<0.100
KQSSKALQR		Wt (I) 117.31	0.728	0.999
SSKALQRPV		NP	0.403	0.999
SKALQRPVA		NP	0.570	<0.100
KALQRPVAS		Wt (I) 118.11	0.022	<0.100

Table 3.2: Proteosomal cleavage prediction for selected peptides:

Proteosomal cleavage prediction of BCR/ABL derived peptides was assessed by three publicly available algorithms. Tumour associated epitopes (hTERT, PR3 and WT1) and the Flu epitope were also included for comparison. [a]Paproc website found at www.paproc.de. Values represent predicted C-terminus cleavage by wild type (wt) proteosome I, II or III. The highest cleavage strength predicted by at least one wt proteosome is shown. NP: peptide cleavage not predicted. [b]NetChop website found at www.cbs.dtu.dk/services/Netchop. Values represent the cleavage probability by C-term 2.0 proteosome. [c]Fragpredict website found at www.mpiib-berlin.mpg.de/MAPPP. Values represent the cleavage probability of the specified fragment.

The experimentally based PaProc algorithm excluded three BCR/ABL derived peptides (ATGFKQSSK, SSKALQRPV and SKALQRPVA, **Table 3.2**) as potential tumour specific epitopes. These peptides were however predicted and given a relatively high score values by both the Netchop and Fragpredict algorithms (with the exception of the SKALQRPVA peptide using Fragpredict algorithm). At least two of these peptides were shown to stabilize HLA-A*0201 (SSKALQRPV) and HLA-A*1101 (ATGFKQSSK) molecules and to sensitize antigen specific T cells *in-vitro* (Yotnda et al. 1998). Conversely, the BCR/ABL

derived KALQRPVAS peptide was not theoretically predicted to be cleaved at the Serine C-terminal residue (low score values obtained by Netchop and Fragpredict algorithms), but experimentally predicted by PaProc to be cleaved by the wild type proteosome I (**Table 3.2**). The proteosomal cleavage of the three BCR/ABL derived peptides selected for binding to the HLA-A*0201 (SSKALQRPV), HLA-A*0301 (KQSSKALQR) and HLA-B*0801 molecules (GFKQSSKAL) were predicted by at least two out of the three algorithms.

The discordance observed between the three different algorithms for the proteosomal cleavage of a given peptide were also found for the control peptides. For example, the Proteinase 3 derived peptide VLQELNVTV was predicted to be cleaved with high probability by the Netchop and Fragpredict algorithms, however the PAPROC algorithm refuted the C-terminal Valine cleavage of this peptide (**Table 3.2**, top panel). The two algorithms that predicted the possible cleavage of this peptide use mathematical rules for prediction whereas PaProc predictive ability is based on experimental data. Nevertheless, the experimental based values predicted by PaProc were not, in some cases, representative of physiological antigen processing. Indeed, the Paproc algorithm did not predict the crucial C-terminal proteosomal cleavage of both the proteinase 3 (VLQELNVTV) and telomerase (ILAKFLHWL) derived peptides where it has been demonstrated that specific T cells for both epitopes are circulating in the peripheral blood of patients and can be functionally expanded *in vitro* (Molldrem et al. 1999; Molldrem et al. 2000; Vonderheide et al. 1999).

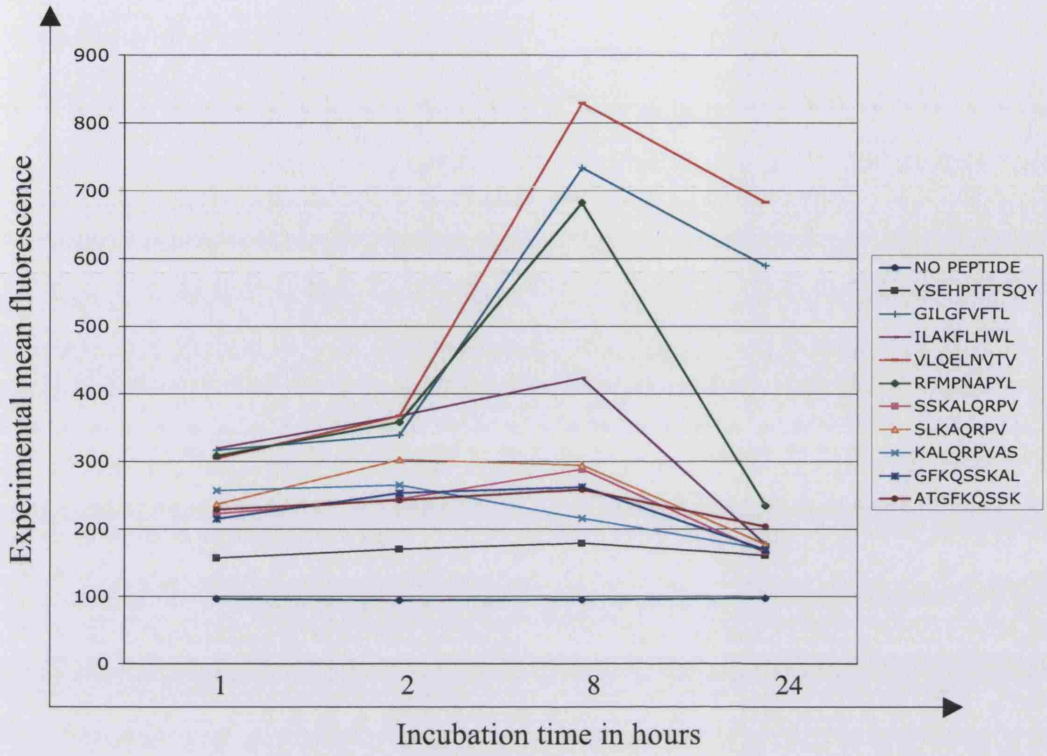
3.1.3 Experimental HLA-A*0201 molecule stabilisation assay:

One of the requirements for an antigenic peptide to be presented on the cell surface is to bind to and sufficiently stabilize the HLA molecule. In order to assess and confirm the HLA binding affinity of the BCR/ABL peptides predicted by the two algorithms, these peptides were evaluated for their ability to stabilize HLA molecule in a standard T2 binding assay. This was performed in the context of HLA-A*0201 molecules, since none of the BCR/ABL derived peptides obtained a high binding score and a comparative study with the other HLA-A*0201 tumour associated peptides could also be undertaken.

T2 cells are HLA-A*0201 positive but TAP deficient, therefore endogenous peptides can not be transported to the endoplasmic reticulum for HLA class I molecule loading and stabilization. This results in low minimal expression of HLA-A*0201 molecules (in association with ER derived peptides) at the cell surface. However if T2 cells are pulsed with an exogenous source of peptide that have enough HLA binding affinity, these peptides will bind and stabilised HLA-A*0201 molecules at the cell surface. The up-regulation of cell surface HLA molecules upon peptides pulsing was measured with the HLA-A*0201 specific BB7.2 monoclonal antibody and analysed by flow cytometry (as described in Chapter 2). A time course assay of HLA stabilisation was performed, as the majority of the BCR/ABL peptides were predicted to bind HLA-A*0201 molecules with moderate or low affinity. The high affinity HLA-A*0201 binding Flu peptide (GILGFVFTL), as well as the control HLA-A*0201 tumour associated epitopes WT1 (RMFPNAPYL), PR1 (VLQELNVTV) and hTERT (ILAKLHWL) were included in this study. An irrelevant HLA-A*0201 peptide (YSEHPTFTSQY CMVpp65 derived peptide associated with HLA-A*0101 molecule (Longmate et al. 2001)) was included in parallel to assess the background mean fluorescence. The up-regulation of HLA-A*0201 molecules expression on T2 cells was assessed following 1, 2, 8 and 24 hours incubation with the different peptides. The level of cell surface HLA molecule up-regulation was expressed as experimental mean fluorescence and is shown in **Figure 3.1**, panel **A**. The peak level of cell surface HLA expression for all the HLA-A*0201 associated peptides was observed and compared after 8 hours incubation.

BCR/ABL derived peptides induced an increase of HLA expression above the background mean fluorescence detected on T2 cells un-pulsed (No peptide) or pulsed with the HLA-A*0101 irrelevant CMV derived peptide (negative control YSEHPTFTS peptide). The BCR/ABL derived SSKALQRPV peptide stabilised the highest level of HLA-A*0201 molecule on the cell surface, as predicted by the Syfpeithi database (**Figure 3.1**, panel **A**, 289 experimental mean fluorescence (EMF), at 8 hours peak level).

A



B

Peptides	*Protein*	*Bimas scoring*	*Syfpeithi scoring*	*Stabilization values*
YSEHPTFTSQY	CMVpp65	0.001	6	0
GILGFVFTL	Flu	550.927	30	3.565±0.093
ILAKFLHWL	hTERT	1745.714	30	1.633±0.109
VLQELNVTV	PR1	484.777	28	4.155±0.121
RMFPNAPYL	WT1	313.968	22	3.242±0.105
SSKALQRPV	BCR/ABL	0.003	12	0.795±0.097
SLKALQRPV	modified	0.487	22	0.832±0.106
KALQRPVAS	BCR/ABL	0.013	10	0.348±0.209
GFKQSSKAL	BCR/ABL	0.001	10	0.633±0.132
ATGFKQSSK	BCR/ABL	0.000	8	0.608±0.144

Figure 3.1: HLA-A*0201 stabilisation assay:

The HLA-A*0201 cell surface expression on T2 cells was assessed after 1, 2, 8 and 24 hours incubation with 10μg/ml of different antigenic peptides as described in chapter 2 (**A**). The HLA expression was measured with BB7.2 antibody and is represented as experimental mean fluorescence. Negative controls (no peptide and irrelevant HLA-A*0101 CMVpp65 peptide, YSEHPTFTS) as well as a positive control (HLA-A*0201 Flu peptide, GILGFVFTL) were also included. Stabilisation values were calculated for each peptide at the peak of HLA expression (8 hours) with the following formula: (experimental mean fluorescence/irrelevant peptide (background) mean fluorescence)-1. The stabilisation values and the HLA-A*0201 binding affinity scores predicted by Bimas and Syfpeithi algorithms for each peptides are shown in **B**. The standard deviation obtained from the triplicate stabilization values are also shown.

The modified SLKALQRPV peptide induced only a slight increase of the binding stability (295 EMF, 8 hours). A comparatively higher mean fluorescence for this peptide was expected as the Syfpeithi predicted score was significantly higher than for the SSKALQRPV peptide, and relatively close to the strong HLA-A*0201 Flu or tumour associated peptides. The KALQRPVAS peptide showed the weakest binding affinity and stabilised the HLA-A*0201 molecule at the cell surface only for the first two hours, after which the peptide dissociated and the level of cell surface HLA expression was similar to the background level (**Figure 3.1**, panel **A**, 217 EMF, 8 hours).

The Flu derived peptide demonstrated a 2 to 3-fold higher binding affinity than the BCR/ABL derived peptides (**Figure 3.1**, panel **A**, 735 EMF calculated at 8 hours incubation). The tumour associated peptides stabilised the HLA-A*0201 molecule to different degrees. The PR1 peptide showed the strongest HLA-A*0201 binding affinity followed by the WT1 peptide (VLQELNVTV and RFMPNAPYL, 830 and 683 EMF at 8 hour respectively). The telomerase derived peptide hTERT (ILAKFLHWL) stabilised the HLA-A*0201 molecule with intermediate affinity, with a mean fluorescence about 1.5-fold higher than the BCR/ABL derived peptides at 424 EMF. All tumour associated antigens, as well as the Flu derived peptides clearly demonstrated a higher HLA-A*0201 binding affinity compared to the different BCR/ABL derived peptides assessed. Thus, it appears that there are no strong HLA-A*0201 binding 9-mer peptide derived from the BCR/ABL b3a2 fusion protein junctional region.

After 24 hours incubation the levels of cell surface HLA molecules detected on T2 cells decreased in the majority of cases (≤240 EMF, to the exception of PR1 and Flu peptides, **Figure 3.1**, panel **A**). At this point, the HLA/peptide complexes may have been internalised and as no excess peptides were used in our system this may have resulted in the decreased of stable HLA/peptide molecules expressed at the cell surface.

The stabilisation values were calculated for each peptide based on the peak level of HLA expression at 8 hours of incubation following the formula: (experimental mean fluorescence / irrelevant peptide or background mean fluorescence) −1 (Schatz 1993). Both the stabilisation values and the binding affinity scores predicted by Bimas and Syfpeithi algorithms for each peptide are shown in **Figure 3.1**, panel **B**. In the majority of cases, peptides which were given a high binding affinity score by Bimas (>300) and by Syfpeithi (>20) algorithms obtained an HLA-A*0201 stabilisation value greater than 1 in our experimental T2 assay. Accordingly, the selected BCR/ABL peptides predicted to be weak

HLA-A*0201 binders by both algorithms obtained a stabilisation value below 1 (**Figure 3.1**, panel **B**). The SSKALQRPV BCR/ABL peptide reproducibly obtained the highest stabilisation value (0.795±0.097), however the modification of an anchor residue at position two failed to significantly increase this value (**Figure 3.1**, panel **B**, SLKALQRPV, 0.832±0.106).

In some cases, the experimental stabilization values are in disagreement with the predicted scores. Both Bimas and Syfpeithi databases predicted the hTERT peptide (ILAKFLHWL) to be the strongest HLA-A*0201 binder, with a score of 1745.71 and 30 respectively, but this peptide was actually a weaker binder than PR1 or Flu peptide in our experimental T2 binding assay. Similarly, the WT1 peptide (RMFPNAPYL) and the modified BCR/ABL peptide (SLKALQRPV) were predicted by Syfpeithi to have the same binding affinity for the HLA-A*0201 molecule. However these peptides showed a significantly different experimental binding affinity, with a mean fluorescence 3-fold higher for the WT1 peptide. Thus the theoretical values are in some cases, in discordance with the experimental values and may not be representative of the physiological observations.

3.1.4 Correlation between the stabilisation values and the algorithms' predicted scores:

The correlation between the peptides binding scores predicted by the two algorithms and their experimental HLA-A*0201 stabilisation values obtained in the T2 binding assay was further assessed and is shown in **Figure 3.2**. In the majority of cases the peptides, which generated a high stabilisation value obtained a high score with the Syfpeithi and to a lesser extend with the Bimas epitope prediction databases (**Figure 3.2**, panel **A** and **B** respectively). A better positive correlation was found between the experimental stabilization values and the Syfpethi epitope predicted scores than with the Bimas predicted scores (Pearson's coefficient r=0.8063 and r=0.3962 respectively, panel **A** and **B**). Paradoxically the epitope prediction algorithm Bimas, which is based on experimental data, demonstrated a lower correlation with our experimental T2 stabilization assay values compared to the Syfpeithi algorithm (p=0.2570 and p=0.0048 respectively, Pearson's correlation test). It is worth mentioning that Bimas scoring values are calculated on a logarithmic scale, which may have account for the greater occurrence of discrepancy.

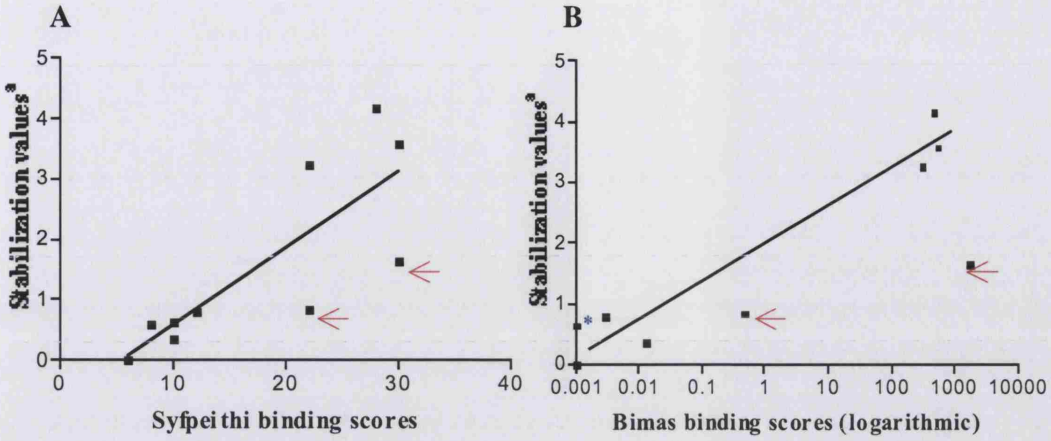


Figure 3.2: Correlation between two epitope prediction algorithms and experimental binding affinity:

HLA-A*0201 predicted binding scores of selected 9-mers peptides (**Table 3.1**) by Syfpeithi (**A**) or Bimas (**B**) databases were correlated with the experimental stabilization values obtained in our experimental T2 binding assay (**Figure 3.1**). Bimas scores were plotted in logarithmic values. [a]Stabilization values were obtained with the formula: (experimental mean fluorescence/ irrelevant peptide (background) mean fluorescence)-1. A Pearson's correlation test was carried out and demonstrated no significant correlation for the Bimas algorithm with a coefficient of r=0.3962 (95% CI, -0.31-0.82; p=0.2570; r^2=0.1570), but a good correlation for the Syfpeithi algorithm with a coefficient of r=0.8063 (95% CI, 0.36-0.95; p=0.0048; r^2=0.6502). Two peptides (GFKQSSKAL and ATGFKQSSK) obtained a value not discernable and are shown with *. Two other peptides (SLKALQRPV and ILAKFLHWL) were predicted to be strong HLA-A*0201 binders by the two algorithms but demonstrated a weak experimental binding, these are shown with the red arrows.

The hTERT (ILAKFLHWL) and the modified BCR/ABL (SLKALQRPV) peptides were predicted to be strong HLA-A*0201 binders but stabilised T2 cell surface HLA molecule only weakly compared to the other strong binding peptides (**Figure 3.2**, red arrows). The HLA stabilisation value obtained for the hTERT peptide in our study was in accordance with Vonderheide et al. group (Vonderheide et al. 1999). Using an alternative formula for the calculation of their experimental stabilisation values, the hTERT peptide obtained a value of 3.1 and with their formula we calculated a value of 3.24 at the 8 hours peak stabilization for the same peptide. These predicted strong, but experimentally weaker HLA binders have also been referred by other groups as "false-positive' epitopes (Andersen et al. 2000; Pelte et al. 2004). Although some of these false-positives may represent irrelevant epitopes, others may actually represent physiological HLA-associated epitopes. For example, the hTERT peptide has been demonstrated to be recognized by and to generate functional antigen specific T cells (Vonderheide et al. 1999; Vonderheide et al. 2001). Thus it remains important to assess the relevance of even weak HLA binding peptides.

3.2 Definition of Naturally presented BCR/ABL derived peptides:

The BCR/ABL derived peptides were predicted and were also demonstrated to have a low binding affinity for the HLA-A*0201 molecules, these peptides however could still represent potential tumour specific antigens. In order to assess and confirm the relevance of these antigens *in-vivo*, HLA-bound peptides were directly eluted from tumour cells and their nature was characterised by tandem mass spectrometry. In this study, we investigated the processing and presentation of BCR/ABL derived peptides in the context of HLA-A*0201 and HLA-B*0801 molecules. K562 cells were selected and transfected with a single relevant HLA class I molecule to reduce the background peptide signals that would be detected using a heterozygous cell, which can present up to six HLA class I molecules. In order to control for the level of antigen expression, HLA-bound peptides were also eluted from primary CML cells derived from patients. The eluted peptides were then sequenced by Electrospray Ionisation Mass Spectrometry (ESI/MS) and matched with the BCR/ABL derived pre-selected peptides as described in material and methods.

3.2.1 HLA class I transfection of K562 cell lines:

The cDNA sequences for HLA-A*0201 and HLA-B*0801 were cloned into the pBJ1Neo vector and K562 cells were transfected by electroporation. Resistant clones were selected with neomycin antibiotic and after two weeks of culture these were further cloned by limiting dilution as described in chapter 2. The specificity and level of HLA expression on transfected cells was verified by cell surface staining of selected K562 clones with W6/32 (HLA class I specific), BB7.2 (HLA-A*0201 specific) and GAP-A3 (HLA-A*0301 specific) primary monoclonal antibodies. HLA expression was detected with a secondary FITC conjugated anti-mouse IgG antibody and cells were analysed by flow cytometry as shown in **Figure 3.3**. The percentages and the levels of HLA expression on transfected cells are represented in **Table 3.3**. For comparison purposes, the level of HLA molecules detected on the surface of HLA-A*0201 (JY) and HLA-A*0301 (TUBO) homozygous control EBV-transformed B cell lines was assessed in parallel (**Table 3.3**).

HLA-A*0201 molecules were specifically detected on the cell surface of transfected K562 clone with both W6/32 and BB7.2 specific antibodies (441 and 406 mean fluorescence intensity (MFI) respectively, **Table 3.3** and **Figure 3.3**, panel **B**). In contrast no FITC fluorescence was detected with the secondary antibody alone (red line) or with the HLA-A*0301 specific GAP-A3 antibody (**Figure 3.3**, panel **B**, <96 MFI, **Table 3.3**).

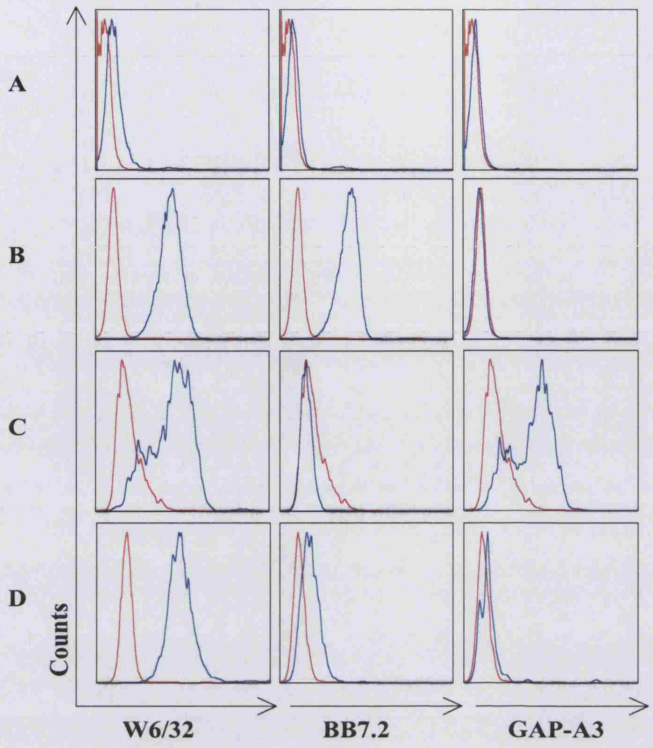


Figure 3.3: HLA class I cell surface staining of transfected K562 cells:
Cell surface staining of HLA class I molecules was performed on K562 cells transfected with pBJ1Neo DNA vector alone (A), or with pBJ1Neo DNA vector encoding HLA-A*0201 (B), HLA-A*0301 (C) or HLA-B*0801 (D) molecules. Cells were stained with W6/32 (HLA class I pan-specific), BB7.2 (HLA-A*0201 specific) and GAP-A3 (HLA-A*0301 specific) primary antibodies, followed by FITC conjugated anti-mouse IgG secondary antibody (–) and analysed by flow cytometry. Cells were also stained with the secondary antibody (–). The percentage of HLA molecules expression as well as the mean fluorescence intensity detected is shown in **Table 3.1**.

Cell lines	Antibodies							
	Control		*W6/32*		*BB7.2*		*GAP-A3*	
	%HLA	MFI	%HLA	MFI	%HLA	MFI	%HLA	MFI
K562 pBJ1Neo	0.50	78	4.47	275	1.13	149	2.18	134
K562-HLA-A*0201	0.33	89.5	98.9	441	96.9	406	0.52	95.6
K562-HLA-A*0301	3.87	176	81.2	487	0.11	153	73.7	455
K562-HLA-B*0801	0.89	137	99.1	497	2.52	209	2.33	177
JY-HLA-A*0201	0.88	89	99.9	698	99.9	709	1.51	98.2
Tubo-HLA-A*0301	1.56	97.5	98.9	586	99.2	609	97.8	506

Table 3.3: Mean fluorescence values of transfected K562 cells surface expression of HLA class I molecules:
The HLA class I cell surface staining of K562 cells transfected with pBJ1Neo DNA vector alone, encoding HLA-A*0201, HLA-A*0301 or HLA-B*0801 molecules was performed as shown in Figure 3.3. The percentage of HLA class I molecules expression and the Mean Fluorescence Intensity (MFI) detected with the W6/32 (HLA class I pan-specific), BB7.2 (HLA-A*0201 specific) and GAP-A3 (HLA-A*0301 specific) are shown for each cell line. Cells were also stained with anti-mouse IgG secondary antibody (Control). The cell surface expression of HLA molecules on JY (HLA-A*0201 homozygous) and Tubo (HLA-A*0301 homozygous) EBV positive cell lines was assessed in parallel and is shown.

The staining of HLA-A*0301 transfected K562 cell line with the HLA class I (W6/32) and the HLA-A*0301 (GAP-A3) specific antibodies also resulted in the detection of a significant FITC signal (**Figure 3.3**, panel **C**, 487 and 455 MFI **Table 3.3**). The expression of HLA-A*0301 molecule on the transfected K562 cells was specific as no significant FITC signals were detected with either the secondary antibody alone or with the HLA-A*0201 specific BB7.2 antibody (≤176 MFI, **Table 3.3**). The HLA-A*0301 transfected K562 cells were not cloned, which results in a more heterogeneous level of HLA class I expression detected (performed prior to my PhD). Finally the expression of HLA molecules on HLA-B*0801 transfected K562 clone was detected with the HLA class I specific W6/32 antibody (**Figure 3.3**, panel **D**, 497 MFI, **Table 3.3**). No FITC signal was however detected after staining these cells with the secondary antibody alone or with the BB7.2 and GAP-A3 non-HLA-B*0801 specific antibodies (≤209 MFI, **Table 3.3**). K562 cells were also transfected with plasmid DNA vector without insert, as an internal control of transfection and showed no detectable levels of HLA staining (**Figure 3.3**, panel **A**, ≤ 275 MFI, **Table 3.3**). Thus, significant and specific levels of HLA molecules were detected on the cell surfaces of the transfected K562 cell line (HLA-A*0301) and clones (HLA-A*0201 and HLA-B*0801).

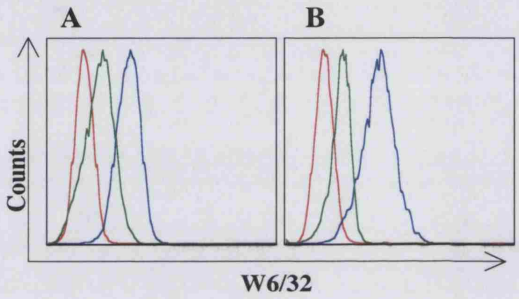
Higher levels of HLA molecules were detected from both JY and TUBO EBV-transformed B cell line controls compared to the transfected K562 cells (from 506 to 709 MFI, **Table 3.3**). BCR/ABL derived peptides were nevertheless detected from the cell surface of HLA-A*0301 transfected K562 cells (Clark et al. 2001). Thus the levels of HLA-A*0201 and HLA-B*0801 molecules expressed on transfected K562 cells should be sufficient to detect HLA-bound BCR/ABL derived peptides.

3.2.2 Peptide elution from transfected K562 cells:

HLA-A*0201 and HLA-B*0801 K562 clones were expanded in large culture flasks and HLA-bound peptides were eluted from 1.5×10^9 HLA-A*0201 cells and 2×10^9 HLA-B*0801 cells as described in chapter 2. In parallel, HLA-associated peptides were eluted from primary CML cells from HLA-A*0201 and HLA-B*0801 positive CML patients following leukapheresis. This was performed by our collaborators in Liverpool (Prof R. Clarke, Royal Infirmary Hospital, Liverpool) and samples were sent to Prof R. Rees at the Nottingham Trent University for analysis.

Cell surface HLA class I expression was monitored prior to and after peptide elution to confirm the efficiency of the elution process. The successful release of bound peptides from

the cell surface of transfected cells following mild acid treatments dissociates the β-2 microglobulin and induces the unfolding of the HLA class I heavy chain, which can be measured by the loss of W6/32 monoclonal antibody binding. The HLA expression on the cell surface of transfected K562 cells was assessed and is shown in **Figure 3.4**. HLA-A*0201 and HLA-B*0801 transfected K562 clones were stained with the W6/32 antibody and the HLA expression was detected with the FITC conjugated anti-mouse IgG secondary antibody. K562 cells were also stained with the secondary antibody alone and showed no background level of HLA detection (**Figure 3.4**, red line, ≤197 MFI).



Cell lines	*Pre-Elution*			*Post-Elution*				*Controls*	
	%Viability	% HLA	MFI	%Viability	% HLA	MFI	%Efficiency	% HLA	MFI
K562-A*0201	87	90.2	375	77	16.3	231	***61.6***	0.97	177
K562-B*0801	78	94	429	65	12.1	244	***56.9***	1.01	197

Figure 3.4: Monitoring the effective peptide elution from transfected K562 cells:

Cell surface staining of HLA class I molecules was performed on HLA-A*0201 (**A**) and HLA-B*0801 (**B**) transfected K562 cells. Cells were stained with the W6/32 (HLA class I specific) primary antibody, followed by FITC conjugated anti-mouse IgG secondary antibody and analysed by flow cytometry. K562 cells were stained prior to (–) and after (–) peptide elution. The percentage of viability and the level of HLA molecules expression (% HLA and mean fluorescence intensity MFI) are also shown in the table. The peptide elution efficiency was calculated using the following formula: (MFI post elution/MFI pre-elution) × 100. Cells were also stained with the secondary antibody pre and post elution (–, Controls).

The levels of HLA expression detected from both HLA-A*0201 and HLA-B*0801 transfected K562 cells prior to the mild acid treatment is shown in blue lines in **Figure 3.4**, panel **A** and **B** respectively (375 and 429 MFI). Following peptide elution, a lower level of FITC signal was detected from the cell surface of both transfectants (231 and 244 MFI, green lines). The reduction of HLA class I molecules on the cell surface of these clones was consistent with the successful elution process. An elution efficiency of 61.6% and 59.9% was calculated for the HLA-A*0201 and HLA-B*0801 K562 cells respectively as shown in **Figure 3.4**. In addition, the transfected K562 cells remained viable after the acid treatment, thus demonstrating that cellular integrity had been maintained following the elution process. Peptides eluates were filtered and were stored at -80°C until required for further use.

3.2.3 Mass spectrometry analysis of cell surface eluates:

The mass spectrometric analysis of the peptides eluates was performed at the Nottingham Trent University following the method described in chapter 2. The definition of BCR/ABL derived epitopes was first assessed in the eluates from HLA-B*0801 cells. The HPLC fraction containing the predicted HLA-B*0801 associated BCR/ABL derived peptide GFKQSSKAL, was primarily defined with a positive control (peptide-pulsed HLA-B*0801 EBV-transformed cells). This allowed us to define the HPLC fraction containing the predicted epitope (m/z 483.27) and to then analyse this particular fraction eluted from transfected K562 cells or from primary leukaemic cells obtained from CML patients. These fractions were subjected to nanospray sequencing by ESI/MS and assessed for the presence of BCR/ABL derived peptides. The tandem mass spectrum of HLA-B*0801 associated BCR/ABL peptides eluted from transfected K562 cells was not obtainable. The GFKQSSKAL peptide was found in a single HPLC fraction at a very low concentration, particularly when compared to the HLA-A*0301-associated KQSSKALQR peptide eluted from transfected K562 cells (Clark et al. 2001).

The nanospray ESI/MS profile obtained from the cell eluate of a HLA-B*0801 CML patient was kindly provided by Joanne Clayton from the Nottingham Trent University and is shown in **Figure 3.5**. The raw tandem mass spectrum of the 483.27 m/z HPLC fraction was exported to Excel for the subtraction of neighbouring background fractions (**Figure 3.5**, panel **A** and **B** respectively). The fragment ion pattern of this mass spectrum was compared with the positive control (peptide-pulsed HLA-B*0801 EBV transformed cells) and confirmed by MS-Tag algorithm to correspond to the BCR/ABL derived GFKQSSKAL peptide. Thus BCR/ABL associated HLA-B*0801 tumour specific peptides are naturally processed and presented by CML cells.

With respect to HLA-A*0201 molecules, the natural processing and presentation of BCR/ABL derived peptides was assessed for the four predicted peptides (SSKALQRPV, GFKQSSKAL, KALQRPVAS and ATGFKQSSK). Positive controls were established by peptide-pulsing HLA-A*0201 homozygous EBV-transformed cells with each of the predicted peptides and eluting HLA-bound peptides. Thus the HPLC fractions, which contained each of the potential HLA-A*0201 BCR/ABL associated epitopes could be assigned. Then, the test sample containing HLA-A*0201-bound peptide eluted from HLA-A*0201 transfected K562 cells was analysed for the presence of these peptides. The Nottingham group was unable to characterise the presence of any of the predicted BCR/ABL peptides from these eluates. These peptides were however detected in the

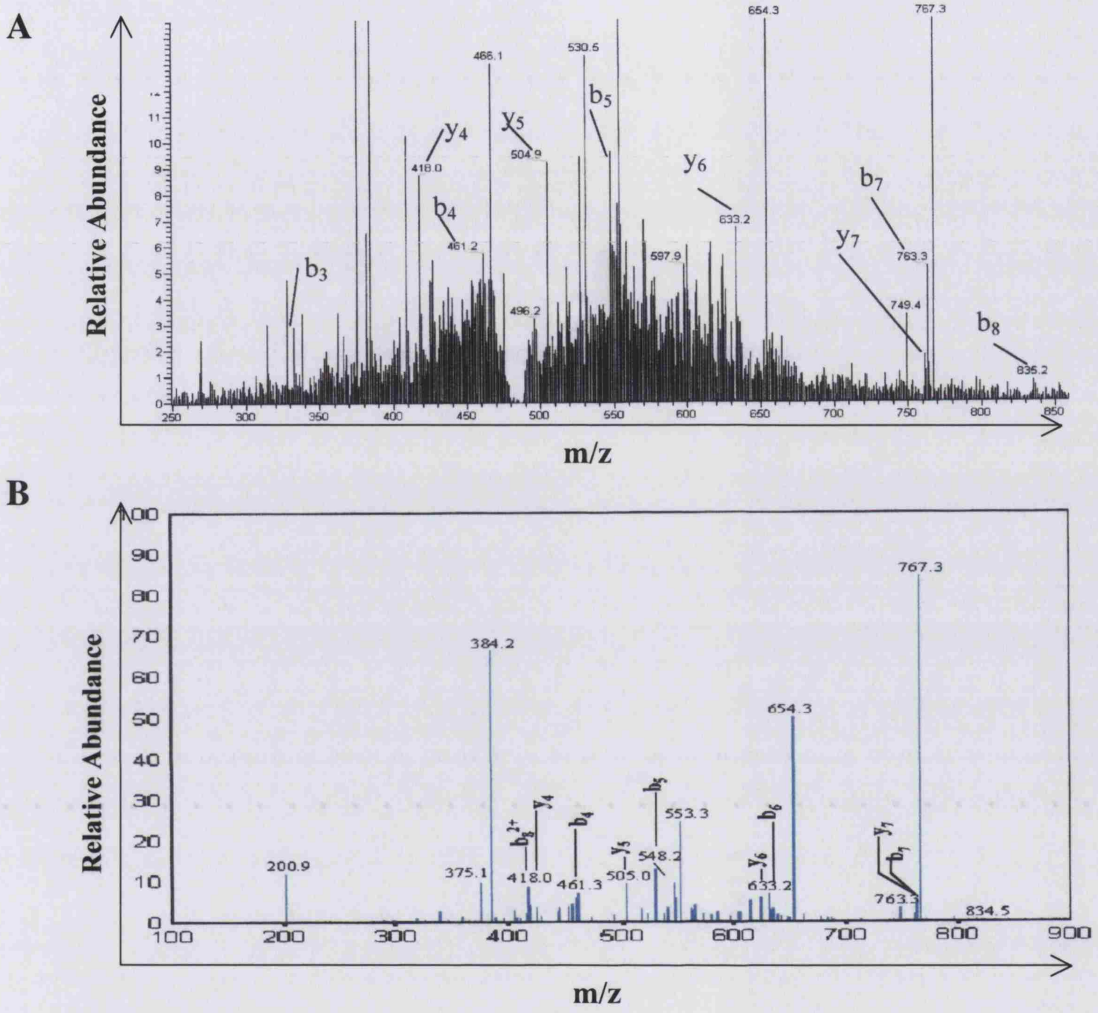


Figure 3.5: Tandem mass spectrum of the HLA-B*0801 associated BCR/ABL peptide eluted from CML patients PBMCs:

Cell surface HLA associated peptides were eluted from PBMCs obtained by leucophoresis from an HLA-B*0801 CML patient. The HPLC fraction containing the predicted HLA-B*0801 associated BCR/ABL peptide GFKQSSKAL was defined through the use of a positive control. This consisted of an HLA-B*0801 matched EBV cell line to which an excess amount of the synthetic peptide was added. These cells were then washed and the cell surface peptides were eluted and fractionated. The fraction containing the GFKQSSKAL peptide was identified and found to be eluted in the m/z 483.27 ion $[M+2H]^{2+}$. The MS/MS spectrum of the HLA-B*0801 CML patient cell surface bound peptide obtained at this m/z 483.27 point is shown in panel **A**. The spectrum was exported to Excel software for background subtractive analysis and is shown in panel **B**. The peptide sequence was confirmed to be GFKQSSKAL using the MS-Tag algorithm by the Nottingham Trent university group.

positive control eluates. Thus the low HLA binding affinities of these predicted epitopes did not account for the lack of signal. The level of expression of these peptides may be too low for the sensitivity of this technique, or these peptides may simply not be presented on the surface of HLA-A*0201 transfected K562 cells.

Additional peptide eluates generated from HLA-A*0201 transfected K562 cells were processed to further investigate and confirm these results. At the end of this study, none of the potential BCR/ABL derived peptides associated with the HLA-A*0201 molecule were detectable from either HLA-A*0201 transfected K562 cells or primary CML cells. A representative tandem mass spectrum of the SSKALQRPV peptide is shown in **Figure 3.6**.

The BCR/ABL derived SSKALQRPV peptide was eluted from the positive control eluate (peptide-pulsed HLA-B*0201 EBV-transformed cells) after 22 to 24 minutes and characterised by ESI/MS (**Figure 3.6**, panel **A**). This peptide was however not detected from the HLA-A*0201 transfected K562 eluates (**Figure 3.6**, panel **B**). The different peptide spikes eluted between 22 and 24 minutes (top panel **B**) did not contain the SSKALQRPV peptide (lower panel **B**) or any of the other potential BCR/ABL derived peptides. Additionally, none of these BCR/ABL peptides were characterised from leucophoresis sample eluates obtained from HLA-A*0201 CML patients (Joanna Clayton personal communication).

These data demonstrate that BCR/ABL derived peptides are naturally processed and presented on the surface of CML cells, at least in the context of HLA-A*0301 (KQSSKALQR peptide, (Clark et al. 2001)) and now HLA-B*0801 molecules (GFKQSSKAL peptide). It appears however that no peptides derived from the BCR/ABL junctional region are presented in the context of HLA-A*0201 molecules. This information was only confirmed at the end of my PhD, thus the feasibility to generate and expand BCR/ABL specific T cells in the context of HLA-A*0201 molecules was still undertaken, targeting the three potential BCR/ABL derived GFKQSSKAL, KALQRPVAS, SSKALQRPV peptides and the modified SLKALQRPV peptide. In the context of HLA-A*0301 and HLA-B*0801 molecules, the characterisation of a BCR/ABL specific T cell repertoire and the feasibility to generate functional immune responses were assessed for KQSSKALQR and GFKQSSKAL peptides respectively.

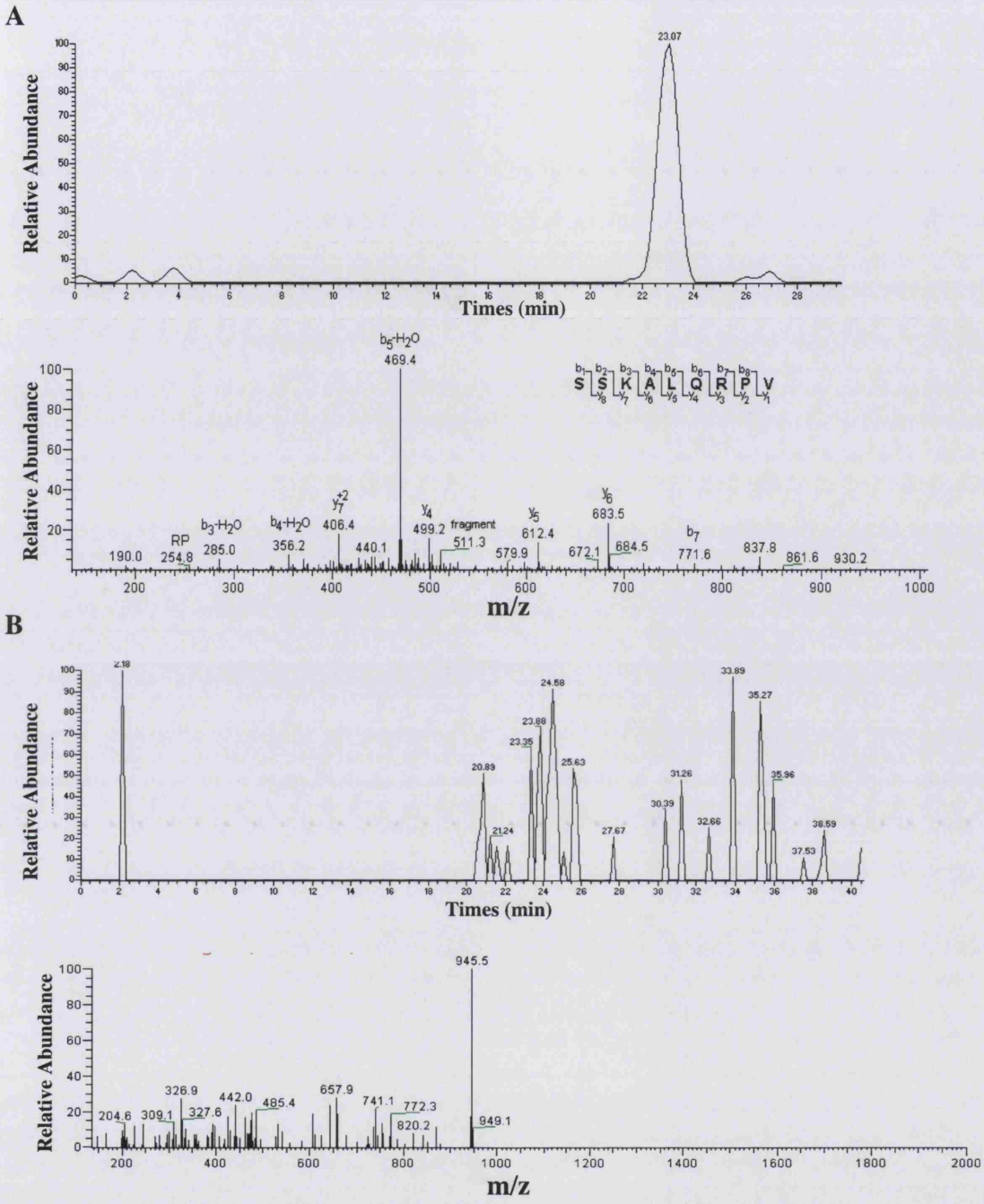


Figure 3.6: Tandem mass spectrum of the HLA-A*0201 associated SSKALQRPV peptide eluted from transfected K562 cells:

The natural processing and presentation of the SSKALQRPV BCR/ABL derived peptide was assessed from HLA-A*0201 transfected K562 cell eluates. The HPLC fraction containing this peptide was first defined with a positive control consisting of an HLA-A*0201 EBV cell line to which an excess amount of the synthetic peptide was added (panel **A**). The HPLC fraction in which the SSKALQRPV peptide was eluted from the positive control is shown (top panel **A**). This fraction was further sequenced and confirmed by ElectroSpray Ionisation tandem Mass Spectrometry (ESI/MS) (lower panel **A**). HLA-bound peptides were also eluted from HLA-A*0201 transfected K562 cells (panel **B**). The peptides fractions eluted between 22 and 24 minutes (top panel **B**) were assessed by ESI/MS (lower panel **B**). The BCR/ABL derived SSKALQRPV peptide was not characterised from the K562 cells (panel **B**). The mass spetrometry analysis was performed and obtained by Joanna Clayton from the Nottingham Trent University group.

3.3 Characterisation of BCR/ABL specific T cells *ex-vivo*:

The relevance of the selected BCR/ABL peptides, i.e. their capacity to be recognized and induce antigen-specific T cell responses, was investigated from both CML patients and healthy donors. The presence of precursor T cells bearing the specific T cell receptor for recognition of BCR-ABL/HLA class I complexes was assessed in the context of HLA-A*0201 (for the three predicted potential epitopes), HLA-A*0301 and HLA-B*0801 molecules. BCR/ABL specific T cells were characterised *ex-vivo* both qualitatively (proliferative responses) and quantitatively (HLA class I tetramer staining).

As mention previously, antigen-specific T cells can be characterised *in-vitro* by assessing their proliferative response following antigen stimulation. The most used assay to monitor T cell proliferation is the measurement of ^{3}H-thymidine incorporation into DNA during cell proliferation. Lymphocytes are cultured with the potential antigenic peptides, which will be presented on HLA class I molecules and upon TCR recognition, antigen-specific T cells will be induced to proliferate. This assay allows the characterisation of antigen-specific T cell precursors in the peripheral blood but their frequencies can not be accurately determined. More recently, fluorescently labelled HLA/tetramers have allowed the estimation of antigen-specific $CD8^+$ T cells frequencies by flow cytometry. Peripheral blood T cells are stained with specific HLA/peptide tetramer complexes and visualised on a FACs calibur (Becton and Dickinson). The frequencies of antigen-specific T cells circulating in the peripheral blood of an individual can be quantified as a proportion of total lymphocytes (expressed as a percentage of total $CD3^+$ T cells) and in the context of HLA class I epitopes, as a proportion of $CD8^+$ T cells (expressed as a percentage of total $CD3^+CD8^+$ T cells).

3.3.1 BCR/ABL specific proliferative responses:

The proliferation of PBMCs isolated from CML patients and healthy individuals following stimulation with BCR/ABL derived peptides was first assessed. In the context of the HLA-A*0301 peptide KQSSKALQR and the HLA-B*0801 peptide GFKQSSKAL, proliferative responses were compared to the CMV pp65 derived epitopes IGDQYVKVY (selected from our in-house CMV epitopes mapping study, unpublished data) and DANDIYRIF (Wills et al. 1996). With reference to the HLA-A*0201 molecule, the immunogenicity of BCR/ABL tumour specific peptides (GFKQSSKAL, KALQRPVAS, SSKALQRPV and the modified SLKALQRPV peptide) were compared with the immunogenicity of the tumour associated antigens WT1, PR1 and hTERT. A negative control (medium and irrelevant HLA-

A*0201/Flu peptide for HLA-A*0301 and/or HLA-B*0801 individuals) and a positive control (polyclonal mitogen stimulation with Phytohaemagglutinin, PHA) were also included in the assay. Lymphocytes were incubated for 5 days with the different peptides at 10 μg/ml and ^{3}H-thymidine incorporation was measured as described in chapter 2. The level of ^{3}H-thymidine incorporation is relative to the specific proliferative responses obtained. The proliferative responses of HLA-A*0301, HLA-B*0801 and HLA-A*0201 CML patients and healthy donors following the different peptide stimulations are represented as the mean of triplicate assays (**Figure 3.7**, panel **A**, **B** and **C** respectively). As expected, the polyclonal PHA mitogen stimulation induced a strong proliferative response from all CML patients and healthy donors. Only lymphocytes derived from CML patient samples harvested at diagnosis (No treatment) showed proliferative responses in the absence of antigenic peptides (Medium). The large numbers of blast cells in these patients may have account for the higher background proliferation observed.

In the context of HLA-A*0301, a single CML patient demonstrated a statistically significant HLA-A*0301 restricted BCR/ABL peptide specific proliferative response compared to both the medium and the irrelevant HLA-A*0201 Flu peptide (**Figure 3.7** panel **A**, b3a2^{+} patient with No treatment, P=0.0324 and P=0.0287 respectively, paired *t*-test). Additionally, the two HLA-A*0301 CMV seropositive CML patients, one with no treatment and one b2a2^{+} CML patient treated with Imatinib mesylate demonstrated a specific proliferative responses following stimulation with the CMVpp65 peptide (**Figure 3.7**, panel **A**). These responses were statistically significant compared to the responses with medium alone (P=0.0263 and P=0.0003 respectively, paired *t*-test) or with the irrelevant HLA-A*0201 Flu peptide (P=0.0198 and P=0.0005 respectively, paired *t*-test).

In the context of HLA-B*0801, a single CML patient showed proliferative responses to the BCR/ABL and to the CMVpp65 specific peptides (**Figure 3.7**, panel **B**, No treatment, CMV seropositive). These responses were however not statistically significant compared to the negative controls, medium alone and irrelevant HLA-A*0201 associated Flu peptide (P>0.05, paired *t*-test).

Finally in the context of HLA-A*0201, the proliferative response was assessed for the three different predicted BCR/ABL peptides, GFKQSSKAL (CML1), KALQRPVAS (CML2) and SSKALQRPV peptide (CML3). The SSKALQRPV peptide, which was modified to contain a dominant anchor residue (SLKALQRPV, CML4), was also included in this assay.

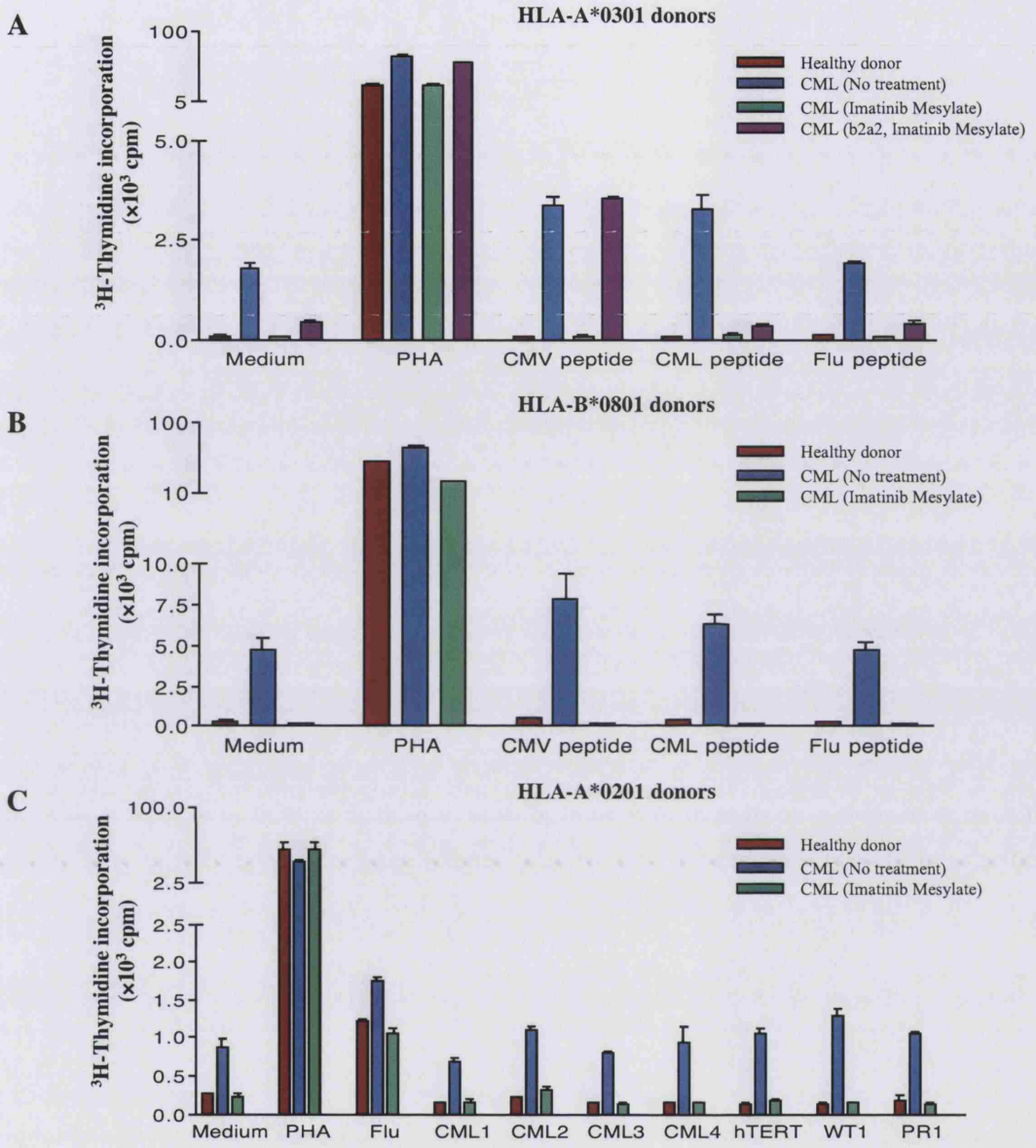


Figure 3.7: *Ex-vivo* lymphocyte proliferation following peptide stimulation:

The proliferative responses to peptide stimulation of PBMCs derived from (**A**) HLA-A*0301, (**B**) HLA-B*0801 and (**C**) HLA-A*0201 CML patients or healthy donors are shown. CML patients were selected for their expression of the b3a2 transcript, except where mentioned. Lymphocytes were incubated with 10μg/ml of either viral (Flu or CMV), or BCR/ABL (CML, CML1, 2, 3 and 4) or tumour (WT1, PR1 and hTERT, only in panel C) peptides for 5 days. Cells were then labelled with ^{3}H-thymidine, incubated for an additional 16 hours and the thymidine incorporation was measured as described in chapter 2. Cells were also incubated without peptide (Medium, negative control) and with the polyclonal mitogen phytohaemagglutinin (PHA, positive control). Peptide sequences are: Flu (GILGFVFTL), CML1 (GFKQSSKAL), CML2 (KALQRPVAS), CML3 (SSKALQRPV), CML4 (modified BCR/ABL peptide SLKALQRPV), WT1 (RMFPNAPYL), PR1 (VLQELNVTV), hTERT (ILAKFLHWL), CMV-HLA*0301 associated (IGDQYVKVY, panel A), and CMV-HLA-B*0801 associated (DANDIYRIF, panel B). HLA-A*0201/Flu associated peptides were used as an HLA-irrelevant peptide control in panel A and B. Error bars represent the standard deviation between triplicate assays. Proliferation is expressed in 10^3 cpm (counts per minute).

The proliferative responses to the BCR/ABL wild type and to the modified peptides were compared with the proliferative responses to the Influenza virus Flu peptide, as well as to the tumour associated hTERT, WT1 and PR1 derived peptides. Antigen-specific T cell proliferative responses to the BCR/ABL peptides were observed from a single HLA-A*0201 CML patient assessed at diagnosis (**Figure 3.7**, panel **C**, No treatment), but these responses were not statistically significant compared to the negative control (medium alone, $p<0.05$, paired *t*-test). This same donor also showed some proliferative responses upon stimulation with the hTERT, WT1 and PR1 tumour associated antigens. However these responses were again not statistically significant compared to the proliferation to medium alone ($p<0.05$, paired *t*-test). In contrast, the stimulation of PBMCs derived from HLA-A*0201 healthy donor and CML patients assessed at diagnosis or treated with Imatinib mesylate with the Flu antigen resulted in significant proliferative responses compared to the negative control (**Figure 3.7**, panel **C**, healthy donor, CML No treatment or Imatinib mesylate; $P=0.0012$, $P=0.018$ and $P=0.0143$ respectively, paired *t*-test).

In conclusion, with the exception of a single HLA-A*0301 CML patient assessed at diagnosis, none of the healthy donors nor the CML patients treated with Imatinib mesylate demonstrated HLA-A*0301 or HLA-B*0801 restricted BCR/ABL peptide specific T cells proliferation above the background of medium alone and irrelevant Flu peptide. In addition, no statistically significant proliferative responses were observed from any HLA-A*0201 donors after short-term stimulation with either BCR/ABL or TAA peptides. The frequencies of these tumour specific $CD8^+$ T cells are expected to be low, especially in CML patients who's immune response has not succeeded in controlling their disease. Thus, the *ex-vivo* proliferation assay may not be sensitive enough for the detection of low frequent BCR/ABL specific T cells.

3.3.2 HLA/peptide tetramer staining:

Antigen specific $CD8^+$ T cells can be detected with HLA class I tetramers from frequencies as low as 10^{-4} to 10^{-5} events (Altman et al. 1996). Thus, the presence and the frequencies of BCR/ABL specific T cells circulating in the peripheral blood of CML patients and healthy donors was further characterised by HLA/tetramer staining.

HLA-A*0201, HLA-A*0301 and HLA-B*0801 molecules were refolded with the appropriate BCR/ABL peptide(s), and in relation to HLA-A*0201 also with the tumour associated antigens WT1, PR3 and hTERT. HLA-A*0201 tetramers were also constructed

with viral peptides from CMV (NLVPMVATV) and Influenza virus protein A (GILGFVFTL). These tetramers served as controls for the detection of higher frequency antigen-specific T cells. Each batch of HLA/peptide monomers was assessed for correct refolding and for efficient biotinylation of the complexes prior to their tetramerisation as described and shown in chapter 2. The tetramerization of these complexes was performed using fluorescently labelled streptavidin, thus allowing the visualisation of single antigen-specific $CD8^+$ T cell by flow cytometry. PBMCs were isolated and stained with 1μg of HLA tetramer, followed by anti-CD3 and anti-CD8 antibodies as described in chapter 2. The fluorescently marked cells were then detected and analysed by flow cytometry.

An example of antigen-specific T cell gating is shown both for high frequency HLA-A*0201/CMV specific T cells and for low frequency HLA-A*0301/BCR-ABL specific T cells (**Figure 3.8**, CMV and CML). Tetramer positive cells were visualised and quantified by first gating on the live lymphocyte population (**Figure 3.8**, panel **A**). As a result, the false tetramer positive cells resulting from apoptotic and/or necrotic cells showing non-specific binding were discriminated and eliminated from the analysis. The lymphocytes were then gated on the $CD3^+$ T cells and on the $CD3^+CD8^+$ T cells to ensure the specificity of the antigen-specific T cell population (**Figure 3.8**, panel **B** and **C**). Finally, antigen-specific $CD8^+$ T cells were gated on the double positive population in the CD8/tetramer dot plots and were expressed as a percentage of $CD3^+$ T cells or as a percentage of $CD3^+CD8^+$ T cells (**Figure 3.8**, panel **D** and **E**).

The CD3 molecule is part of the TCR complex and the binding of HLA/peptide tetramers to $CD8^+$ T cells was shown to interfere with the subsequent binding of anti-CD3 antibody (Hoffmann et al. 2000). The tetramer positive T cells therefore exhibit a lower CD3 mean fluorescence than the tetramer negative or false-positive T cells population as shown in **Figure 3.8**, panel **F**. This binding "competition" allows for the discrimination between antigen-specific T cells and T cells binding the tetramer non-specifically. The magnitude of the CD3 mean fluorescence shift was greater for the high frequency HLA-A*0201/CMV specific T cells (**Figure 3.8**, panel CMV **F**) than for the low frequency HLA-A*0301/CML specific T cells (**Figure 3.8**, panel CML **F**). These data are consistent with the observation of Hoffman *et al.* that the magnitude of CD3 competition with HLA/tetramer is proportional to the frequency and to the HLA/peptide affinity for the TCR of tetramer positive cells (Hoffmann et al. 2000).

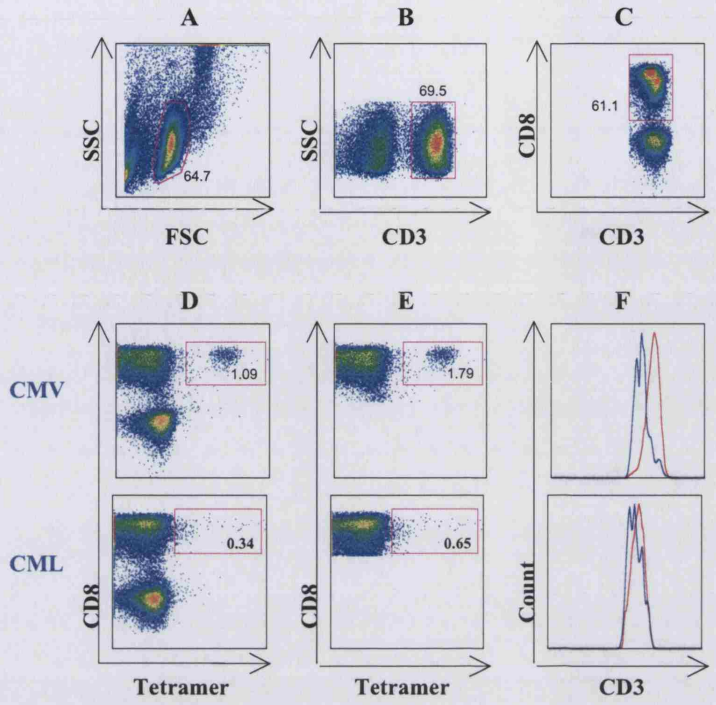


Figure 3.8: Example of HLA/peptide tetramer analysis by flow cytometry and the gating strategy applied:

Lymphocytes were stained with the corresponding tetramer, anti-CD3 and anti-CD8 antibodies and analysed by flow cytometry. Lymphocytes were first gated from dead cell debris on the forward and side scatter plot (panel **A**, pink gate). $CD3^+$ T cells were gated on a second dot plot, the CD3/ side scatter plot (panel **B**) as well as $CD8^+$ T cell fraction (panel **C**). Tetramer specific cells were then gated and expressed as a percentage of total $CD3^+$ T cells (panel **D**) or as a percentage of $CD3^+CD8^+$ T cells (panel **E**). The specific binding of $CD8^+$ T cells to the HLA/peptide tetramers interferes with the subsequent binding of anti-CD3 antibody. This results in a lower CD3 mean fluorescence of the $CD8^+$ tet^+ (blue) than the $CD8^+$ tet^- (red) population as shown in panel **F**. An example of high frequency of HLA-A*0201/CMV (**CMV**) and low frequency HLA-A*0301/CML (**CML**) tetramers staining performed on PBMCs derived from an HLA-A*0201 CMV seropositive donor and an HLA-A*0301 CML patients treated with Imatinib mesylate respectively is shown.

The frequencies of antigen-specific T cells circulating in the peripheral blood of CML patients and healthy donors was first assessed in the context of HLA-A*0301 and HLA-B*0801 molecules, as the BCR/ABL epitopes associated to these alleles were demonstrated to be naturally processed and presented.

3.3.3 BCR/ABL specific T cells frequencies in the context of HLA-A*0301 and HLA-B*0801 molecules:

The frequencies of HLA-A*0301 and HLA-B*0801 BCR/ABL specific T cells detected in the peripheral blood of our CML patient cohort, as well as in healthy individuals and control CML patients lacking expression of the HLA-A*0301 or HLA-B*0801 alleles was compared and is shown in **Figure 3.9**, panel **A** and **B** respectively. A representative HLA/tetramer staining analysis is also shown in **Figure 3.10** and the details of HLA type, bcr/abl transcript type and treatment received by these patients are described in **Table 3.4**.

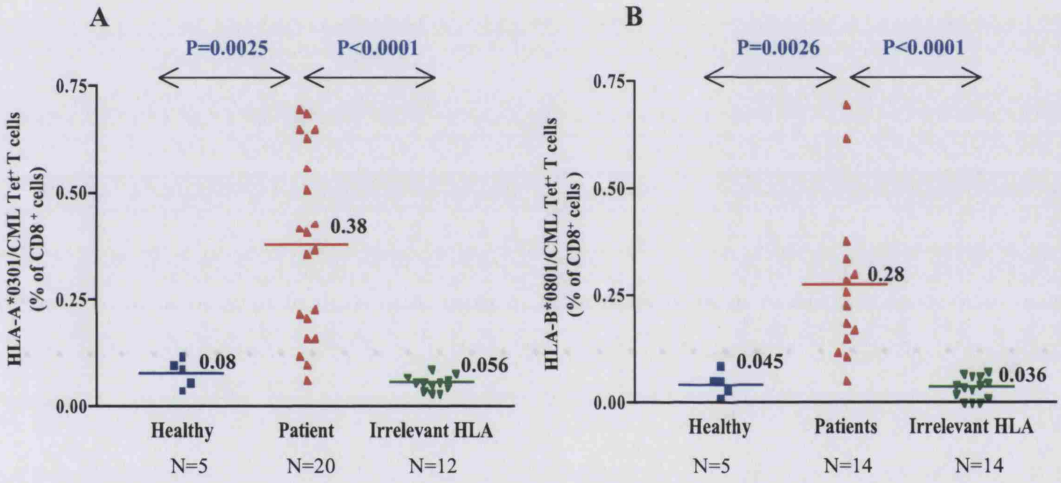


Figure 3.9: Frequencies of *ex-vivo* HLA-A*0301 and HLA-B*0801 BCR/ABL specific T cells:

The frequencies of HLA-A*0301/KQSSKALQR (**A**) and HLA-B*0801/GFKQSSKAL (**B**) specific $CD8^+$ T cells detected in the peripheral blood of CML patients (red), as well as in healthy individuals (blue) and CML patients bearing an irrelevant HLA types (non HLA-A*0301 and/or non HLA-B*0801, green) are represented. Tetramer positive cells are expressed as a percentage of $CD3^+$ $CD8^+$ T cells. Bars indicate the mean values for each category. The frequencies of BCR/ABL specific T cells circulating in the peripheral blood of CML patients are in both the HLA-A*0301 and the HLA-B*0801 group significantly higher than in healthy individuals (P=0.0025 and P=0.0026 respectively, Mann-Whitney U test) or in patients bearing irrelevant HLA types (P<0.0001, Mann-Whitney U test). N represents the number of samples.

The frequency of these antigen specific T cells circulating in CML patients or healthy individuals was expected to be low and as no positive BCR/ABL specific CTL line controls were available, it was difficult to determine the threshold limit of detections for the HLA/BCR-ABL tetramers. The limit of detection of tetramer positive $CD8^+$ T cells using the HLA-A*0201/Flu tetramer was established at 1 positive cell per 8000 (0.0125% of $CD8^+$

T cells) based on the upper 99th percentile of tetramer positive CD8$^+$ T cells in HLA-A2$^-$ individuals (Hoffmann et al. 2000). Recently a comparative study between tetramer staining, Elispot assay and intracellular cytokine flow cytometry has reported the limit of detection for HLA/tetramers to be at 1 positive cell per 10,000 (0.01% of CD8$^+$ T cells) (Whiteside et al. 2003).

Individual	*Disease*	*HLA types*	*Transcript type*	*Treatment*	*Clinical responses*
1	Healthy	A3, 29; B13, 58	NA	NA	NA
2	CML	A2, 25; B27, 55	b3a2	IM	PR
3	CML	A2, 3; B44, 53	b3a2	IM	PR
4	CML	A2, 3; B8, 44	b3a2	AutoHSCT/IM	PR
5	CML	A3, 29; B8, 35	b3a2	AutoHSCT/IM	PR
6	CML	A2, 3; B51, 60	b3a2	AutoHSCT/IM	CR
7	CML	A1, -; B8, 18	b3a2	AlloHSCT	PR
8	CML	A3, -	b3a2	AlloHSCT	PR
9	CML	A1, 3; B8, 14	b2a2	AlloHSCT	CR

Table 3.4: Patients Details:

Summary of the HLA phenotypes and the bcr/abl transcripts types of the CML patients analysed for the presence of BCR/ABL specific CD8$^+$ T cells as shown in **Figure 3.10**. The treatments received as well as the clinical responses at the time of the blood harvest are also shown. NA: not applicable. IM: Imatinib Mesylate. Auto and Allo HSCT: Autologous or Allogeneic Haematopoietic Stem Cell Transplantation. PR: Partial clinical response where patients demonstrate a complete cytogenetic response but bcr/abl transcripts can be detected at a molecular level of 5 to 10%. CR: Complete clinical response including both cytogenetic and molecular responses.

In this study, very low levels of tetramer positive cells were detected in patients bearing irrelevant HLA types with the HLA-A*0301/BCR-ABL tetramer (mean of 0.056%, **Figure 3.9**, panel **A** and **Figure 3.10**, row 2) and with the HLA-B*0801/BCR-ABL tetramer (0.036%, **Figure 3.9**, panel **B** and **Figure 3.10**, row 2, 3 and 6). In addition, few or no BCR/ABL specific T cells were detected in the peripheral blood of CML patient bearing the b2a2 transcript type (**Figure 3.10**, row 9). Thus, we are confident that the binding of T cells to our HLA/BCR-ABL tetramers is specific.

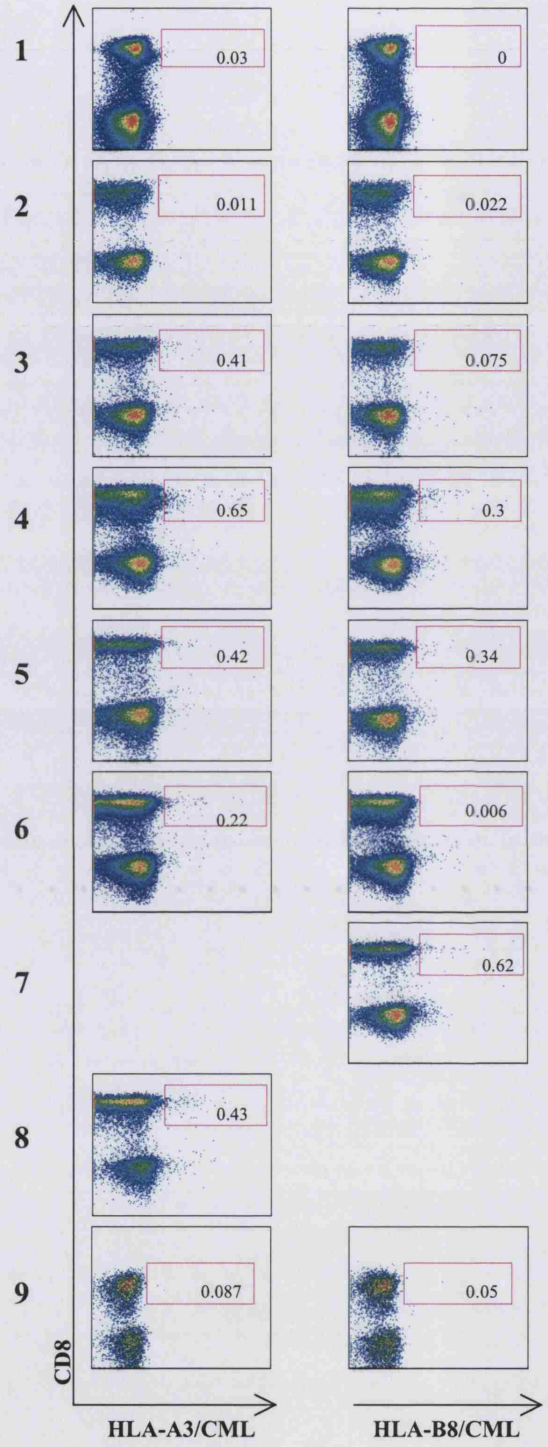


Figure 3.10: Representative staining of BCR/ABL specific T cells detected *ex-vivo*: PBMCs were isolated from CML patients (2-9) and healthy individual (1) and stained with HLA-A*0301/KQSSKALQR tetramers (left panel) and/or HLA-B*0801/GFKQSSKAL tetramers (right panel) and analysed by flow cytometry. Lymphocytes were gated on $CD3^+$ T cells and the CD8/HLA-peptide tetramer dot plots are shown. Tetramer positive cells are represented in the top right gate and expressed as a percentage of $CD8^+$ T cells. The HLA types, bcr/abl transcripts and treatments of the patients are described in Table 3.4. Patients 2, 3 and 6 were also stained with the irrelevant HLA tetramer as controls. Patients 7 and 8 could not be stained with the irrelevant tetramer as there was insufficient number of cells.

The levels of BCR/ABL specific $CD8^+$ T cells detected in CML patients was very heterogeneous in the context of both HLA-A*0301 and HLA-B*0801 molecules (**Figure 3.9** and **Figure 3.10**). Higher frequencies were found in the context of HLA-A*0301 than in HLA-B*0801 positive CML patients (means of 0.38% versus 0.28% respectively, **Figure 3.9**, panel **A** and **B**). Additionally, CML patients bearing both HLA-A*0301 and HLA-B*0801 molecules have circulating BCR/ABL specific T cells recognizing both HLA/peptide tetramers with reproducibly higher frequencies in the context of HLA-A*0301 molecule than HLA-B*0801 molecule (**Figure 3.10**, row 4 and 5). These data demonstrate that BCR/ABL specific T cells can be detected in CML patients using these HLA/tetramers. The frequency of these tumour specific T cells were in the majority of cases low, but this can be expected as these patients failed to mount an effective immune response and subsequently to control their disease.

The majority of the CML patients included in this study were demonstrating at least a cytogenetic remission following auto HSCT, allo HSCT and/or Imatinib mesylate treatment. It was not possible to correlate the frequency of BCR/ABL specific T cells detected in the peripheral blood of patients with their respective treatment and subsequent clinical responses, as the number of patients assessed in each category was too small. However higher frequencies of BCR/ABL specific T cells were detected in patients experiencing at least a complete cytogenetic response following treatment (as shown in **Table 3.4** and **Figure 3.10**) than in patients remaining in active chronic phase of the disease (≤0.21%, **Figure 3.9**). Thus these data tend to suggest that the large tumour burden in CML patients may affect the generation of BCR/ABL specific T cell responses.

Additionally, the frequency of BCR/ABL specific T cells detected in all CML patients was significantly higher than in healthy individuals with both HLA-A*0301 tetramers and HLA-B*0801 tetramers (**Figure 3.9**, panel **A** and **B** respectively, P=0.0025 and P=0.0026, Mann-Whitney U test). This demonstrated that HLA-A*0301 and/or HLA-B*0801 CML patients, who express and present BCR/ABL derived peptides to cells of the immune system, have significantly higher frequencies of circulating BCR/ABL specific T cells than healthy donors.

3.3.4 BCR/ABL specific T cell frequencies in the context of HLA-A*0201 molecules:

The frequencies of tumour antigen-specific T cells were then assessed in the context of HLA-A*0201 molecules. As the associated BCR/ABL derived epitope had not been defined (at the time of the study), HLA-A*0201 molecules were refolded with three predicted BCR/ABL derived peptides (GFKQSSKAL, KALQRPVAS SSKALQRPV) and with the modified BCR/ABL peptide (SLKALQRPV). Tumour associated antigen tetramers were also generated and refolded with WT1 (RFMPNAPYL), PR1 (VLQELVTNV) and hTERT (ILAKFHTWL) associated peptides. These combinations of tetramers enabled us to compare the frequencies of tumour specific and tumour associated antigens-specific T cells. Tetramers for the HLA-A*0201 Influenza virus derived Flu peptide (GILGFVFTL) were also generated and included in the study as a control.

The frequencies of BCR/ABL and tumour associated antigens-specific T cells detected in the peripheral blood of 20 HLA-A*0201 CML patients and 4 HLA-A*0201 healthy donors were assessed and are shown in **Figure 3.11**, panel **A** and **B** respectively. A representative HLA/tetramer staining is also shown in **Figure 3.12**.

The levels of HLA-A*0201 restricted BCR/ABL specific T cells detected in the peripheral blood of CML patients were consistently low (mean of CML1, CML2 and CML3 tetramer positive cells from 0.12% to 0.18%, **Figure 3.11**, panel **A** and **Figure 3.12**). Very little binding of HLA-A*0201 tetramers was observed from the CML patient bearing irrelevant HLA alleles (non HLA-A*0201, from 0% to 0.05%, column 5, **Figure 3.12**), and again is indicative of the validity of our HLA/tetramers specificity.

The lowest frequencies of BCR/ABL specific T cells were detected with the HLA-A*0201 tetramer refolded with the peptide that showed the lowest score for HLA binding, i.e. the KALQRPVAS peptide (mean of 0.12% for CML patients, **Figure 3.11**, panel **A**). This is consistent with the fact that peptides failing to stabilize HLA molecules are unlikely to be presented to the immune system long enough for the generation of antigen-specific T cells. Slightly higher level of tetramer positive T cells were detected with the HLA-A*0201/GFKQSSKAL tetramer (mean of 0.14%) but the highest frequencies of tetramer positive T cells were detected with the HLA-A*0201/SSKALQRPV (mean of 0.18%, **Figure 3.11**, panel **A** and **Figure 3.12**).

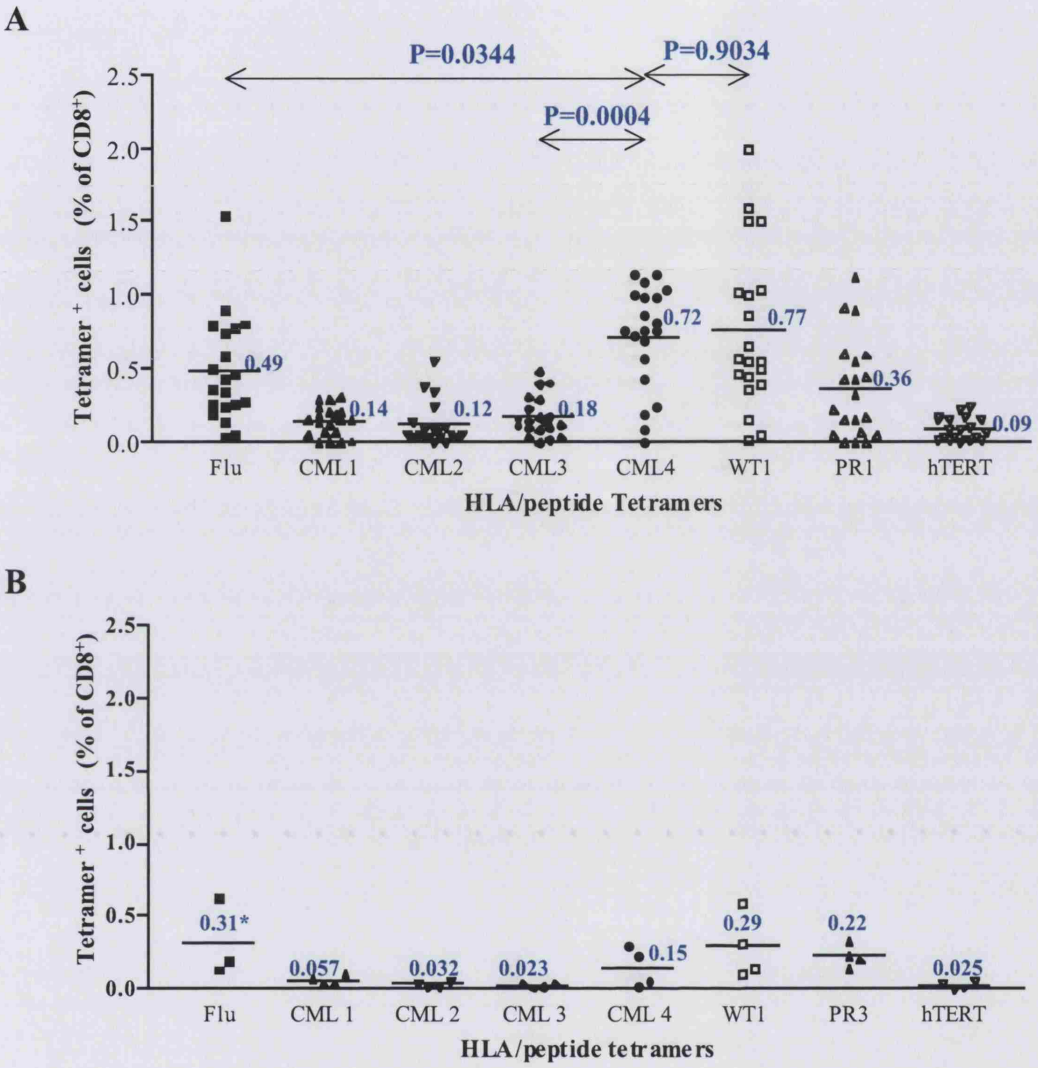


Figure 3.11: Comparative analysis of HLA-A*0201 BCR/ABL and Tumour associated antigen specific T cells frequencies in CML patients and healthy donors detected *ex-vivo*:

The frequencies of HLA-A*0201 associated Flu, CML1 (GFKQSSKAL), CML2 (KALQRPVAS), CML3 (SSKALQRPV), CML4 (modified peptide SLKALQRPV), WT1, PR1 and hTERT specific $CD8^+$ T cells in the peripheral blood of 20 CML patients (panel **A**) and 4 healthy donors (panel **B**) are shown. Tetramer positive cells are expressed as a percentage of $CD3^+$ $CD8^+$ T cells. Bars indicate the mean values for each HLA/tetramers. The frequencies of tetramer specific $CD8^+$ T cells detected in CML patients with the modified BCR/ABL peptide CML4 is significantly higher than the frequencies of the wild type peptide CML3 (P=0.0004, panel **A**), bordering the significant level of Flu specific $CD8^+$ T cells (P=0.0344), but not significantly different from the frequency of circulating WT1 specific $CD8^+$ T cells (P=0.9034). P values are calculated using the Wilcoxon signed ranks test. No statistical analysis could be performed for healthy donors due to the limited number of donors assessed. The frequency of Flu specific T cells was assessed from three healthy donors.

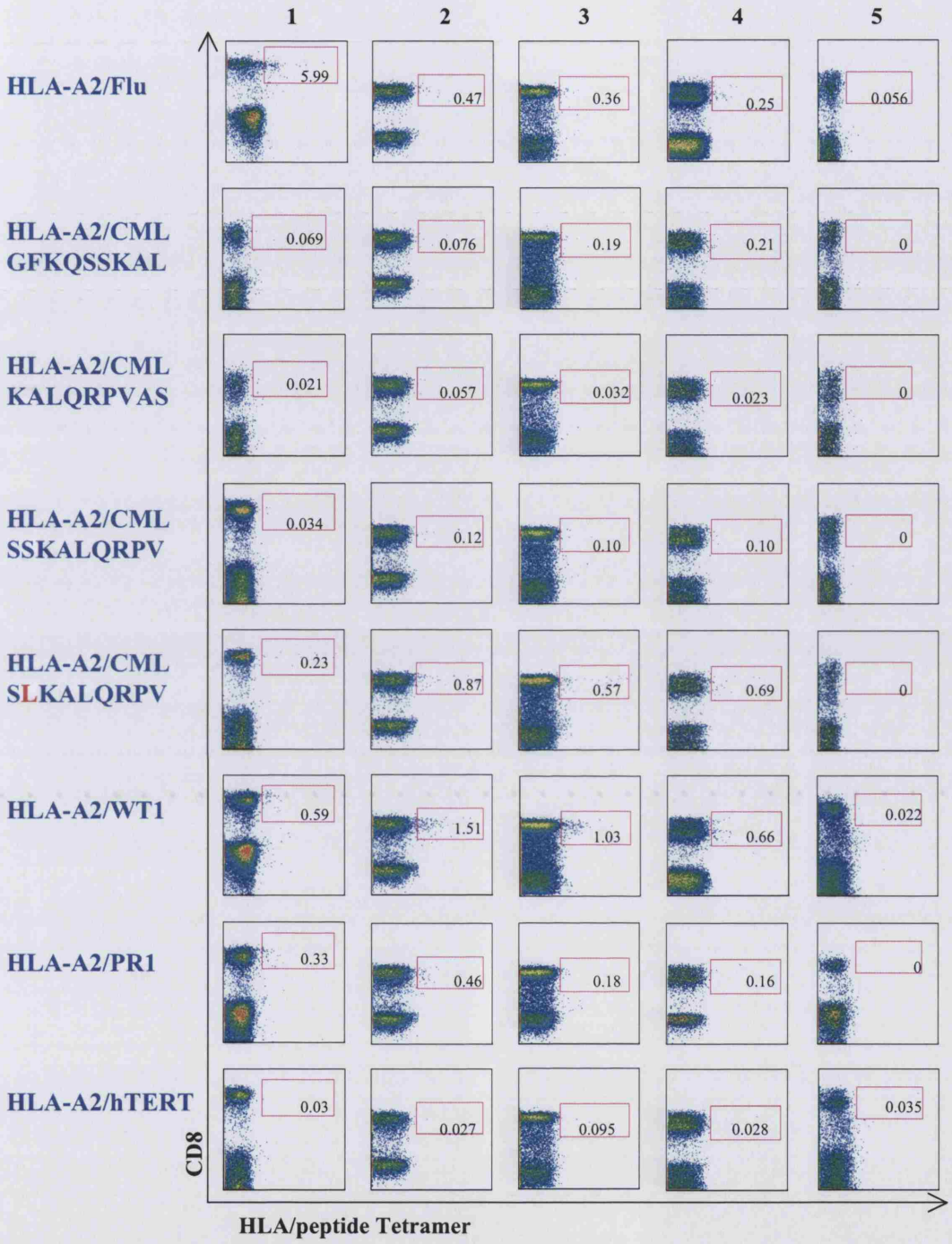


Figure 3.12: Representative staining of HLA-A*0201 BCR/ABL and tumour associated antigen specific T cells detected *ex-vivo*:

PBMCs from HLA-A*0201 healthy individual (1) CML patients (2, 3, 4) and non HLA-A*0201 CML patient (5) were isolated and stained with different HLA-A*0201/peptide tetramers (Flu, BCR/ABL derived peptides, WT1, PR3 and hTERT) for flow cytometry analysis. Lymphocytes were gated on $CD3^+$ T cells and the representative CD8/tetramer dot plots are shown. Tetramer positive cell frequencies are shown in the top right gate and expressed as a percentage of $CD8^+$ T cells. Patient treatments included alloHSCT (2, 5), Imatinib mesylate (3) or autoHSCT and Imatinib mesylate treatment (4). Three patients demonstrated a partial clinical response (2, 4, 5) and the remaining patient had a complete clinical response (both cytogenetic and molecular response, 3).

The frequencies of HLA-A*0201/BCR-ABL specific T cells detected in healthy donors were lower than in CML patients, however the differences were not found to be statistically significant (mean from 0.023% to 0.057% versus mean from 0.12% to 0.18%, P>0.05 Mann-Whitney U test, **Figure 3.11**, panel **A** and **B** respectively).

In addition, the frequencies of BCR/ABL specific T cells detected from the peripheral blood of CML patients were found to be lower in the context of HLA-A*0201 molecules (mean from 0.12% to 0.18%, **Figure 3.11**, panel **A**) than in the context of HLA-A*0301 or HLA-B*0801 molecules (mean of 0.38% and 0.28% respectively, **Figure 3.9** panel **A** and **B**). The differences were however only statistically significant when compared to the HLA-A*0301 molecule (P<0.0064, Wilcoxon ranked test).

The modification of a dominant anchor residue in the SSKALQRPV peptide (Leucine for Serine in position 2, SLKALQRPV) was not shown to significantly increase its HLA binding affinity (shown in **Figure 3.1**). In contrast, the HLA-A*0201/SLKALQRPV tetramers detected significantly higher frequencies of tetramer positive cells in CML patients compared to the levels detected with the unmodified BCR/ABL peptide tetramers (mean of 0.72% versus 0.18% respectively, P=0.0004, Wilcoxon signed ranks test, **Figure 3.11**, panel **A** and **Figure 3.12**). This was also observed in healthy donors (mean of 0.15% versus 0.023%, **Figure 3.11**, panel **B**). However in this case the differences were not found to be statistically significant (P=0.1250, Wilcoxon signed ranks test). In addition, the level of SLKALQRPV specific T cells detected in healthy donors was significantly lower than the level detected in CML patients (mean of 0.149%, **Figure 3.11**, panel **B** and mean of 0.72%, panel **A**, P=0.0182 Mann-Whitney U test).

The frequencies of antigen-specific T cells detected with this modified peptide/HLA tetramer were significantly higher than the frequencies of BCR/ABL specific T cells detected in the context of both HLA-A*0301 and HLA-B*0801 molecules, at least in CML patients (mean of 0.72% versus 0.38% and 0.28%, P=0.004 and P=0.0022 respectively, Wilcoxon signed ranks test, **Figure 3.11**, panel **A** and **Figure 3.9**). Thus despite the lack of strong peptide binding affinity for HLA-A*0201 molecules, this modified peptide significantly increases the detection sensitivity of these HLA/peptide complexes. The functionality of these SLKALQRPV specific T cells remains to be assessed to confirm their BCR/ABL tumour specificity (will be discussed in chapter 5).

In contrast to tumour specific T cells, tumour associated antigen-specific T cells were detected in both CML patients and in healthy donors (**Figure 3.11**, panel **A** and **B**

respectively). These tumour associated antigens can also be expressed in normal cells and therefore could be presented and generate antigen specific precursor T cells in healthy donors.

The lowest frequencies of tumour associated antigen-specific T cells were detected with the HLA-A*0201/hTERT tetramer (mean of 0.09% in CML patients and 0.0.25% in healthy donors, **Figure 3.11** and **Figure 3.12**). Higher levels of PR1 specific $CD8^+$ T cells were found in both CML patients and healthy donors (mean of 0.36% and 0.22%, **Figure 3.11**, panel **A** and **B** respectively). The highest frequencies of tetramer positive T cells were however detected with the HLA-A*0201/WT1 tetramer (mean of 0.77% and 0.29% in CML patients and healthy donors **Figure 3.11**). Because the frequencies of these tumour associated antigen-specific T cells detected in CML patients were very heterogeneous (especially for PR1 and WT1 peptides), these were not found to be statistically different to the frequencies detected in healthy donors ($P>0.05$, Mann-Whitney U test).

Finally the frequencies of tumour associated antigen-specific T cells were compared to the frequencies of BCR/ABL specific T cells detected in this cohort of donors. Higher frequencies of tetramer positive T cells were detected with the tetramers refolded with PR1 and WT1 peptides compared to the frequency of BCR/ABL specific T cells (**Figure 3.11**, panel **A** and **B**). The differences were only found to be statistically significant for CML patients in the context of WT1 and PR1 antigens (P values from 0.0002 to 0.036, Wilcoxon signed ranks test). The level of BCR/ABL specific T cells detected in these 20 CML patients with the modified peptide SLKALQRPV/HLA tetramer was however not significantly different to the level of WT1 specific T cells (mean of 0.72% versus 0.77%, $P=0.9034$ Wilcoxon signed ranks test) and was bordering the level of Flu specific T cells (mean of 0.72% versus 0.49%, $P=0.0344$, Wilcoxon signed ranks test).

Due to the variety of treatments and subsequent clinical responses of the patients included in this study, it was again not possible to assess the correlation between the frequencies of antigen specific T cells detected in the peripheral blood of these patients and their clinical status. The frequencies of HLA-A*0201/BCR-ABL specific T cells detected in patients being homogeneous and negligible, no noticeable differences were found between patients responding to treatment and those remaining in active disease. In contrast, higher frequencies of PR1 and WT1 specific T cells were detectable in patients experiencing at least a complete cytogenetic response. As observed in the context of HLA-A*0301 and HLA-B*0801, the diminution of the tumour burden in treated patients may allow the development of tumour specific T cell responses.

DISCUSSION

The definition of antigenic peptides associated with HLA molecules on the surface of tumour cells remains one of the first requirements for the generation of antigen specific T cell responses and the subsequent development of therapies aiming to increase the frequency of these cells.

The binding affinity of BCR/ABL (b3a2) derived peptides for HLA-class I molecules was assessed using the two publicly available epitope prediction algorithms, Syfpeithi and Bimas, and the three publicly available proteosomal cleavage prediction algorithms, Paproc, Netchop and Fragpredict. We and others have characterised potential BCR/ABL b3a2 derived epitopes predicted to bind with high or intermediate affinity to HLA-A*0301 (ATGFKQSSK, KQSSKALQR), HLA-B*0801 (GFKQSSKAL, KALQRPVAS) and HLA-A*0201 (SSKALQRPV and the modified SLKALQRPV) using the Syfpeithi algorithm (Bocchia et al. 1995; Buzyn et al. 1997; Yotnda et al. 1998). The more recently developed proteosomal cleavage prediction algorithms were expected to increase the sensitivity of the theoretical epitope definition. The PaProc prediction algorithm leads to the exclusion of three potential BCR/ABL specific tumour antigens (ATGFKQSSK, SSKALQRPV and SKALQRPVA). However the PR1 (VLQELNVTV) and the hTERT (ILAKFLHWL) derived epitopes were also excluded, despite the fact that these were demonstrated to be recognized by specific $CD8^+$ T cells (Molldrem et al. 1999; Vonderheide et al. 1999). In addition, the experimental stabilization values obtained in our T2 binding assay were in some cases, in disagreement with the peptide binding scores given by the two epitope prediction algorithms Bimas and Syfpeithi. The prediction algorithms based on experimental data (Bimas and Paproc) were themselves in discord with the other "more" theoretical algorithms (Syfpeithi, Netchop and Fragpredict). Such lack of correlation has also been described by the group of Andersen *et al.* (Andersen et al. 2000). Antigen processing and presentation is a very regulated process that are not yet completely understood and can therefore not be taken into account by the theoretical prediction algorithms (review in chapter 1, (Rock and Goldberg 1999)). The prediction of "false-positives" by the Bimas and/or the Syfpeithi databases, although problematic, is less of a concern than the omission of a relevant antigenic epitope. Thus it becomes critical to confirm the *in-vivo* relevance of any predicted epitopes, even weaker HLA binders.

One method to confirm that antigens are naturally processed and presented is to elute and sequence HLA-bound peptides from the surface of infected or cancerous cells. Using this technique, it has been possible to demonstrate that the HLA-B*0801 associated BCR/ABL derived peptide GFKQSSKAL is presented on the cell surface of HLA transfected K562 cells (although at low level) and on cells from CML patient. No HLA-A*0201 associated BCR/ABL specific peptide(s) have been characterised, although this is still under investigation. Tumour specific antigens, derived from the BCR/ABL (b3a2) fusion protein region were however demonstrated to be presented in the context of both HLA-A*0301 and now HLA-B*0801 molecules.

In a recent publication by the Falkenburg group, the HLA-A*0301 associated BCR/ABL KQSSKALQR peptide was not detected on the surface of CML cells (Posthuma et al. 2004). It is important to mention that in their study, HLA-bound peptides were eluted from HLA-A*0301 transduced and not transfected K562 cells, and that no information regarding the level of HLA molecule expression was described. In addition the mass spectrometry method was slightly different and may have accounted for the discrepant result. Nevertheless, in order to confirm that BCR/ABL peptides are natural processed and presented on HLA-A*0301 CML cells, it would be important to have these results confirmed.

On the assumption that CML patients are presenting BCR/ABL tumour specific peptides at their cell surface, at least in the context of HLA-A*0301 and HLA-B*0801 alleles, it remains to be defined if these tumour antigens can select and induce the expansion of BCR/ABL specific T cells. Only a few groups have assessed the frequencies of de-novo circulating BCR/ABL specific T cells, without prior antigen priming and/or expansion. Berke et al. failed to detect INFγ production by BCR/ABL (b2a2) or ABL/BCR specific T cells in HLA-A*0201, -A*1101 and –A*0101 CML patients (Berke et al. 2000). Rezvani et al. quantified the frequencies of *ex-vivo* BCR/ABL specific T cells by measuring INFγ production of peptide-pulsed PBMCs using a highly sensitive quantitative PCR technique (Rezvani et al. 2003). They were only able to detect BCR/ABL specific T cells in the peripheral blood of CML patients (HLA-A*0201 patients were tested only for the GFKQSSKAL peptide) and they found higher frequencies after, rather than prior to, HSCT. In our study we were only able to detect a statistically significant BCR/ABL specific proliferative response in an HLA-A*0301 CML patient assessed at diagnosis (without prior treatment). The proliferation and Elispot assays used are too insensitive to detect such low antigen specific T cell frequencies and the quantitative PCR assay performed by Rezvani et al. would certainly be a more appropriate system to do so.

HLA/peptide tetramers have become an invaluable tool for the detection of frequencies as low as 10^{-4} to 10^{-5} antigen specific T cells and have allowed us to monitor the low frequency of BCR/ABL specific T cells. The BCR/ABL tetramers used in our study have been shown to be both HLA and peptide specific, as no BCR/ABL specific T cells were detected in donors with irrelevant HLA phenotype or in patients bearing the alternate BCR/ABL translocation such as $b2a2^{+}$ CML patients.

The frequencies of BCR/ABL specific T cells detected in the peripheral blood of HLA-A*0301 and HLA-B*0801 CML patients were low and very heterogeneous. Higher frequencies of tetramer positive T cells were detected with the HLA-A*0301/BCR-ABL tetramer than with the HLA-B*0801/BCR-ABL tetramer. This can result from a number of factors, including differences in the peptides processing by the proteosome and in the peptides binding affinity for HLA class I molecules.

In relation to HLA-A*0201, BCR/ABL derived peptides were predicted and demonstrated to be very low HLA binders, with some peptide (KALQRPVAS) only being capable of stabilizing HLA-A*0201 molecule for less than 2 hours. The generation of refolded HLA tetramers with these peptides was difficult and these tetramers were only stable for up to 3 months. It is still uncertain if these peptides are actually presented, or presented for sufficiently long enough, on the surface of BCR/ABL positive cells to prime and/or expand specific BCR/ABL T cell responses. None of the predicted BCR/ABL peptides induced *ex-vivo* proliferative responses and although BCR/ABL peptide specific T cells were detected in CML patients, their frequencies were lower than the frequencies detected in HLA-A*0301 patients. The highest percentage of tetramer positive T cells in HLA-A*0201 CML patients were detected with the HLA/CML3 tetramer (SSKALQRPV peptide). This low affinity BCR/ABL derived peptide has been shown to be capable of generating specific CTL responses *in-vitro* (Yotnda et al. 1998).

The levels of BCR/ABL specific T cells detected in CML patients were consistently low and in some patients absent. Tumour specific T cells are not known to circulate at high frequency in the peripheral blood of patients. These patients failed to mount specific and efficient T cell responses and subsequently control their tumour growth. The low avidity of BCR/ABL peptides deduced from the binding affinities obtained in the context of HLA-A*0201 molecules and the large tumour burden may have accounted for the lack of development of specific immune responses in these patients. On the other hand, the antigen expression demonstrated by cell surface presentation of BCR/ABL derived epitopes in CML patients, possibly accounted for the higher frequencies of BCR/ABL specific T cells

detected in the peripheral blood of patients than of healthy individuals, as the latter do not present the antigen to the immune cells.

A number of HLA-A*0201 associated epitopes derived from tumour associated antigens (TAA) have been described and for comparison purposes, were included in the study. We found heterogeneous levels of TAA specific T cells in the peripheral blood of CML patients. Very low level of hTERT specific T cells were detected in healthy individuals and CML patients and no proliferative responses to this antigen were observed. Our results are in accordance with the group of Vonderheide, who did not detect hTERT specific T cells in the peripheral blood or in Tumour Infiltrating Lymphocytes (TIL) samples of cancer patients above a frequency of 0.03% (Vonderheide et al. 1999). They also assessed INFγ responses and failed, without stimulating antigen-specific T cells with hTERT peptide, to detect any INFγ producing cells. Variable levels of PR1 specific T cells were detected in our cohort of CML patients. Molldrem *et al.* characterised high frequencies of PR1 specific T cells in patients in cytogenetic remission following INFα or allogeneic HSCT, but not in patients relapsing, untreated or receiving chemotherapy (Molldrem et al. 2000). In our cohort of patients, higher frequencies of BCR/ABL and TAA specific T cells also tended to be detected from patients experiencing at least a complete cytogenetic response rather than in patients remaining in active chronic phase of the disease.

The highest levels of TAA specific T cells in CML patients were detected with the WT1 tetramer. WT1 peptide has also been shown to induce the production of INFγ by antigen specific T cells *ex-vivo* in CML patients, pre or post HSCT and also in AML patients (Rezvani et al. 2003). The major concern with these tumour associated antigens is that they are derived from endogenous self proteins. It is therefore not surprising to detect TAA specific T cells in the peripheral blood of healthy individuals. We and others have detected a frequency of 0.03% up to 0.59% of TAA specific T cells from the peripheral blood of healthy individuals (Molldrem et al. 2003; Rezvani et al. 2003; Vonderheide et al. 1999). Thus one significant advantage in the development of immunotherapy using tumour specific antigens is that these are only expressed by tumour cells. Tumour specific antigens are not always the best candidates for the development of T-cell based immunotherapy. They usually demonstrate a low peptide binding affinity to HLA class I molecules, as we have shown for the HLA-A*0201 molecule.

The substitution of a single amino acid in the antigenic peptide sequence to increase the HLA class I/peptide affinity and thereafter the TCR avidity was however demonstrated to

significantly enhance the avidity of the specific T cell responses and to maintain the wild type peptide-restricted cytotoxic activity (Slansky et al. 2000; Tangri et al. 2001). The modification of the SSKALQRPV peptide to contain an anchor residue at position 2, SLKALQRPV, failed to increase the peptide binding affinity. However, HLA-A*0201 tetramers refolded with SLKALQRPV significantly increased the level of antigen specific T cells detected in the peripheral blood of CML patients compared to the level detected with the putative peptide. In addition, the frequencies of tetramer positive cells detected in CML patients with the modified peptide were significantly higher than the levels of hTERT and PR1 specific T cells and similar to the levels of WT1 specific T cells. The specificity and the functionality of these cells were further assessed and will be further discussed in chapter 5.

Overall the level of BCR/ABL specific T cells circulating in the peripheral blood of CML patients was low and in some cases fewer than a hundred $CD8^+$ $tetramer^+$ events were collected. The successful expansion of these antigen specific T cells will not only prove the authenticity of their antigen specificity, but also demonstrate the basis for the development of T-cell based specific immunotherapy for CML patients.

CHAPTER 4

In-vitro stimulation and expansion of BCR/ABL specific $CD8^+$ T cells

INTRODUCTION

In the previous chapter, BCR/ABL derived peptides were shown to be presented on the surface of $b3a2^+$ CML cells, at least in the context of HLA-A*0301 and HLA-B*0801 molecules. Additionally, BCR/ABL specific $CD8^+$ T cells were detected in the peripheral blood of CML patients using HLA class I tetramers. The frequency of these BCR/ABL specific T cells was low and may reflect the inability of these patients to mount a specific immune response and control their disease. In order to confirm the authenticity and specificity of these tetramer positive T cells, different *in-vitro* stimulation protocols were assessed for their feasibility to expand or generate functional BCR/ABL specific T cells from CML patients and healthy donors.

A number of groups have examined the efficiency of generating BCR/ABL specific T cells in the context of both HLA class I and class II molecules and a summary of their results is shown in **Table 4.1**. In these studies, the BCR/ABL specific responses were obtained from a limited number of donors who were mainly healthy individuals. The BCR/ABL specific T cell responses were generated *in-vitro* after at least three rounds of stimulation with peptide-pulsed autologous PBMCs, PHA lymphoblasts or DCs. Although PHA expanded T cells are not natural APCs, they have been shown to have the capacity to present antigens and stimulate specific T cells responses. PHA treatment of T cells has the advantage of providing an unlimited source of APCs, however these cells may not be optimal APCs as they may lack the expression of crucial costimulatory molecules. Conversely, DCs are known to be potent APCs, with the capacity to both prime naïve and boost memory T cell responses. Their use to generate BCR/ABL specific T cells has also been widely described **(Table 4.1)**.

Donor type	*APCs*	*BCR/ABL Antigen*	*Assessment of functionality*	*HLA-restriction*	*Reference*
11 healthy donors	DC	9-mer and 25-mer peptide	Cytotoxic activity against peptide-pulsed CML cell lines or PBMCs. HLA-class II clones were assessed by proliferative responses	HLA-A*0301 HLA-DRB1*1101	(Bocchia et al., 1996)
5 Healthy donors	PBMCs	23-mer peptide	Proliferative and cytokine responses to peptide-pulsed PBMCs	HLA-DRB1*1101 HLA-DRB1*0301/0402	(Pawelec et al., 1996)
1 healthy donor	DC	16-mer peptide	Cytotoxic activity against CML cells, but not HLA-restricted	HLA-A*2401 and/or HLA-B*52/-B*59	(Nieda et al., 1998)
1 healthy donor	DC	12-mer peptide	Cytotoxic activity against HLA-matched CML cells	HLA-A*0201/-A*24, HLA-B*48/-B*70 and/or HLA-DR4/DRB1*0403/05	(Osman et al., 1999)
7 healthy donors	DC	17-mer peptide	Proliferative response to peptide-pulsed autologous PBMCs	HLA-DR*0401	(Bertazzoli et al., 2000)
1 CML patient	DC	Endogenous presentation or CML cell lysate	Proliferative and cytokine responses to autologous CML cells	HLA-DRB1	(Muller et al., 2001)
1 CML patient	DC	Endogenous presentation	Growth inhibition and cytotoxic activity against HLA-matched CML cells	HLA-class I restricted	(Choudhury et al., 1997)
5 Healthy donors	PHA blast	9-mer peptide	Cytotoxic activity against HLA-matched CML cells and/or peptide-pulsed PHA blasts	HLA-A*0301 HLA-B*0801	(Norbury et al., 2000)
2 CML patients	PHA blast	9-mer peptide	Cytotoxic activity against peptide-pulsed PHA blasts and CML cells	HLA-A*0301	(Clark et al., 2001)
1 healthy donor	PHA blast	10-mer peptide	Proliferative response to peptide-pulsed (or cell lysate) EBV targets	HLA-DRB*0101-02	(Mannering et al., 1997)
1 healthy donor	PBMCs	17-mer peptide	Proliferative response to HLA-matched CML cells	HLA-DRB1*1501 HLA-DRB1*0401/0101	(Bosch et al., 1996; ten Bosch et al., 1995)
2 healthy donors	PHA blast	17-mer peptide	Cytotoxic activity against peptide-pulsed EBV targets and HLA-matched CML blasts	HLA-DRB1*0901	(Yasukawa et al., 1998; Yasukawa et al., 2001)
9 Healthy donors and 7 CML patients	DC and/or PHA blast	9-mer peptide	Cytotoxic activity against HLA-matched CML cells and/or peptide-pulsed EBV targets	HLA-A*0201/-A*0301 HLA-A*1101/-B*0801	(Yotnda et al., 1998)

Table 4.1: BCR/ABL specific T cell responses described in the literature:
This table summarized the BCR/ABL specific T cell responses successfully generated from CML patients and/or healthy donors, which are documented in the literature. APC: Antigen presenting cells; DC: dendritic cells, PHA: Phytohaemagglutinin treated T cells; PBMCs: Peripheral Blood Mononuclear Cells.

Leukaemic DCs derived from CML patients have been demonstrated to carry the Philadelphia chromosome, which has been associated with their defective adherence properties, capacity to migrate, capture and process exogenous antigen, as well as producing cytokines (Dong et al. 2003; Eisendle et al. 2003; Gordon et al. 1987; Smit et al. 1997; Verfaillie et al. 1992). Thus their use as APCs may be questionable. However upon *in-vitro* maturation, no differences in the levels of costimulatory marker expression was seen between CML patients and healthy donors (Dong et al. 2003).

One advantage of using leukaemic DCs as a source of APCs is that they can process and present endogenous tumour antigens to T cells. Some groups have reported the efficient generation of antigen specific T cells from CML patients using leukaemic DCs (Choudhury et al. 1997; Muller et al. 2001; Nieda et al. 1998). The specificity of these responses is however not clear. The Choudhury and the Muller groups described anti-leukaemic responses relying on the endogenous antigen presentation by the leukaemic DCs or pulsing the DCs with CML cell lysates (Choudhury et al. 1997; Muller et al. 2001). The nature of the antigen recognized by their CTL lines was not defined and may not be BCR/ABL specific. The HLA-A*2401 restricted BCR/ABL specific $CD8^+$ T cell clone generated from a CML patient by Nieda et al. was not demonstrated to be HLA restricted in its cytotoxic activity against CML cells (Nieda et al. 1998). They postulated that their highly specific clone did not require HLA recognition, which raises the issue of their T cell clones specificity.

The BCR/ABL specific T cells described in these studies were further assessed by proliferative responses to peptide-pulsed autologous PBMCs or HLA-matched CML cells (**Table 4.1**). Other groups have defined their successful BCR/ABL specific responses by assessing growth inhibition or cytokines production upon stimulation with HLA-matched CML cells. The functionality of these antigen specific T cells was only demonstrated in some cases by assessing their cytotoxic activities against peptide-pulsed autologous and allogeneic PBMCs, PHA blasts or EBV-transformed cells. The clinical relevance of these specific anti-leukaemic T cells surely requires the demonstration of their specific cytotoxic activity against HLA-matched CML cells and this was only assessed by some groups (Choudhury et al. 1997; Clark et al. 2001; Norbury et al. 2000; Osman et al. 1999; Yotnda et al. 1998).

Thus, it can be argued that functional BCR/ABL specific T cell response can be successfully generated from any CML patients or even healthy donors.

In addition to PHA blasts or monocytes-derived DCs (mDCs), plasmacytoid DCs (pDCs) represent an alternative source of APCs. In contrast to mDCs, pDCs derive from lymphoid progenitors, lack myeloid lineage markers and are characterised by the expression of blood DC antigen markers BDCA-2, BDCA-4, CD123 (IL-3 receptor α chain) and HLA-DR (Briere et al. 2002; Dzionek et al. 2000; Grouard et al. 1997). The infiltration of pDCs into tumour lesions has been associated with a worse clinical outcome and this has been related to their suggested function in immune tolerance induction (Salio et al. 2003; Vakkila et al. 2004; Zou et al. 2001). However the quantitative and functional impairment of mDCs and/or pDCs in patients with myeloid malignancies has been hypothesized to contribute to the leukaemic clones escape from immune surveillance (Clark et al. 2003; Mohty et al. 2001). The lymphoid origin of pDCs suggests that they may not be derived from Philadelphia positive myeloid progenitors in AML and CML, yet their quantitative and functional relevance in CML remains to be investigated.

The aims of this chapter are to evaluate the feasibility of generating and/or expanding BCR/ABL specific $CD8^+$ T cells, in the context of HLA-A*0301 and HLA-B*0801 molecules. Different *in-vitro* stimulation protocols, including the use of autologous PHA lymphoblasts, monocytes derived DCs and plasmacytoid DCs as APCs, were assessed for their ability and reproducibility in generating functional BCR/ABL responses from both CML patients and healthy donors. Antigen specific T cell responses were monitored by HLA/tetramer staining and their frequencies were correlated with their specific cytotoxic activity against HLA-matched CML targets.

RESULTS

4.1 Characteristics of CML patients and healthy donors:

CML patient blood samples were obtained from six different hospital centres. Patients were selected for the expression of HLA-A*0201, HLA-A*0301 and/or HLA-B*0801 alleles and for their b3a2 bcr/abl transcript type. In the majority of cases, this information was provided by the CML patients originating hospital centre. When this information was not available, the HLA typing was performed at the histocompatibility laboratories of the Anthony Nolan Trust and the bcr/abl typing was performed following the protocol kindly provided by Dr Letizia Foroni (Haematology Department, Royal Free Hospital, London) and Dr Kaeda Jaspal (Haematology Department, Hammersmith Hospital, London). As the detection of bcr/abl DNA requires the presence of the Philadelphia chromosome, such typing was only feasible when samples from CML patients prior to molecular remission were available.

mRNA was extracted from PBMCs derived from nine CML patients and reverse transcribed into cDNA for bcr/abl PCR typing as described in chapter 2. A gel example of such typing is shown in **Figure 4.1**, panel **A**. The bcr/abl gene amplification products assessed from CML patients 29, 41, 43 and 48 are shown in lane 9, 8, 6 and 7 respectively. Distinct bands are visible for patient 41 and patient 43, corresponding to and confirming their b3a2 and b2a2 bcr/abl transcript types respectively (**Figure 4.1**, panel **A** lane 8 and 6 respectively). No bcr/abl bands were observed in lane 7 and 9. For one of these patients (CML patient 48, lane 7) no β-actin amplification product was detected demonstrating that the generation of either mRNA or cDNA from this patient failed. For the other patient (CML patient 29, lane 9), a successful amplification of the housekeeping β-actin gene was obtained (**Figure 4.1**, panel **B**), however no bcr/abl product was detected (**Figure 4.1**, panel **A**). This patient has been treated with Imatinib Mesylate and may have reached a molecular remission, which resulted in the reversion to a Philadelphia chromosome negative profile. As no bcr/abl type was definable, these patients were excluded from the study (CML patients 29 and 48).

The characteristics of the CML patients included in this study are detailed in **Table 4.2**. Four $b2a2^+$ bcr/abl CML patients were also included and used as negative controls in cytotoxic assays. The generation of BCR/ABL specific T cells was also assessed from healthy donors. These donors were selected from laboratory staff for their HLA types.

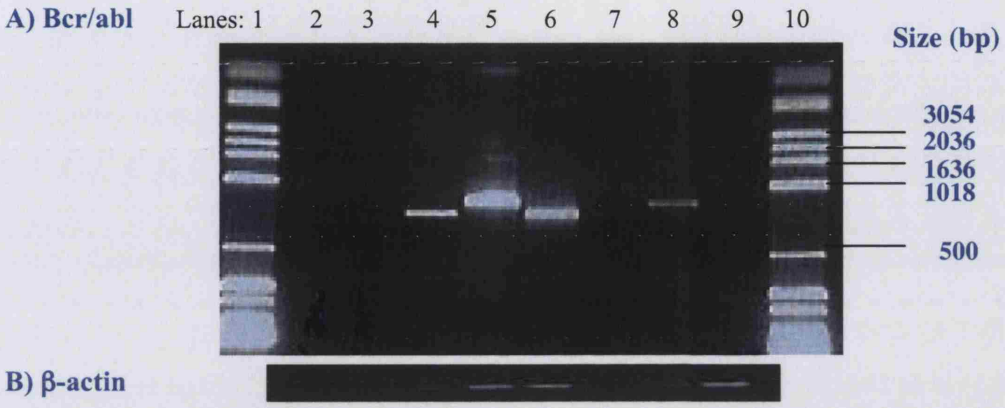


Figure 4.1: Bcr/abl PCR typing:

PCR typing for bcr/abl (**A**) was performed from the cDNA generated from CML patients as described in chapter 2. A 1kb DNA ladder was loaded in lane 1 and lane 10. A PCR negative control (no primers, lane 2) and cDNA negative control (no cDNA, lane 3) were also included. The cDNA was also generated from the KCL22 (b2a2 positive line, lane 4) and K562 (b3a2 positive line, lane 5) and the bcr/abl gene was amplified in parallel. This allowed us to characterize the position of the single b2a2 (595 bp) and the b3a2 (670 bp) bcr/abl product on the DNA gel. The bcr/abl PCR typing for the CML patients 29, 41, 43 and 48 are shown in lanes 9, 8, 6 and 7 respectively. To assess the quality of the generated cDNA, PCR for the house-keeping β-actin gene was performed in parallel and is shown in **B**.

A

Patients	*Centres**	*HLA types*$^{+}$	*Transcript type***	*Treatment****	*Clinical responses*****	*Study******
1	LRI	A2, **3**; B44, 53	b3a2	IM	PR	DC
2	LRI	**A3, -**; B13, -	b3a2	AutoHSCT	-	PHA
3	LRI	**A3**, 11; B35, 50	b3a2	IM	-	PHA
4	LRI	A2, **3**;B51, 60	b3a2	AutoHSCT/IM	PR	DC
5	UCH	**A3**	b3a2	AlloHSCT	CR	DC
6	UCH	**A3**	b3a2	AlloHSCT	CR	DC
7	LRI	**A3**, 11;B35, 44	b3a2	AlloHSCT	CR	PHA
8	LRI	**A3**, 68;B35, 41	b3a2	IM	PR	PHA / PDC
9	LRI	**A3, -**; B62, 65	b3a2	AlloHSCT/DLI/IM	CR	PHA / PDC
10	LRI	**A3**, 29; B7, 44	b3a2	AlloHSCT/IM	No Response	PHA
11	LRI	**A3, -**; B57, 35	b3a2	IM	-	PHA
12	LRI	**A3**	b3a2	IM	PR	PHA / PDC
13	LRI	A2, **3**; **B8**, 44	b3a2	AutoHSCT/IM	PR	PHA / DC
14	LRI	**A3**, 29; **B8**, 35	b3a2	Auto HSCT/IM	PR	DC
15	LRI	A1, **3**; **B8, -**	b3a2	Auto HSCT/IM	CR	PHA / PDC
16	LRI	A1, 2; **B8**, 35	b3a2	IM	PR	DC
17	LRI	A1, 2; **B8**, 35	b3a2	AlloHSCT/DLI	CR	DC
18	LRI	A1, 31; **B8**, 60	b3a2	AutoHSCT/IM	PR	PHA
19	LRI	A1, -; **B8**, 18	b3a2	IM	PR	PHA
20	UCH	A1, -; **B8**, 37	b3a2	AlloHSCT	PR	PHA
22	HH	A1, **3**; B7, 17	b3a2	At diagnosis	CP	DC / PDC / Target
23	KCH	**A3**, 26; B7, 51	b3a2	At diagnosis	CP	PDC / Target
24	HH	**A3**, 33; B14, 15	b2a2	At diagnosis	CP	PDC / Target
25	HH	A1, **3**; **B8**, 27	b3a2	At diagnosis	CP	PDC / Target
26	HH	B8	b2a2	At diagnosis	CP	Target

B

Patients	*Centres**	*HLA types*$^{+}$	*Transcript type***	*Treatment****	*Clinical responses*****	*Study******
27	LRI	**A2**, 30; B39, 57	b3a2	IM	No Response	PDC / TAA DC
28	LRI	A1, **2**; B60, -	b3a2	AutoHSCT	PR	TAA DC
29	LRI	**A2**, 24; B7, 60	-	IM	No Response	PDC
30	LRI	A1, **2**; B35, 44	b3a2	AutoHSCT/IM	PR	BCR-ABL DC
31	LRI	**A2**, 25; B27, 55	b3a2	AlloHSCT	PR	BCR-ABL DC / TAA DC
32	LRI	**A2**, -; B18, 60	b3a2	AlloHSCT	PR	BCR-ABL DC / TAA DC
33	LRI	**A2**, 23; B18, 60	b3a2	AlloHSCT	PR	BCR-ABL DC /TAA DC
34	LRI	**A2**, -; B27, 44	b3a2	IM	PR	BCR-ABL DC/ PDC/TAA DC
35	LRI	**A2**, 24; B7, 62	b3a2	AlloHSCT/IM	CR	BCR-ABL DC/ PDC/TAA DC
36	RFH	**A2**, 11; B35, 52	b3a2	AlloHSCT	-	BCR-ABL DC / TAA DC
37	RFH	**A2**, 26; B51, 7	b3a2	AlloHSCT	-	BCR-ABL DC/ TAA DC
38	BARTS	**A2**, 31; B51, 44	MDS, b3a2	At diagnosis	CP	PDC/Targets
39	HH	**A2**, 24; B7, 62	b3a2	At diagnosis	CP	PDC/Targets
40	HH	**A2**, 25; B51, 55	b3a2	At diagnosis	CP	PDC/ Targets
41	HH	A24, 29; B7, 44	b3a2	At diagnosis	CP	Targets
42	HH	**A2**, -; B40, 47	b2a2	At diagnosis	CP	PDC / Targets
43	HH	**A2**, 24; B7, 62	b2a2	At diagnosis	CP	Targets

Table 4.2: CML patients' characterisitics:

CML patients were selected for their HLA types, including 26 HLA-A*0301 and/or HLA-B*0801 patients (Table **A**) and 16 HLA-A*0201 patients (Table **B**, described in chapter 5) and for their b3a2 bcr/abl transcript types. For control purposes, one irrelevant HLA bearing CML patient (patient 41) and b2a2^{+} CML patients (patients 24, 26, 42 and 43) were also included. The centre of blood harvest, HLA class I types, bcr/abl transcript types, treatments received and clinical responses at the time of blood harvest, and study in which each of these patients were included are detailed. * Centres included LRI: Liverpool Royal Infirmary, Liverpool; UCH: University College Hospital, London; HH: Hammersmith hospital, London; KCH: Kings' college hospital, London; RFH: Royal Free Hospital, London; BART: Barts' hospital, London. $^{+}$ The HLA types were obtained from the hospital centres or from the Histocompatibility laboratories, Anthony Nolan Research Institute, London. ** Transcript type: Most of the bcr/abl transcript types were obtained from the hospital centres. Some samples were screened in house as described in chapter 2. The bcr/abl type for patient 29 was not available. MDS: Myelodysplasia syndrome. *** Patients' treatments included: IM: Imatinib Mesylate drug, AutoHSCT or AlloHSCT: autologous or allogeneic Haematopoietic Stem Cell Transplantation; DLI: Donor Lymphocytes Infusion. Some samples were obtained from leucopheresis harvested at diagnosis. **** Clinical responses: PR: Partial clinical response where patients demonstrated a complete cytogenetic response but bcr/abl transcripts were still detected at the molecular level. CR: Complete clinical response, including cytogenetic and molecular remission. CP: Chronic Phase. -: The clinical information was not obtainable from the patient originating hospital centre. ***** Study: lymphocytes were purified and used in the following studies: **PHA**: Use of PHA lymphoblastoid cells as antigen presenting cells (APC); **DC**: Use of Dendritic cells as APCs; **PDC**: Plasmacytoid Dendritic cells enumeration and use as APCs; **BCR-ABL DC**: HLA-A*0201 potential BCR/ABL peptides pulsed Dendritic cells as APCs; **TAA DC**: Comparison of wild type and modified HLA-A*0201 BCR/ABL specific peptides with Tumour associated peptides using Dendritic cells as APCs; **Targets**: HLA matched or unmatched CML cells used as targets in cytotoxic assays.

4.2 Generation of BCR/ABL specific T cells using PHA lymphoblasts as APCs:

The generation of BCR/ABL specific T cells was first assessed using PHA treated T cells as APCs. These lymphoblasts were pulsed with the HLA-restricted BCR/ABL peptide (KQSSKALQR in the context of HLA-A*0301 or GFKQSSKAL in the context of HLA-B*0801) and incubated with autologous responder cells (PBMCs) at a ratio of 1 to 5 respectively as described in chapter 2. Lymphocytes were re-stimulated every seven days for up to five weeks and the proliferation of antigen specific T cells was supported by the addition of IL-2 cytokine three days following each antigen stimulation. The frequency of BCR/ABL specific $CD8^+$ T cells expanded in these cultures was monitored by HLA-BCR/ABL tetramer staining three or four days after each stimulation. Thirteen CML patients (described in **Table 4.2**, PHA study*****) and six healthy donors were assessed with this protocol. The highest frequencies of HLA-A*0301 and HLA-B*0801 BCR/ABL specific T cells for all the donors assessed were detected after the fourth antigen stimulation. A representative tetramer staining performed three days after the fourth stimulation (day 31) is shown in **Figure 4.2**. The frequencies of BCR/ABL specific T cells generated from HLA-A*0301 (**Figure 4.2**, top panel) and HLA-B*0801 (**Figure 4.2**, middle panel) CML patients and healthy donors are represented in the upper right gate of each dot plot. Lymphocytes were also incubated with peptide un-pulsed autologous PHA lymphoblasts (**Figure 4.2**, No peptide).

In order to assess the efficiency of these PHA lymphoblasts as APCs and the capacity of CML patients T cells to respond to antigenic stimulation *in-vitro*, HLA-A*0201 restricted CMV responses were stimulated in parallel following the same protocol. This was only possible when the CML patients were also HLA-A*0201 positive and were CMV seropositive. As an example, CML patient 13 was selected (HLA-A*0201, CMV seropositive) and stimulated with both CMV and BCR/ABL antigens in parallel. The representative HLA/tetramer dot plots obtained after four stimulations with autologous PHA lymphoblasts un-pulsed or pulsed with the CMV derived peptide are shown in **Figure 4.2**, bottom panel. The frequency of HLA-A*0201 CMV specific T cells detected in the culture after four weeks was about 0.36% (**Figure 4.2**, bottom panel, No peptide). CMV peptide-pulsed PHA lymphoblasts induced a 5-fold increase of tetramer positive cells after four stimulations (1.95%, **Figure 4.2**, bottom panel, Peptide). Thus, T cells derived from CML patients are capable of responding and generating an antigen-specific immune response, at least in the context of a viral antigen stimulus (CMV).

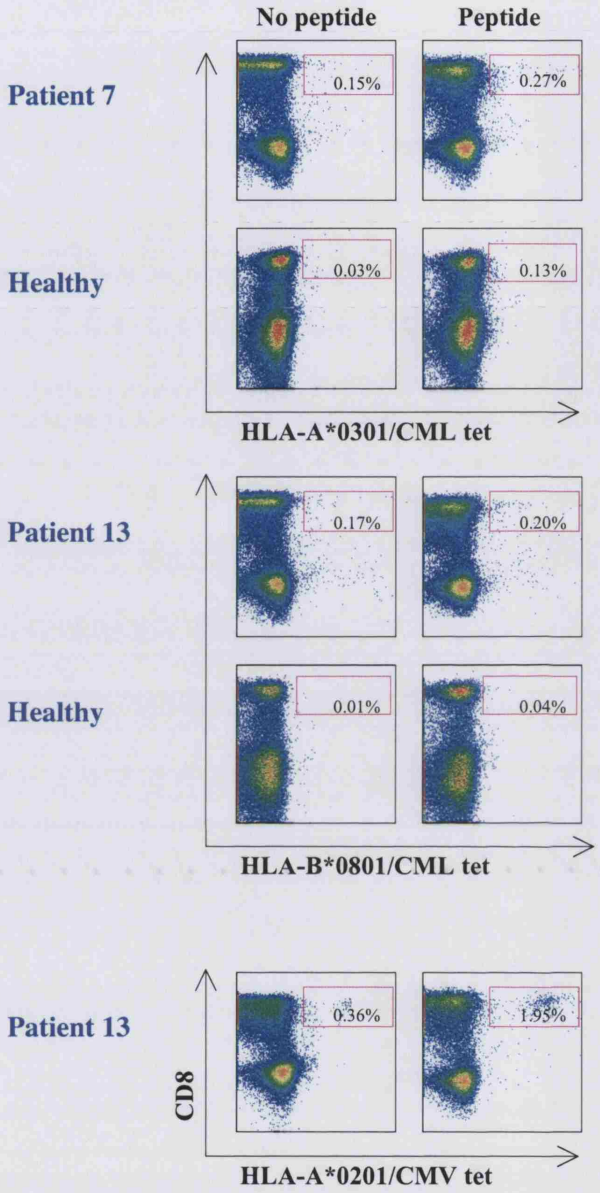


Figure 4.2: Generation of BCR/ABL specific T cells with autologous PHA lymphoblasts:

BCR/ABL specific T cells were generated from CML patients and healthy donors using autologous PHA lymphoblasts as APCs, in the context of HLA-A*0301 (top panel) and HLA-B*0801 (middle panel). PHA lymphoblasts were also pulsed with the HLA-A*0201 CMV associated peptide to assess the capacity of CML patients to respond to a viral antigen (lower panel). Antigen specific T cells were stimulated every seven days for four weeks as described in chapter 2. The frequencies of antigen specific $CD8^+$ T cells were monitored by HLA/tetramer staining three days after the fourth stimulation (day 31) without (**No peptide**) or with the antigen (**Peptide**). Antigen specific T cells are expressed as a percentage of $CD3^+$ $CD8^+$ T cells and are shown in each dot plots gates.

The frequencies of BCR/ABL specific T cells detected in cultures without peptide stimulation were reproducibly higher from CML patients than from healthy donors in the context of both HLA-A*0301 and HLA-B*0801 molecules (0.15% and 0.17% versus 0.03% and 0.01% respectively, **Figure 4.2**, No peptide). In this case however, it is unlikely due to the endogenous presentation of BCR/ABL antigen, as the APCs used in this protocol were T cells and it has been demonstrated that CML patient peripheral T lymphocytes, especially mature T cells do not carry the Ph chromosome nor the bcr/abl mRNA transcript (Ariad et al. 1993; Cho et al. 2000; Takahashi et al. 1998). In addition, the majority of patients assessed with this protocol were at least in complete cytogenetic remission, with only low levels of bcr/abl transcripts detectable at a molecular level (8 out of 13 patients, **Table 4.2**).

After four stimulations with peptide-pulsed PHA lymphoblasts, the highest percentage of tetramer positive T cells were still detected from both HLA-A*0301 and HLA-B*0801 CML patients cultures (0.27% and 0.20% respectively, **Figure 4.2**, Peptide). These frequencies remained however low and decreased further after the fifth stimulation in all the CML patients assessed (< 0.2% tetramer positive cells). Although a significant tetramer staining background ($CD8^-$ $Tetramer^+$) was observed from CML patients' cultures, the percentages of these non-specific cells were always lower than the percentages of $CD8^+$ $Tetramer^+$ cells (<0.1%). The frequencies of BCR/ABL specific T cells detected in the cultures from healthy donors after four consecutive antigen stimulations were lower than in CML patients, especially in the context of HLA-B*0801 (0.04%, **Figure 4.2**, Peptide).

Thus, using this protocol we were not able to generate or expand BCR/ABL specific T cells from both CML patients and healthy donors. PHA lymphoblasts were however demonstrated to successfully present CMV peptide and induce the expansion of antigen-specific T cells from CML patients. These T lymphoblast cells are nevertheless not professional APCs and may lack expression of some crucial costimulatory receptors required for the generation and expansion of tumour antigen-specific T cells, particularly of low frequent BCR/ABL specific T cells.

4.3 Generation of BCR/ABL specific T cells using monocytes-derived Dendritic cells (mDCs) as APCs:

Dendritic Cells (DCs) represent the most powerful natural APCs, capable of both priming naïve T cell responses and boosting memory T cell responses, thus their efficiency to stimulate and expand BCR/ABL specific T cells was further evaluated.

4.3.1 Generation of mDCs from CML patients and healthy donors:

The feasibility of generating fully matured monocytes-derived DCs *in-vitro* from both CML patients and healthy donors was first assessed. Monocytes are normally enriched from non-adherent cells based on their property of adhering to plastic supports. It has been demonstrated, and we also observed, that Philadelphia chromosome positive dendritic cells and monocytes derived from CML patients have a defect in their adherence capacity (Dong et al. 2003; Gordon et al. 1987; Verfaillie et al. 1992). Thus monocytes obtained from patient leukapheresis samples or from patients demonstrating a high blast cells count were selected by purifying CD14 positive cells. Matured DCs were generated from monocytes in 8 to 10 days culture as described in chapter 2.

Prior to use as APCs, the expression of costimulatory molecules on the cell surface of matured DCs was assessed and analysed by flow cytometry. A representative DCs maturation profile is shown in **Figure 4.3**. The levels and percentages of maturation markers expressed by the mDCs generated from our cohort of donors are represented in **Table 4.3**. Monocytes, immature and mature DCs were first gated from the side and forward scatter dot plots (**Figure 4.3,** panel **I**). This was possible as the differentiation of monocytes into immature DCs and then mature DCs induces progressive changes of size and granularity (Side Scatter and Forward Scatter respectively).

The histogram plots for CD14 (monocyte marker), HLA-DR and costimulatory molecules CD80, CD83 (B7.1) and CD86 (B7.2) expression on the cell surface of monocytes, immature and mature DCs are represented in **Figure 4.3**, panel **II**. The level of costimulatory molecule expression on each population was compared between healthy donors (**Figure 4.3**, panel **II, A**) and CML patient donors (panel **II, B** and **C**) and also between the adherence method (panel **II, A** and **B**) and the CD14 positive cell selection method (panel **II, C**).

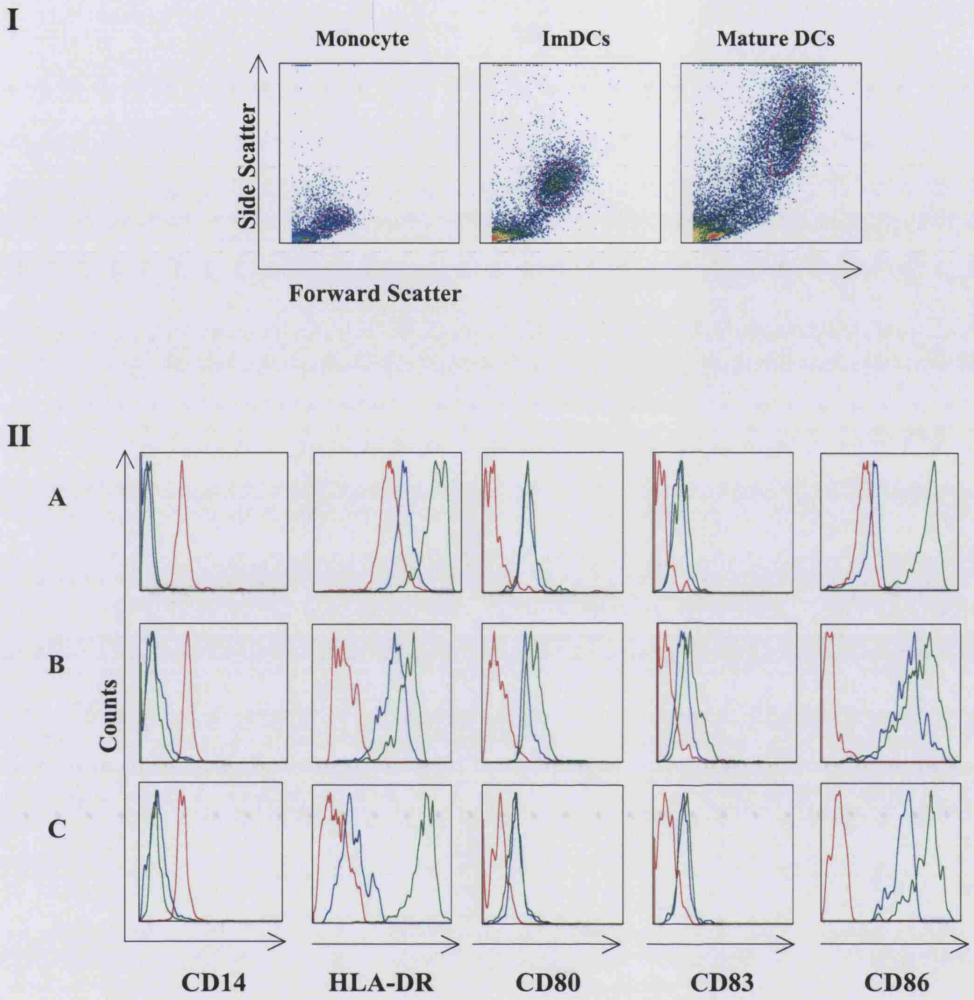


Figure 4.3: Representative maturation profile of monocyte-derived DCs generated from CML patients and healthy donors:

Monocytes-derived DCs were generated *in-vitro* from both CML patients and healthy donor. Prior to their use as APCs, these DCs were assessed for the cell surface expression of maturation markers by flow cytometry. Monocytes (**I**, Monocyte), immature (**I**, ImDCs) and mature DCs (**I**, Mature DCs) were first gated on the forward and side scatter dot plots according to their size and granularity. The maturation profiles of mDCs generated by adherence method from a healthy donor (panel **II, A**) and a treated CML patient 14 (panel **II, B**) are shown. DCs were also generated from $CD14^+$ purified monocytes, derived from untreated CML patient 22 (panel **II, C**). The expression of CD14, HLA-DR and of costimulatory molecules CD80, CD83 and CD86 on the cell surface of monocytes (—) immature (—) and mature DCs (—) were assessed by flow cytometry and are represented for each sample.

	Healthy n=15		*CML patients n=6 Adherence*		*CML patients n=3 CD14 selection*	
Markers	**MFI ± SD**	**% ± SD**	**MFI ± SD**	**% ± SD**	**MFI ± SD**	**% ± SD**
CD14	71± 22	3.5±3	75±15	2.5±1.5	80±15	5±2
CD80	304±50	87±6	266±62	87±9	251±49	80±5
CD83	265±60	88±9	256±50	88±10	246±43	85±8
CD86	615±104	94±7	586±77	90±9	533±67	86±6
HLA-DR	623±79	96±10	604±100	90±5	569±70	90±8

Table 4.3: Levels of maturation markers expression on fully matured mDCs:

The levels of CD14 (monocyte marker), HLA-DR and costimulatory molecules CD80, CD83, CD86 expressed on fully matured mDCs were assessed by flow cytometry as described in chapter 2. The levels and percentages of mDCs expressing these molecules were compared between healthy donors and CML patients and also between the adherence method and the $CD14^+$ cells selection method. The levels are expressed in Mean Fluorescence Intensity (MFI) ± standard deviation (SD) between the different donors. N represents the number of donors in each category.

CD14 is expressed on the cell surface of monocytes (red lines) but become down regulated as these cells differentiated into DCs (blue and green lines, **Figure 4.3** panel **II**, <80±15 MFI, **Table 4.3**). Monocytes expressed low or medium levels of HLA-DR, CD80, CD83 and CD86 (red lines, **Figure 4.3**, panel **II**). A significant up-regulation of HLA-DR and CD86 markers was observed on the cell surface of mDCs, especially matured mDCs (green lines, **Figure 4.3**, panel **II**). High levels of these markers were detected on more than 80% of mature mDCs generated from both healthy and CML patient donors with both methods (from 533±67 to 623±79 MFI, **Table 4.3**). CD80 and CD83 are also up regulated, though at a lower level, on the cell surface of both immature and mature mDCs (**Figure 4.3**, panel **II**). Similar level of CD80 and CD83 molecules were detected on 75% to 98% of mature mDCs generated from all the donors (246±43 to 304±50 MFI, **Table 4.3**).

The level of expression of maturation markers on monocytes and immature mDCs varied between the different donors. In this example the level of HLA-DR and CD86 expression on monocytes derived from the healthy donor was higher than from CML patients (**Figure 4.3**, panel **II**). The levels of expression for all the costimulatory molecules on matured mDCs were not found to be statistically different between healthy donors and CML patients ($P>0.05$, Mann-Whitney test) or between the adherence and the $CD14^+$ cell selection methods ($P>0.05$, Wilcoxon signed rank test). Thus fully matured monocyte-derived DCs can be generated from CML patients and these expressed similar levels of costimulatory molecules to mDCs generated from healthy donors.

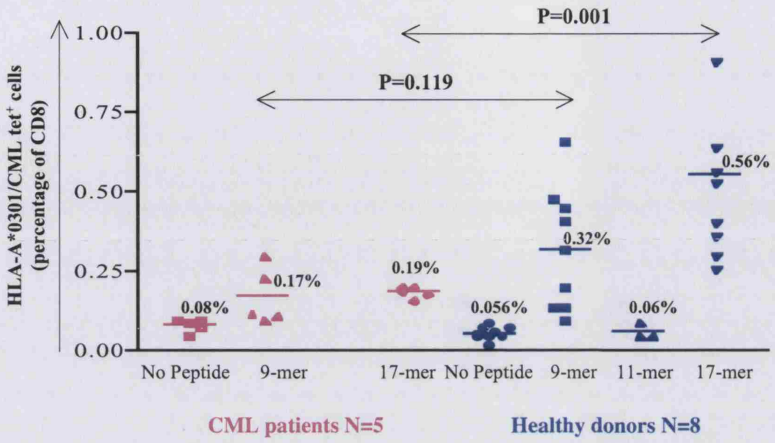
4.3.2 Stimulation of BCR/ABL specific T cell using mDCs:

Fully matured mDCs generated from CML patients and healthy donors were pulsed with the 9-mer HLA-A*0301 or HLA-B*0801 associated BCR/ABL derived peptides, or with the BCR/ABL derived 11-mer (ATGFKQSSKAL) and 17-mer (ATGFKQSSKALQRPVAS) peptides. These longer peptides may contain other HLA-class I and/or HLA-class II epitopes, which subsequently could be processed and presented on the cell surface of DCs. This could enhance the generation and expansion of BCR/ABL specific $CD8^+$ T cell responses. Responder cells (non-adherent lymphocytes) were stimulated with irradiated autologous peptide-pulsed DCs on day 1 and day 9, and a final stimulation was performed with irradiated autologous peptide-pulsed PBMCs, on day 18. IL-2 and IL-15 cytokines were added to the cultures three days following each stimulation as described in chapter 2 (section 2.3.18). Lymphocytes were also stimulated without antigen (un-pulsed APCs) to assess the frequency of BCR/ABL specific T cells remaining in these cultures.

The efficiency of peptide-pulsed mDCs in generating BCR/ABL specific T cells was assessed from nine CML patients (five HLA-A*0301 and four HLA-B*0801, described in **Table 4.2**, DC study*****) and fifteen healthy donors (eight HLA-A*0301 and seven HLA-B*0801). The antigen specific T cell responses were monitored by HLA/tetramer staining three days after each stimulation. The highest frequencies of BCR/ABL specific T cells were observed after the third antigen stimulation (day 21) and are represented in **Figure 4.4**. The frequencies of BCR/ABL specific T cells generated with this particular protocol were compared in the context of HLA-A*0301 (**Figure 4.4**, panel **A**) and HLA-B*0801 (**Figure 4.4**, panel **B**) between CML patients (pink dots) and healthy donors (blue dots). A representative tetramer staining is also shown in **Figure 4.5**.

In order to establish the capacity of CML patient T cells to respond to antigenic stimulation and the efficiency of their mDCs to generate antigen-specific T cell responses with this protocol, responses to the HLA-A*0201 associated CMV peptide were assessed in parallel when possible (with HLA-A*0201 and CMV seropositive patient donors). Four CML patients fulfilled the correct criteria and were selected for this study (patients 1, 4, 13 and 17, **Table 4.2**). In all cases, the stimulation protocol induced the proliferation of CMV specific $CD8^+$ T cells. A representative HLA/CMV tetramer staining obtained from the CML patient 1, before any stimulation and after the third stimulation (day 21) is shown in **Figure 4.5**, panel **C**.

A



B

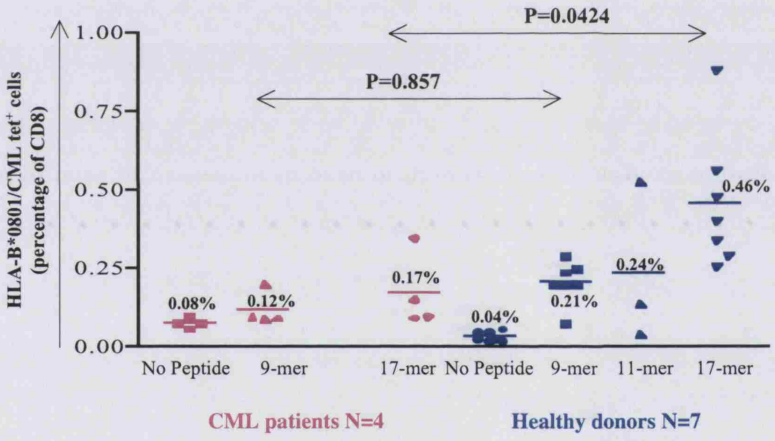


Figure 4.4: BCR/ABL specific $CD8^+$ T cell responses generated with peptide-pulsed autologous mDCs:

The generation of BCR/ABL specific T cells using monocytes-derived DCs as APCs was assessed from HLA-A*0301 (panel **A**) and HLA-B*0801 (panel **B**) CML patients (**pink**) and healthy donors (**blue**). Lymphocytes were stimulated with autologous irradiated DCs pulsed with no peptide (**No Peptide**) or BCR/ABL derived **9-mer**, **11-mer** and **17-mer** peptides following the protocol described in chapter 2. Activated T cells were harvested three days after the last stimulation (day 21) and stained with the appropriate HLA/CML tetramer. BCR/ABL specific T cells are expressed as a percentage of $CD3^+CD8^+$ T cells, with the bar mean values shown for each condition. The P values differences between CML patients and healthy individuals were calculated with the Mann-Whitney U test.

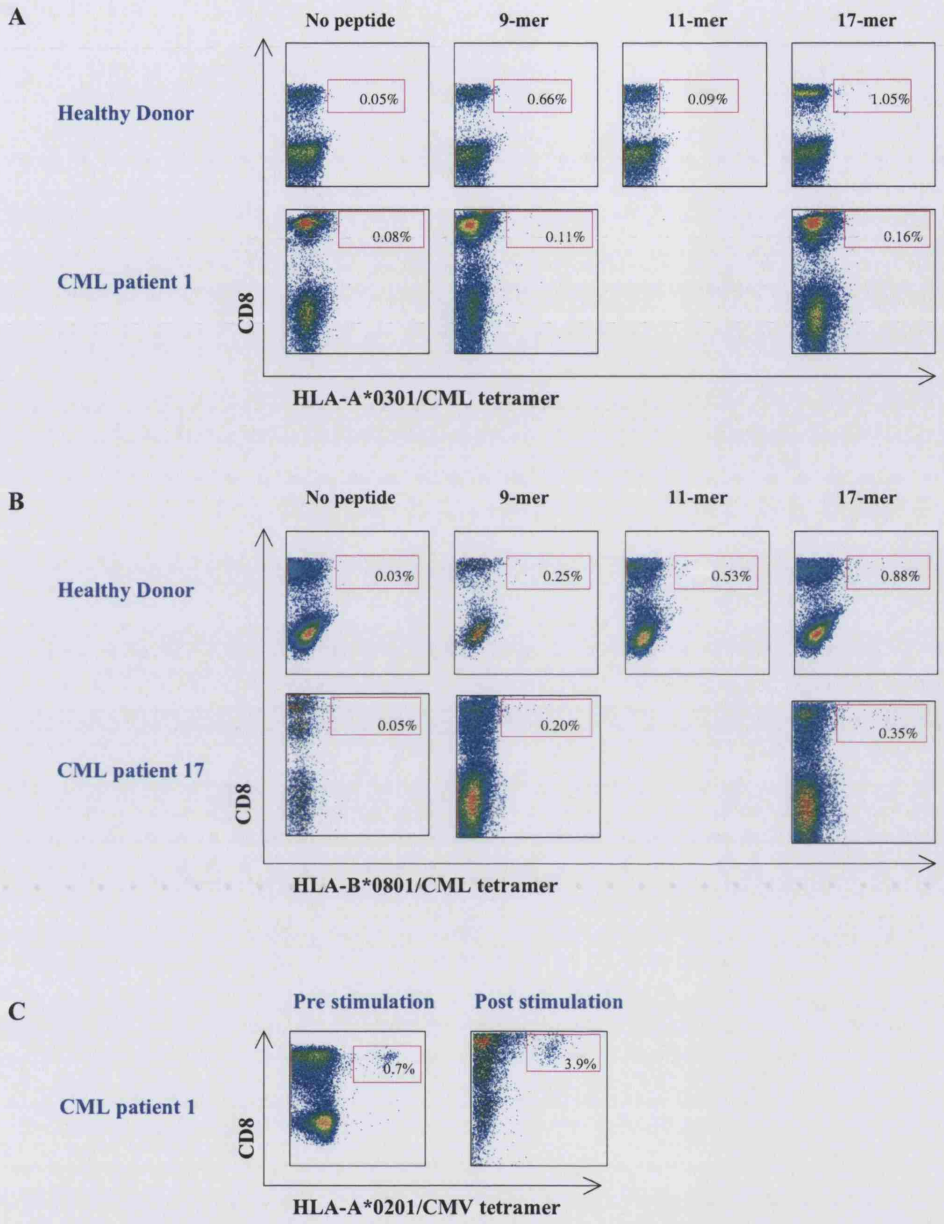


Figure 4.5: Representative HLA/tetramer staining of antigen specific T cells stimulated with peptide-pulsed autologous mDCs:

Non adherent lymphocytes derived from HLA-A*0301 (**A**) and HLA-B*0801 (**B**) CML patients (patients 1 and 13, described in **Table 4.2**) and healthy donors were stimulated on day 1 and day 9 with autologous mDCs, pulsed with the BCR/ABL (b3a2) derived **9-mer**, **11-mer** or **17-mer** peptides. A third stimulation with autologous irradiated peptide-pulsed PBMCs was performed on day 18. Lymphocytes were stimulated with un-pulsed APCs as controls (**No peptide**). Lymphocytes derived from CML patient 1 were also stimulated with autologous CMV peptide-pulsed mDCs in parallel (**C**). Activated T cells were harvested on day 21, stained with the appropriate HLA/peptide tetramers and analysed by flow cytometry. The frequencies of tetramer positive cells are expressed as a percentage of $CD3^+$ $CD8^+$ T cells and are represented in the upper right gates. The frequencies of CMV specific T cells were monitored on day 0 (panel **C**, **Pre stimulation**) and three days after the third stimulation (day 21, **Post stimulation**).

The frequency of CMV specific T cells detected from the peripheral blood of this patient was 0.7% of $CD8^+$ T cells (**Figure 4.5**, panel **C**, Pre-stimulation). Following stimulation, the CMV specific T cells derived from this patient were successfully expanded to up to 3.9% of $CD8^+$ T cells (**Figure 4.5**, panel **C**, post-stimulation). Thus, CML patients are capable of responding to peptide-pulsed mDCs and generating an antigen-specific T cell response, at least in the context of the CMV antigen.

The stimulation of CML patient lymphocytes with BCR/ABL peptide-pulsed mDCs induced a small increase of tetramer positive T cells in the context of both HLA-A*0301 and HLA-B*0801 (from mean of 0.08% to 0.19% and from mean of 0.08% to 0.17% respectively, **Figure 4.4**, panel **A** and **B**, pink dots and **Figure 4.5**, CML patients). The frequencies of BCR/ABL specific T cells detected after stimulation with the 9-mer and 17-mer peptides were not found to be statistically significant compared to the frequencies of BCR/ABL specific T cells detected in cultures without antigen stimulation (No peptide, $P>0.05$, Wilcoxon signed ranked test). The majority of the patients assessed here were at least in complete cytogenetic remission (8 out of 9 patients, **Table 4.2**). Among these patients, five remained in partial remission with the molecular detection of bcr/abl transcript of 5 to 10% and only one patient was in active chronic phase of the disease. The highest frequencies of tetramer positive T cells were detected in patients receiving an alloHSCT and experiencing complete cytogenetic and molecular responses compared to patients treated with autoHSCT and/or Imatinib mesylate. This was observed both in the context of HLA-A*0301 and HLA-B*0801 (patients 5, 6 and 17, **Figure 4.4** and **4.5**). These tetramer positive T cells may be donor-derived and therefore more capable of generating an anti-leukaemic response. The numbers of BCR/ABL specific T cells detected from all the CML patient cultures remained however low, with or without peptide stimulations (<0.2% **Figure 4.4**, panel **A** and **B**, pink dots and **Figure 4.5**, CML patients). Thus, BCR/ABL specific T cells could not be generated and/or expanded from these CML patients using this protocol.

In contrast, in healthy donors this protocol induced very heterogeneous antigen specific T cell responses from both HLA-A-A*0301 and HLA-B*0801 donors (from 0.1% to 1.05% and from 0.08% to 0.88% respectively, **Figure 4.4**, panel **A** and **B**, blue dots and **Figure 4.5**, healthy donors). The frequencies of BCR/ABL specific $CD8^+$ T cells detected after stimulation with the 9-mer BCR/ABL peptides increased significantly compared to the frequencies detected in the cultures without antigen stimulation (No peptide). This was observed in healthy donors both in the context of HLA-A*0301 (from mean of 0.056% to 0.32%, $P=0.0078$ Wilcoxon signed ranked test, **Figure 4.4**, panel **A**) and HLA-B*0801 (from mean of 0.04% to 0.21%, $P=0.0156$, panel **B**). The frequencies of BCR/ABL specific

T cells generated after stimulation with the 17-mer BCR/ABL peptide were also found to be statistically significant compared to the negative control (mean of 0.56%, P=0.0039 for HLA-A*0301 and mean of 0.46%, P=0.0125 for HLA-B*0801, Wilcoxon signed ranked test, **Figure 4.4**, panel **A** and **B**).

The degree of the BCR/ABL specific $CD8^+$ T cell responses generated was also shown to be affected by the length of the BCR/ABL peptides used as a source of antigen to stimulate these responses. The frequencies of tetramer positive T cells detected in healthy donors after stimulation with the 17-mer peptide were significantly higher when compared to the 9-mer peptide (P=0.0391 for HLA-A*0301 and P=0.0156 for HLA-B*0801, Wilcoxon signed ranked test, **Figure 4.4** and **Figure 4.5**). This longer 17-mer BCR/ABL peptide may contain other HLA-class I and/or HLA-class II epitopes that may enhance the activation of HLA-A*0301 and HLA-B*0801 associated BCR/ABL specific T cells. The 11-mer BCR/ABL derived peptide was also included when a sufficient number of lymphocytes were available from these donors (usually healthy donors). This peptide contains the HLA-B*0801 associated BCR/ABL epitope but not the HLA-A*0301 associated epitope. Accordingly, the stimulation of HLA-A*0301 healthy donors with the 11-mer BCR/ABL peptide failed to induce an antigen-specific T cell response (mean of 0.06%, **Figure 4.4**, panel **A**, 11-mer and **Figure 4.5**, panel **A**). On the other hand, the stimulation of HLA-B*0801 healthy donors with this 11-mer BCR/ABL peptide induced heterogeneous antigen specific T cell responses (mean of 0.24%, **Figure 4.4**, panel **B**, 11-mer and **Figure 4.5**, panel **B**). These observations provide some evidence of the authenticity of the BCR/ABL specific T cells generated after stimulation with the BCR/ABL derived 9-mer and 17-mer peptides. The frequencies of BCR/ABL specific T cells generated with the 11-mer peptide in these donors were however not statistically significant compared to the frequencies of BCR/ABL specific T cells detected in the controls (No peptide, P=0.5, Wilcoxon signed ranked test). As only three donors could be assessed with this 11-mer peptide due to the limited amount of mDCs that could be generated from these donors, no firm conclusions can be drawn.

Although the increase of tetramer positive T cells detected after stimulation with the BCR/ABL 9-mer and 17-mer peptides were statistically significant compared to the negative controls, the frequencies of BCR/ABL specific T cells detected remained low even after three consecutive stimulations. The disparity in the frequencies of BCR/ABL specific T cells observed between the different healthy donors may probably reflect the diversity in the antigen specific T cell repertoire between individuals. In one donor it has been possible to demonstrate a significant expansion of BCR/ABL specific T cells to up to 1.05% in the context of the HLA-A*0301 molecule (**Figure 4.5**, panel **A**).

The BCR/ABL specific T cell responses generated using this protocol were compared between CML patients and healthy donors. Higher frequencies of antigen-specific T cells were generated from both HLA-A*0301 and HLA-B*0801 healthy donors than from CML patients (mean of 0.21% to 0.56% versus mean of 0.12% to 0.19% respectively, **Figure 4.4,** blue and pink dots). Because the responses obtained in healthy donors after stimulation with the BCR/ABL derived 9-mer peptide were very heterogeneous, these were not found to be statistically different to the responses obtained in CML patients (P=0.119 for HLA-A*0301 and P=0.857 for HLA-B*0801, Mann-Whitney U test; shown in **Figure 4.4**, panel **A** and **B**). However the numbers of tetramer positive T cells detected following stimulation with the BCR/ABL derived 17-mer peptide were significantly higher from healthy donors than from CML patients in the context of both HLA-A*0301 and of HLA-B*0801 molecules (P=0.001 and P=0.0424 respectively, Mann-Whitney U test, **Figure 4.4**, panel **A** and **B**).

HLA-A*0201 positive CML patients were shown to be capable of generating CMV specific T cell responses following the same protocol (**Figure 4.5**, panel **C**). Therefore the lack of BCR/ABL specific responses is unlikely due to the inefficiency of CML patient DCs to prime an immune response. Additionally, this demonstrates that CML patients are fully capable of generating an antigen specific T cell response, at least in the context of a viral antigen. Thus, these data suggest that CML patients have a specific immune deficit with respect to the BCR/ABL antigen.

Furthermore, the BCR/ABL peptide presented by HLA-A*0301 was demonstrated to induce a greater expansion of antigen-specific T cells than the HLA-B*0801 associated peptide. The frequencies of BCR/ABL specific T cells detected after stimulation with the BCR/ABL 9-mer peptide in both HLA-A*0301 CML patients and healthy donors were higher than those detected in HLA-B*0801 donors (mean of 0.17% and 0.32% versus 0.12% and 0.21% respectively, **Figure 4.4**, panel **A** and **B**, 9-mer). The expansion of these BCR/ABL specific T cells after peptide stimulation was also statistically more significant in the context of the HLA-A*0301 molecule than of the HLA-B*0801 molecule compared to the control (No peptide stimulation). This was observed only for healthy donors (P=0.0078 versus P=0.0156, Wilcoxon signed ranked test). The expansion of these cells was not significantly different to the controls for CML patients, though there were a trend towards a greater expansion in the context of HLA-A*0301 than HLA-B*0801 molecule (P=0.1250 versus P=0.250). Thus, these data also suggest that the HLA-A*0301 BCR/ABL associated epitope is more immunogenic than the HLA-B*0801 BCR/ABL associated epitope.

4.3.3 Assessment of the cytotoxic activity of BCR/ABL specific $CD8^+$ T cells:

The BCR/ABL peptide specific $CD8^+$ T cells generated with autologous peptide-pulsed mDCs were further assessed for their cytotoxicity against HLA-matched CML cells. Antigen activated T cells were harvested after the third and last stimulation (day 21) and incubated for four hours with ^{51}chromium labelled CML target cells at a ratio of 10 to 1 respectively. Both the HLA and the peptide specificity were tested by assessing the cytotoxic activity against HLA-unmatched CML targets as well as HLA-matched $b2a2^+$ CML target cells in parallel. The cytotoxic activity of the BCR/ABL specific T cells was determined by measuring the chromium released by the CML target cells as described in chapter 2.

In total the cytotoxic activity of BCR/ABL specific T cells was assessed from 6 CML patients (3 HLA-A*0301 and 3 HLA-B*0801) and 10 healthy donors (6 HLA-A*0301 and 4 HLA-B*0801). None of the BCR/ABL peptide activated T cells from the CML patients demonstrated specific cytotoxic activities against CML target cells above background (HLA-unmatched targets). The frequencies of BCR/ABL specific T cells detected by HLA/tetramers were shown to be low in these patients (**Figure 4.4 and 4.5**) and might have accounted for the lack of BCR/ABL specific cytotoxic activity.

Out of the 10 healthy donors tested, a single HLA-A*0301 donor showed specific cytotoxic activity against HLA-matched $b3a2^+$ CML targets and is shown in **Figure 4.6**. The lymphocytes derived from this donor stimulated with the BCR/ABL 9-mer or 17-mer peptides demonstrated specific killing of HLA-matched $b3a2^+$ CML targets cells of 5% and 9.1% respectively (**Figure 4.6**, 9-mer and 17-mer). This cytotoxicity was specific as these BCR/ABL activated T cells showed no cytotoxic activity against HLA-mismatched or HLA-matched $b3a2^-$ CML targets cells above the background of 3%. In addition, lymphocytes incubated with un-pulsed mDCs showed no specific cytotoxic activity against HLA-matched CML targets above the background (**Figure 4.6**, No peptide). Thus, the activation of antigen-specific T cells with the BCR/ABL derived peptides induced the specific recognition and killing of native CML cells in an HLA-restricted manner, at least from one donor. This also indicates that HLA-A*0301 CML cells present the BCR/ABL derived peptide on the cell surface.

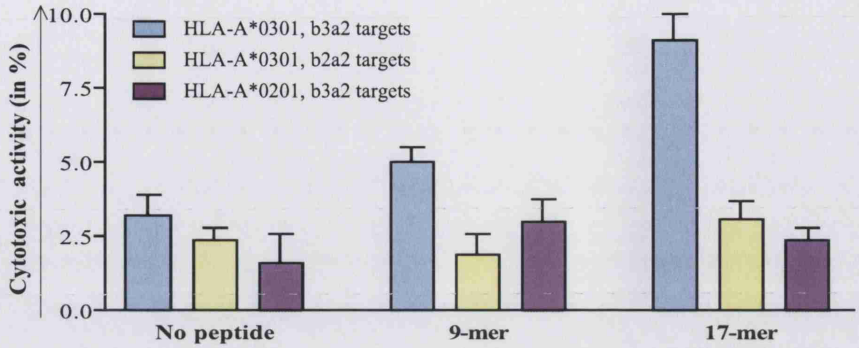


Figure 4.6: Cytotoxic activity of BCR/ABL specific $CD8^+$ T cells generated with mDCs:

PBMC derived from an HLA-A*0301 healthy donor were stimulated with autologous mDCs un-pulsed (**No peptide**) or pulsed with the BCR/ABL derived **9-mer** or **17-mer** peptides, following the protocol described in chapter 2 and shown in **Figure 4.5**, panel **A**. These cells were assessed for their cytotoxic activity by Cr^{51} release. Activated T cells were harvested three days after the last stimulation (day 21) and incubated with Cr^{51} labeled HLA-matched $b3a2^+$ or $b2a2^+$ CML targets and HLA-unmatched $b3a2^+$ CML targets. The target cells were obtained from CML patients 26, 25 and 42 leucopheresis samples respectively. The specific cytotoxic activity was measured after four hours incubation of CML target cells at a ratio of 1:10 with effector cells. The percentage of specific lysis was calculated using the following formula: 100× (cpm of experimental release - cpm of spontaneous release) / (cpm of maximal release - cpm of spontaneous release) where cpm represents the count per minutes. The error bars represent the standard deviation between triplicate samples.

Specific cytotoxicity was only observed in the donor who demonstrated the highest percentage of BCR/ABL specific $CD8^+$ T cells following peptide-pulsed mDCs stimulation (1.05% of tetramer positive T cells detected after stimulation with the 17-mer, **Figure 4.5**, panel **A**). Also, the levels of specific cytotoxicity observed in this donor were found to be proportional to the percentages of BCR/ABL specific T cells detected after *in-vitro* stimulations. Thus the 0.66% of BCR/ABL specific $CD8^+$ T cells generated from this donor after stimulation with the 9-mer BCR/ABL peptide showed a 5% level of cytotoxicicity against HLA-matched CML targets (**Figure 4.6**, 9-mer). The increase of the BCR/ABL specific T cell numbers following stimulation with the 17-mer BCR/ABL peptide (1.05% of total $CD8^+$ T cells, **Figure 4.5**, panel **A**) resulted in an increase of the specific cytotoxicity against the CML target cells (9.1% of killing, **Figure 4.7**, 17-mer). Thus, these data would suggest that the BCR/ABL specific T cells detected with our HLA/peptide tetramers are specific and functional, at least from one HLA-A*0301 healthy donor. In the majority of cases however, no functional BCR/ABL specific T cells were generated from CML patients or healthy donors using this protocol. This may be related to many factors, which will be discussed subsequently.

4.4 Plasmacytoid DCs as APCs:

Plasmacytoid DCs (pDCs) have been shown to originate from lymphoid progenitors and thus may not carry the Philadelphia chromosome (Briere et al. 2002). These pDCs could therefore represent an ideal APCs in CML patients, in contrast to the myeloid derived DCs, which have been shown to be deficient in a number of their functions (Dong et al. 2003). In addition, pDCs have been suggested to play a major role in anti-tumour responses, at least in melanoma patients (Salio et al. 2003). Thus their significance and efficiency in generating BCR/ABL specific T cell responses were assessed from both CML patients and healthy donors.

4.4.1 Frequency of pDCs in CML patients and healthy donors:

The frequency of circulating pDCs was first assessed from the peripheral blood of sixteen CML patients (described in **Table 4.2**, pDC*****) and ten healthy donors. Plasmacytoid DCs can be characterised by flow cytometry using the two newly identified Blood DC Antigen markers, BDCA-2 and BDCA-4 (Dzionek et al. 2000). Plasmacytoid DCs have also been described as to expressing high levels of HLA-class II molecules (HLA-DR) and IL-3 receptors (CD123) on the cell surface. Thus, lymphocytes derived from our donors were stained with BDCA-2 and CD123 (IL-3 receptor α chain) antibodies and analysed by flow cytometry as described in chapter 2. The frequencies of double BDCA-2 and CD123 positive pDCs detected from the peripheral blood of healthy donors and CML patients assessed at diagnosis or receiving Imatinib mesylate treatment are represented in **Figure 4.7**, panel **A**. A representative BDCA-2/CD123 dot plot obtained from each type of donor is also shown in **Figure 4.7**, panel **B**.

A distinct double BDCA-2/CD123 positive population of pDCs was characterised from the peripheral blood of healthy donors, at a frequency of 0.23% of total lymphocytes (mean pDC, **Figure 4.7**, panel **A** and **B**). In contrast, reduced numbers of circulating blood pDC were found in CML patients assessed at diagnosis (**Figure 4.7**, panel **B**). The frequency of pDCs detected from these patients was significantly lower than from healthy donors (mean of 0.04%, P=0.0006, Mann-Whitney U test, **Figure 4.7**, panel **A**). As CML mainly affects the myeloid progenitor compartment and it is known that pDCs originate from a lymphoid progenitor, their deficiency may result from a simple dilutional effect consequent upon uncontrolled myeloid proliferation.

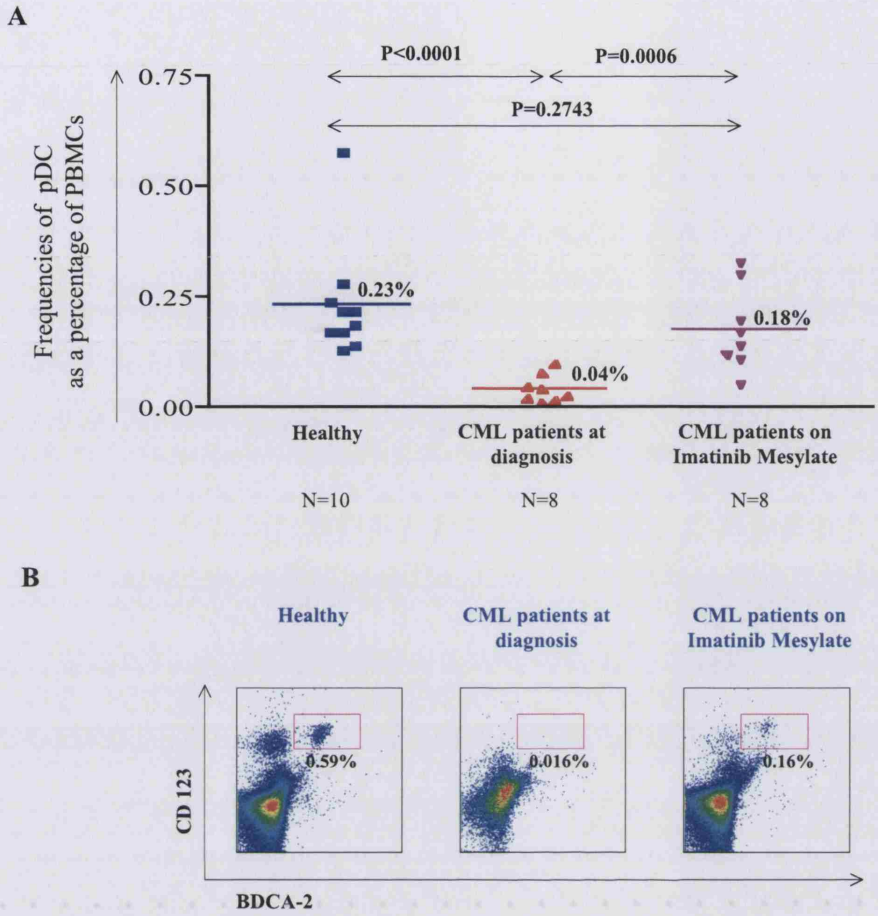


Figure 4.7: Frequencies of circulating plasmacytoid DCs in CML patients and healthy donors:

The frequencies of plasmacytoid DCs (pDC) circulating in the peripheral blood was assessed (**A**) from healthy donors (■), CML patients at diagnosis (▲) or after receiving Imatinib mesylate treatment (▼). Double positives BDCA-2$^+$ CD123$^+$ (pDCs) were quantified by flow cytometry and are expressed as a percentage of total live PBMCs. The mean percentages of pDCs are shown for each category. An example of the CD123/BDCA-2 dot plot staining obtained from the different donors are shown (**B**) and the percentages of pDCs are given in the upper right gate. The P values were calculated using the Mann-Whitney test. N represent the number of donors tested in each category.

Moreover the frequency of circulating pDCs in patients whose CML was controlled with Imatinib mesylate therapy was not found to be statistically different to the frequencies detected in healthy donors (mean of 0.18% ±0.15%; P=0.2743, Mann-Whitney U test, **Figure 4.7**). Thus, the eradication of the myeloid transformed progenitor by Imatinib mesylate appears to restore the frequency of circulating pDCs in treated patients. In some patients, the frequency of pDCs remained very low, despite Imatinib mesylate treatment (<0.1%, **Figure 4.7**, panel **A**). These patients actually failed to respond to the treatment and remained in active chronic phase of CML (patients 27 and 29, **Table 4.2**).

4.4.2 Purification of pDCs from the peripheral blood of healthy donors:

Since the frequency of circulating pDCs was shown to be low, or even absent in CML patients, the selection of a sufficient number of pDCs for the assessment of their APCs functions would have required a large amount of blood from these patients, which could not be provided from the hospital centres. Therefore, the capacity of pDCs to generate and/or expand BCR/ABL specific $CD8^+$ T cells could only be investigated from healthy donors.

Plasmacytoid DCs can be purified from the peripheral blood of donors using the BDCA-2 or BDCA-4 specific markers. The BDCA-2 marker, unlike BDCA-4, is strictly expressed on pDCs and is preferentially used for their characterisation from blood or bone marrow samples. BDCA-2 was, however, demonstrated to be involved in antigen capture by pDCs (Dzionek et al. 2002). Thus its ligation during positive selection may undesirably affect their APC functions. Therefore pDCs were purified by selecting BDCA-4 positive cells. As BDCA-4 can be upregulated on monocytes and myeloid blood DCs in culture, pDCs were selected from healthy donors derived fresh blood (Dzionek et al. 2000).

Plasmacytoid DCs were purified from three HLA-A*0301 and two HLA-B*0801 healthy donors using the BDCA-4 cell isolation kit as described in chapter 2. The purity of the isolated pDCs was evaluated by flow cytometry. pDCs were characterised with BDCA-2, HLA-DR and CD123 markers before and after selection. A representative dot plot staining of pDCs purification from an HLA-A*0301 healthy donor is shown in **Figure 4.8**. The percentages of double positive BDCA-2/HLA-DR and BDCA-2/CD123 cells detected from the peripheral blood of this donor were 0.12% and 0.13% of total lymphocytes (**Figure 4.8**, panel **A** and **B** respectively). The selection of BDCA-4 positive cells from our donors successfully enriched pDCs to at least 95% purity. In the example shown in **Figure 4.8**, pDCs were enriched to up to 98.5% purity (panel **B**, Post selection). Thus, pDCs were successfully purified from the peripheral blood of healthy donors using the BDCA-4 cell isolation kit. These cells were then cultured for 72 hours in the presence of IL-3 cytokine and matured for an additional 24 hours with soluble $CD40^L$ and BCR/ABL peptides as described in chapter 2. Plasmacytoid DCs were then assessed for their APC capacity to stimulate BCR/ABL specific T cells.

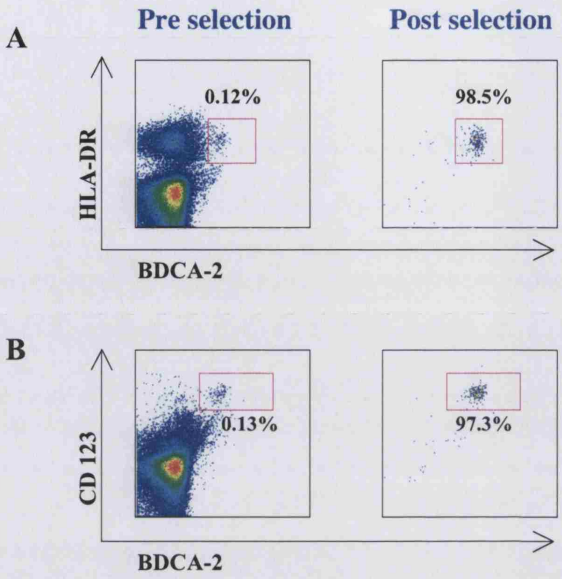


Figure 4.8: Purification of plasmacytoid DCs from healthy donors:

Plasmacytoid DCs were selected from the peripheral blood of healthy donors using the positive BDCA-4 cell isolation kit as described in chapter 2. pDCs were stained with BDCA-2, HLA-DR and CD123 (IL-3 receptor) markers and analysed by flow cytometry. Cells were gated on live PBMCs based on the Forward/Side scatter dot plots. pDCs were characterised on BDCA-2/HLA-DR (**A**) and BDCA-2/CD123 (**B**) dot plots. The numbers of pDCs pre and post selection are expressed as a percentage of total PBMCs and are shown in each gate.

4.4.3 Stimulation of BCR/ABL specific T cells using pDCs as APCs:

Lymphocytes derived from HLA-A*0301 or HLA-B*0801 healthy donors were stimulated with autologous peptide-pulsed pDCs. pDCs were pulsed with the HLA-associated BCR/ABL derived 9-mer or 17-mer peptides and incubated at a ratio of 1 to 10 with responder cells. As a control, responder T cells were also incubated with un-pulsed pDCs. Lymphocytes were stimulated on day 1 and day 9 and antigen activated T cell proliferation was supported by the addition of IL-2 and IL-15 on day 4 and day 11. The ability of this stimulation protocol in generating CMV specific T cell responses could not be assessed due to the limited number of purified pDCs available and the CMV seronegative status of the majority of the healthy donors. The efficiency of pDCs to generate BCR/ABL specific T cell responses was monitored by HLA/CML tetramer staining on day 13 and is shown in **Figure 4.9**. The frequencies of BCR/ABL specific $CD8^+$ T cells detected after two antigen stimulations from HLA-A*0301 and HLA-B*0801 donors are shown in panel **A** and **B** respectively. The stimulation of lymphocytes with peptide-pulsed pDCs induced variable antigen-specific T cell responses from these donors (mean from 0.091% to 0.37% for HLA-A*0301 and mean from 0.055% to 0.31% for HLA-B*0801, **Figure 4.9**). Low numbers of BCR/ABL specific T cells were detected in the cultures incubated with un-pulsed pDCs (≤0.03%, No peptide **Figure 4.9**). Thus pulsing the pDCs with BCR/ABL peptides induced the expansion of antigen specific T cells; however the number of BCR/ABL specific T cells detected in these cultures still remained low (≤0.4% of tetramer positive T cells).

In contrast to mDCs, pDCs pulsed with the 9-mer BCR/ABL peptide reproducibly generated higher numbers of BCR/ABL specific T cells than the pDCs pulsed with the 17-mer peptide (mean from 0.063% to 0.37% versus mean from 0.055% to 0.21%, **Figure 4.9**). It has been demonstrated that pDCs are capable of processing and presenting antigenic peptides associated with HLA-class I molecules (Salio et al. 2003). The frequencies of BCR/ABL specific T cells detected after stimulation with the 17-mer BCR/ABL peptide pulsed pDCs were in fact higher than those detected in the controls (mean from 0.055% to 0.21%, versus mean ≤0.03%, **Figure 4.9**). According to the limited numbers of pDCs purified from these donors, only two stimulations could be performed and may not have been sufficient to stimulate BCR/ABL specific T cells.

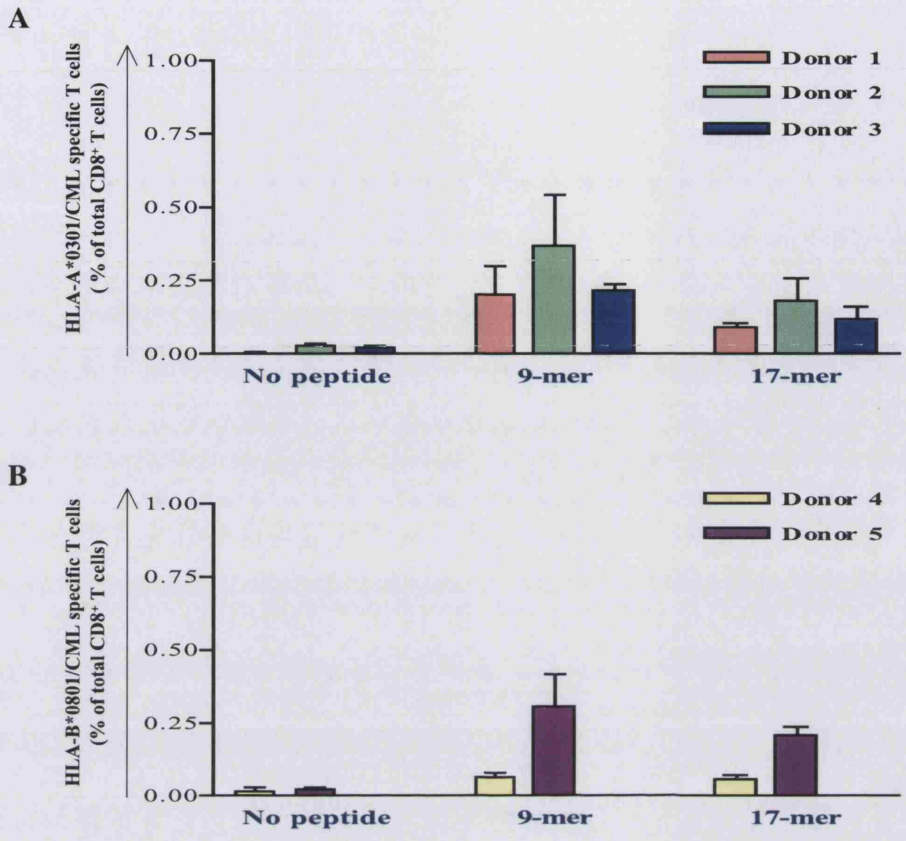


Figure 4.9: Generation of BCR/ABL specific T cells from healthy donors using pDCs as APCs:

PBMCs derived from three HLA-A*0301 (panel **A**) and three HLA-B*0801 healthy donors (panel **B**) were stimulated on day 1 and day 9 with autologous pDCs pulsed with the BCR/ABL **9-mer** or **17-mer** peptides. PBMCs were also incubated with un-pulsed pDCs (**No peptide**) and included as negative controls. pDCs were incubated at a ratio of 1:10 with responder cells and IL-2 as well as IL-15 cytokines were added on day 4 and day 11. Lymphocytes were harvested and the frequency of BCR/ABL specific $CD8^+$ T cells was assessed on day 12 by flow cytometry. Activated T cells were stained with the appropriate HLA/CML tetramers and BCR/ABL specific T cells are expressed as a percentage of $CD3^+CD8^+$ T cells. The error bars represent the standard deviation between duplicate samples.

The cytotoxic activity against HLA-matched CML target cells of the BCR/ABL specific T cells stimulated with pDCs was also assessed from two donors (donor 2 and 3) following the protocol described in chapter 2 and section 4.3.3. As expected from the low frequencies of tetramer positive T cells expanded in these cultures (≤0.4% of tetramer positive cells, **Figure 4.9**), none of the activated BCR/ABL specific T cells generated from these donors demonstrated specific cytotoxicity against HLA-matched CML target cells above background (HLA-unmatched target cells).

DISCUSSION

In this chapter the feasibility of generating functional BCR/ABL specific T cells in the context of HLA-A*0301 and HLA-B*0801 molecules from CML patients and healthy donors was evaluated using different *in-vitro* stimulation protocols.

Peptide-pulsed PHA lymphoblasts were unable to stimulate and expand BCR/ABL specific T cells from HLA-A*0301 and HLA-B*0801 donors, even after four consecutive antigen stimulations. The capacity of these APCs to generate antigen-specific immune responses was however demonstrated in the context of a viral antigen (CMV). The stimulation of T cells from CML patients with CMV peptide-pulsed PHA lymphoblasts was shown to induce over a 5-fold expansion of antigen specific T cells. However no BCR/ABL specific responses were generated with this protocol from CML patients or healthy donors. As mention previously, T cells are not professional APCs and may lack the expression of crucial costimulatory molecules for the stimulation of tumour specific T cell responses. In healthy donors, the BCR/ABL specific T cell responses are expected to be naïve immune responses, as these donors have not been exposed to the BCR/ABL oncogenic protein. Lymphoblasts may not be efficient for priming BCR/ABL responses from these donors. In contrast, in CML patients, the BCR/ABL specific T cells may represent a memory response. However it was also not possible to expand these antigen specific T cells from patients using this protocol. As a significant frequency of BCR/ABL specific T cells was detected from the peripheral blood of CML patients (chapter 3), it is unlikely that the lack of antigen specific T cell responses is due to the *in-vivo* deletion of BCR/ABL specific T cells. On the other hand, it is possible that these tumour specific T cells are tolerized and/or anergized.

Several groups have nevertheless reported the efficient generation of BCR/ABL specific T cells using such APCs. In some of these studies, the autologous T lymphoblasts were pulsed with longer peptides (11 or 17-mers) and were shown to induce the generation of low frequency BCR/ABL specific $CD4^+$ T cells (only 4 clones out of 192 tested) from a limited number of healthy individuals (Mannering et al. 1997; Yasukawa et al. 1998). Yontda et al., have reported the generation of BCR/ABL specific $CD8^+$ T cells able to recognize peptide-pulsed EBV-transformed targets in some CML patients and healthy donors (Yotnda et al. 1998). In this case, the BCR/ABL peptides were loaded by peptide-stripping the lymphoblast cells and thereby may have increased the levels of HLA/peptide complexes presented on the cell surface of these APCs (Yotnda et al. 1998). In the majority of these studies, the BCR/ABL responses were monitored by proliferation assays or cytotoxic

activity against peptide-pulsed targets (Mannering et al. 1997; Yasukawa et al. 1998; Yasukawa et al. 2001), and only few groups demonstrated cytotoxic activity against HLA-matched CML cells (Clark et al. 2001; Norbury et al. 2000; Yotnda et al. 1998). The use of HLA/tetramers to monitor the frequencies of BCR/ABL specific T cells is recent and has only been documented by our group, in collaboration with Prof R. Clark at the Liverpool Royal Infirmary Hospital. The successful expansion of BCR/ABL specific T cells frequencies, in the context of HLA-A*0301, was reported after stimulation with peptide-pulsed PHA lymphoblasts from two CML patients (Clark et al. 2001). However it was later observed that these responses were difficult to reproduce from a larger panel of patients (Prof R. Clark personnel communication). Thus, PHA lymphoblasts may not represent the optimal APCs for the generation of functional BCR/ABL specific T cells.

As DCs are the most potent natural APCs capable of boosting memory and priming naïve responses, their efficiency in expanding BCR/ABL specific T cells was also assessed from our cohort of donors. Fully matured monocyte-derived DCs were successfully generated from CML patients and expressed similar levels of costimulatory molecules as mDCs generated from healthy donors. In addition, these APCs were shown to be capable of generating CMV specific T cell responses from CML patients. In the context of BCR/ABL, this protocol induced an increase of tetramer positive cells from the stimulated cultures compared to the controls (No peptide stimulation). The responses were however only found to be statistically significant in healthy donors. Thus, in accordance with the group of Bertazzoli et al., we found that CML patients were not capable to respond to BCR/ABL pulsed mDCs as compared to the responses obtained from healthy donors (Bertazzoli et al. 2000).

CML patient derived DCs are known to contain the bcr/abl genetic defect and this has been shown to affect their functions as APCs (Dong et al. 2003; Eisendle et al. 2003). The use of CML patient mDCs to generate anti-leukaemic responses has been reported, however as discussed in the introduction the antigen specificity of these responses was unclear (Choudhury et al. 1997; Muller et al. 2001). In our study, the majority of patients were at least in cytogenetic remission with little or no bcr/abl transcript detection at the molecular level and their mDCs were capable of stimulating CMV specific T cell responses. Thus it appears that CML patients have a specific immune deficit for the BCR/ABL antigen. It is possible that BCR/ABL specific T cells are tolerized and/or anergized by autologous mDCs. In fact, mDCs have been reported to induce antigen-specific T cell tolerance, especially in the context of tumour antigens (den Boer et al. 2001; Staveley-O'Carroll et al. 1998).

In healthy donors, the numbers of BCR/ABL specific T cells detected after stimulation with BCR/ABL peptides were found to be statistically significant compared to the negative controls. The use of the 17-mer BCR/ABL longer peptide as a source of antigen to stimulate antigen specific T cell responses induced a higher frequency of tetramer positive T cells compared to the use of the 9-mer BCR/ABL peptide. This peptide may therefore contain an unidentified HLA-class I and/or HLA-class II epitope, which resulted in the enhancement of the stimulation of BCR/ABL specific $CD8^+$ T cell responses. This also indicates that our protocol could generate functional mDCs, with the capacity of processing and presenting BCR/ABL peptides. However the frequencies of tetramer positive cells detected from these donors remained low. In addition, with the exception of a single HLA-A*0301 donor, none of these donors demonstrated specific cytotoxic activity against HLA-matched CML target cells. Thus we were unable to generate functional BCR/ABL specific T cells from both CML patients and healthy donors using this protocol.

The generation of BCR/ABL specific T cells was also attempted using another type of DC as APCs, namely plasmacytoid DCs. pDCs, in contrast to mDCs have been shown to originate from lymphoid progenitors and thus may remain unaffected by the tumour pathogenesis (Briere et al. 2002; Grouard et al. 1997; Res et al. 1999). The role of pDCs in mediating anti-tumour responses is still controversial. Zou et al have shown that the infiltration of pDCs in ovarian carcinoma inhibited the generation of tumour specific T cells (Zou et al. 2001). However, pDCs have also been shown to induce melanoma specific $CD8^+$ T cell responses *in-vitro* (Salio et al. 2003).

A significantly reduced frequency of circulating pDCs was found in CML patients compared to healthy donors. Both quantitative and qualitative impairments of DCs, including pDCs have been described in AML patients (Mohty et al. 2001). We were unable to determine if this depletion is an active process or a dilutional effect resulting from the uncontrolled proliferation of myeloid cells. However the frequencies of pDCs detected from the peripheral blood of Imatinib mesylate treated patients were found to be restored and not significantly different to the frequencies detected in healthy donors. These data are in accordance with the recently published work of Mothy et al. and may suggest a role of pDCs in the control of CML disease and long-term anti-tumour responses (Mohty et al. 2004).

In our experiments the stimulation of lymphocytes with peptide-pulsed pDCs did not succeed in generating and expanding BCR/ABL specific T cells. Due to the limited numbers of pDCs, only two successive stimulations could be performed. Additionally because of the low frequency of pDCs detected in the peripheral blood of CML patients, it was only

possible to assess responses in healthy donors. It is unlikely that two stimulations are sufficient to stimulate tumour specific responses from these donors. In order to confirm their significance in anti-tumour surveillance, a larger number of donors should be assessed, including CML patients. This was unfortunately not possible within the time of this study.

Our data also indicates that the BCR/ABL peptide associated with the HLA-A*0301 molecule was more immunogenic than the BCR/ABL peptide associated with the HLA-B*0801 molecule. The expansion of antigen specific T cells was found to be reproducibly higher in the context of HLA-A*0301 than HLA-B*0801 molecules in healthy donors and to a lesser extent in CML patients. In addition, specific cytotoxicity against HLA-matched CML cells was only detectable from a HLA-A*0301 healthy donor. Some groups, including ourselves, have demonstrated the successful generation of BCR/ABL specific T cells in the context of HLA-A*0301 (Bocchia et al. 1996; Clark et al. 2001; Norbury et al. 2000; Yotnda et al. 1998), however only two groups have also shown BCR/ABL specific T cell responses in the context of HLA-B*0801 (Norbury et al. 2000; Yotnda et al. 1998). Thus, it appears that the HLA-A*0301 BCR/ABL associated epitope is more immunogenic.

Finally, the different stimulation protocols assessed in this chapter were not capable of stimulating and expanding functional BCR/ABL specific T cells from CML patients and from most of the healthy donors tested. The lack of antigen specific T cell responses may be a consequence of the BCR/ABL antigens poor immunogenicity. To analyse this, a comparative analysis between the responses to the BCR/ABL tumour specific peptides and the responses to other tumour associated peptides described in the context of CML was performed, this will be discussed in chapter 5. Conventional antigen stimulation protocols may also not be efficient enough for the expansion of low frequent tumour specific T cells *in-vitro*. Thus, artificial systems for the generation of functional BCR/ABL specific T cells were also developed and assessed, these will be discussed in chapter 6.

CHAPTER 5

Assessment of HLA-A*0201 restricted BCR/ABL tumour specific T cell responses and comparative analysis with other tumour associated antigens

INTRODUCTION

HLA-A2 is the most common allele within the caucasian population (i.e. average frequency of 30%, ANBMT database, Hazael Maldonado Torres personal communication). Therefore, BCR/ABL specific T cell responses were assessed in the context of this allele, namely HLA-A*0201. No BCR/ABL derived peptide(s) have yet been demonstrated to be processed and presented on the cell surface of CML cells in the context of the HLA-A*0201 molecule. Thus, three potential peptides were selected from the BCR/ABL (b3a2) fusion protein on the basis of their predicted binding affinity for the HLA-A*0201 molecule (as discussed in chapter 3) and from previous published data (Rezvani et al., 2003; Yotnda et al., 1998). These are GFKQSSKAL (referred to as CML1), KALQRPVAS (CML2) and SSKALQRPV (CML3). The feasibility of generating BCR/ABL specific T cells to these peptides was assessed from HLA-A*0201 CML patients and healthy donors. As the majority of these peptides were demonstrated to have a low binding affinity for the HLA-A*0201 molecule (chapter 3), the potential increase of peptide affinity for HLA-A*0201 by modifying an anchor residue in the sequence of the CML 3 peptide was also assessed. This peptide was selected from the study by Yontda et al. and was modified to contain a primary HLA-A*0201 anchor residue at position two (Leucine replacing a Serine) (Yotnda et al., 1998). This modified peptide was assessed for its capacity to induce specific immune responses in parallel to the wild type BCR/ABL peptide. Finally, to assess the immunogenicity of the BCR/ABL tumour specific peptides, a comparative analysis with a number of tumour-associated peptides that have been described in the context of CML was performed. As peptides derived from these tumour associated antigens have only been described in the context of the HLA-A*0201 molecule, such comparative analysis could only be performed for this allele.

Very few studies have assessed the relevance of the BCR/ABL (b3a2) tumour specific antigen in the context of HLA-A*0201. Recently however, a low frequency of GFKQSSKAL specific T cell precursors has been characterised from the peripheral blood of HLA-A*0201 CML patients at diagnosis and these were shown to significantly increase after HSCT (Rezvani et al., 2003). Also Yontda et al. demonstrated the capacity of the SSKALQRPV peptide to generate functional antigen specific $CD8^+$ T cells from both healthy donors and CML patients, which recognised HLA-matched primary tumour cells (Yotnda et al., 1998).

The substitution of a dominant anchor residue in the sequence of tumour associated antigens has been demonstrated in some cases, to significantly increase their HLA binding affinity and subsequently enhance their antigenic immunogenicity without losing the specificity for the wild-type tumour. Such modified peptides have been described for melanoma, colorectal or tumour associated antigens (Denkberg et al., 2002; Graff-Dubois et al., 2002; Keogh et al., 2001; Parkhurst et al., 1996; Scardino et al., 2002; Slansky et al., 2000; Tangri et al., 2001; Valmori et al., 1998; Vertuani et al., 2004). Although most of these studies have clearly demonstrated a correlation between the HLA/peptides affinity and the resulting peptide immunogenicity, some groups have also shown that modified peptides, which failed to enhance the binding affinity for the HLA molecules and thus were expected to have no effect on the peptide immunogenicity, still induced an increase in the magnitude of the antigen specific T cell response observed (Tangri et al., 2001; Vertuani et al., 2004). The slow dissociation rate of these peptides from the HLA molecules was shown to increase the half-live of the HLA/peptide complexes, which in turn was beneficial for the induction of antigen specific T cell responses. Thus, although the modified BCR/ABL peptide (SLKALQRPV) failed to increase significantly the binding affinity for the HLA-A*0201 molecule (described in Chapter 3), it may demonstrate a higher immunogenicity and maintain specificity for native CML cells recognition. The immunogenicity of this modified BCR/ABL peptide was assessed and compared to the wild type peptide.

As described in the previous chapter, the different antigen stimulation protocols, including the use of DCs failed to generate functional BCR/ABL specific T cells in CML patients and in most of the healthy donors assessed. The lack of responses could have been due to the poor immunogenicity of BCR/ABL tumour specific antigens. To investigate these issues, we assessed the immunogenicity of the BCR/ABL tumour specific peptides in comparison to various tumour associated peptides. As no tumour associated antigens have been described in the context of either HLA-A*0301 or HLA-B*0801 molecules at the time of this study, this comparative study could not be performed for these HLA molecules. A number of

tumour associated antigens have however been described in the context of HLA-A*0201 molecules, including the Wilms' tumour (WT1), proteinase 3 (PR1) and human telomerase catalytic sub/unit (hTERT) antigens (Molldrem et al., 1996; Oka et al., 2000; Vonderheide et al., 1999). These antigens have been demonstrated to be expressed by leukaemic cells and induce antigen specific T cell responses in leukaemic patients. WT1 and PR1 tumour specific $CD8^+$ T cells have been shown to arise spontaneously in AML patients (Scheibenbogen et al., 2002). More recently, a low frequency of both WT1 and PR1 specific T cells was detected in the peripheral blood of CML patients at diagnosis (Molldrem et al., 2000; Rezvani et al., 2003). Their significant increase after HSCT and correlation with clinical cytogenetic responses, at least in the context of the PR1 peptide, strongly suggests their contribution to the GvL response. Additionally, both antigens have been shown to be immunogenic as these generated functional antigen specific $CD8^+$ T cells from healthy donors (Bellantuono et al., 2002; Molldrem et al., 1997; Molldrem et al., 1999; Oka et al., 2000). Thus, these tumour associated peptides are ideal for comparative analysis with the BCR/ABL tumour specific peptides in the context of CML. Another HLA-A*0201 associated tumour antigen, the telomerase catalytic sub/unit hTERT, has been demonstrated to efficiently stimulate and expand anti-tumour specific T cells from both healthy donors and cancer patients and was also included in the study (Vonderheide et al., 2001).

The aims of this chapter are to assess BCR/ABL specific T cell responses in the context of HLA-A*0201. The increase of the SSKALQRPV peptide immunogenicity following the modification of one of its anchor residue (SLKALQRPV) was also examined. Finally a comparative analysis of the efficiency of BCR/ABL derived peptides and WT1, PR1 or hTERT peptides to sensitize $CD8^+$ T cells from both CML patients and healthy donors was performed to assess the immunogenicity and subsequently the relevance of the tumour specific BCR/ABL antigen for the development of immunotherapeutic strategies to treat CML in the context of HLA-A*0201.

RESULTS

5.1 Assessment of BCR/ABL tumour specific responses in HLA-A*0201 CML patients and healthy donors:

In the context of HLA-A*0201, the BCR/ABL specific immune responses were assessed for the three selected 9-mer peptides, which are GFKQSSKAL (refer to as CML1 peptide), KALQRPVAS (CML2 peptide) and SSKALQRPV (CML3 peptide). HLA-A*0201 tetramers, refolded with these three candidate epitopes were generated and assessed for their correct refolding as described and shown in chapter 2. A low frequency of BCR/ABL specific $CD8^+$ T cells were detected from the peripheral blood of both HLA-A*0201 CML patients and healthy donors (shown in Chapter 3). To determine the relevance of these BCR/ABL candidate epitopes *in-vivo*, HLA-A*0201 restricted T cell responses to autologous mDCs pulsed with the 17-mer BCR/ABL derived peptide (ATGFKQSSKALQRPVAS) were assessed. In this case, the presentation of BCR/ABL epitope(s) associated with the HLA-A*0201 molecule relies totally on endogenous antigen processing. In addition, this longer peptide was shown to enhance BCR/ABL specific T cell responses in the context of HLA-A*0301 and HLA-B*0801, possibly via the presentation of other BCR/ABL derived epitopes in the context of HLA class I and/or HLA class II molecules. Antigen specific $CD8^+$ T cell responses were monitored by staining the activated T cells with the three different HLA-A*0201/BCR-ABL tetramers.

Monocyte-derived DCs were generated from HLA-A*0201 CML patients and healthy donors as described in chapter 2. Prior to their use as APCs, these mDCs were assessed for the expression of maturation markers as shown in chapter 4, section 4.3.1. Fully matured mDCs, pulsed with the 17-mer BCR/ABL peptide were incubated with autologous responder cells (non-adherent lymphocytes) at a ratio of 1 in 10 on day 1 and 9. A third and last stimulation was performed on day 18 with autologous peptide-pulsed PBMCs, irradiated and incubated at a ratio of 1 in 5. IL-2 and IL-15 cytokines were added to the cultures three days after each stimulation as described in chapter 2. Responder cells were also stimulated without antigen (No peptide, negative control). Antigen specific T cells responses were monitored by staining the activated T cells with the CML1, CML2 and CML3 HLA-A*0201/tetramers three days after each stimulation. The feasibility of APCs pulsed with the 17-mer BCR/ABL peptide to generate antigen specific T cells was assessed from eight CML patients (described in **Table 4.2**, BCR-ABL DC study*****) and five healthy donors. The highest frequencies of tetramer positive T cells generated from these donors were observed

after three antigen stimulations and are shown in **Figure 5.1**. The frequencies of BCR/ABL specific T cells generated with this protocol were compared between CML patients and healthy donors (**Figure 5.1**, panel **A** and **B**). A representative HLA/tetramer staining obtained from a CML patient and from a healthy donor is shown in **Figure 5.2**, panel **A** and **B** respectively.

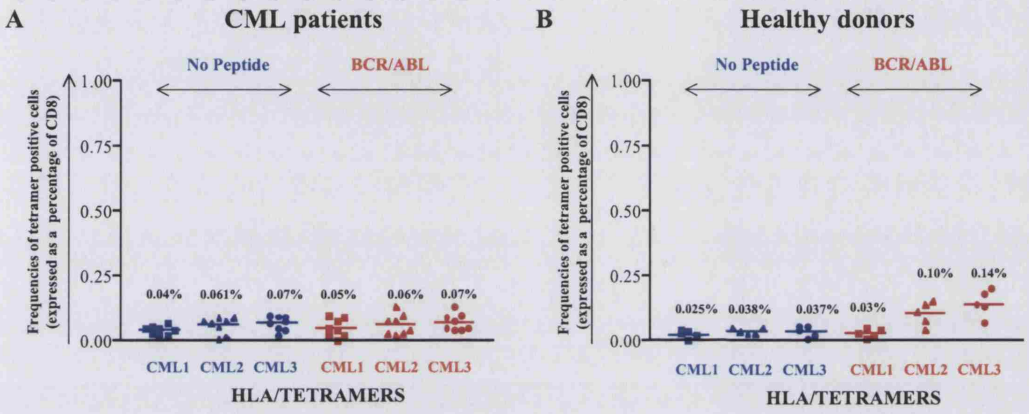


Figure 5.1: BCR/ABL peptide specific T cell responses stimulated from HLA-A*0201 CML patients and healthy donors:

Non-adherent lymphocytes derived from eight HLA-A*0201 CML patients (panel **A**) and five HLA-A*0201 healthy donors (panel **B**) were stimulated with autologous mDCs pulsed with the 17-mer BCR/ABL peptide (**BCR/ABL**) on day 1 and day 9. A third and last stimulation was performed with autologous irradiated peptide-pulsed PBMCs on day 18. IL-2 and IL-15 was added three days after the first two stimulations and two days after the last stimulation. Lymphocytes were also stimulated with un-pulsed APCs (**No Peptide**). Activated T cells were harvested three days after the last stimulation (day 21) and stained with the **CML1** (GFKQSSKAL), **CML2** (KALQRPVAS) and **CML3** (SSKALQRPV) tetramers. The frequency of antigen specific T cells following stimulation without antigen (**No Peptide**) or with antigen (**BCR/ABL**) was assessed by flow cytometry. BCR/ABL peptide specific T cells are expressed as a percentage of $CD3^{+}CD8^{+}$ T cells and the bar mean values are shown for each HLA/tetramer.

HLA-A*0201 restricted CMV specific or Flu specific T cells (from CMV seropositive and seronegative donors respectively) were primed in parallel following the same protocol when a sufficient number of mDCs were available. In this example, the efficiency of the CML patient mDCs as APCs, as well as the capacity of CML patient T cells to respond to antigenic stimulation were assessed in the context of the HLA-A*0201 Flu associated peptide, as this patient was CMV seronegative. The expansion of Flu or CMV specific T cells was assessed with the appropriate HLA/tetramer after the last antigen stimulation and is shown in **Figure 5.2**, panel **A** and **B** respectively, fourth panel. High frequencies of Flu

and CMV specific $CD8^+$ T cells were detected after stimulation with peptide-pulsed mDCs from both the CML patient and the healthy donor (1.45% and 2.17% respectively, **Figure 5.2**, panel **A** and **B**). This stimulation protocol successfully induced over 4.8 and 7 fold expansion of Flu and CMV specific T cells compared to the frequencies detected in the negative controls (**Figure 5.2**, No Peptide). Thus, the mDC generated from these donors, including CML patients are capable of presenting HLA/peptide complexes and inducing the expansion of antigen specific T cells, at least in the context of viral antigens. These data also demonstrate that CML patient T cells are able to respond to antigenic stimulation and generate virus specific T cell responses.

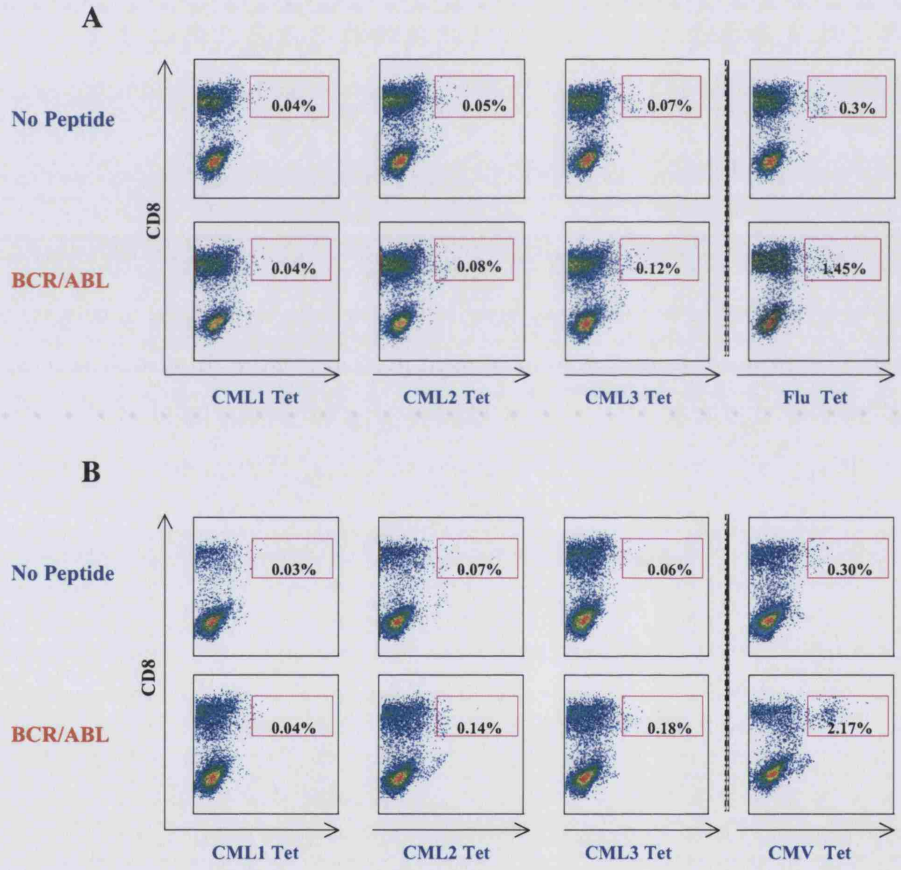


Figure 5.2: Representative HLA/tetramer staining of stimulated HLA-A*0201 BCR/ABL peptide specific T cells:

PBMCs derived from HLA-A*0201 CML patient (panel **A**, CML patient 35) and healthy donor (panel **B**) were stimulated with autologous APCs un-pulsed (**No Peptide**) or pulsed with the 17-mer BCR/ABL peptide (**BCR/ABL**) as described in chapter 2. The frequency of antigen specific T cells generated after three stimulations (day 21) was assessed with HLA-A*0201 tetramers refolded with the GFKQSSKAL (**CML1 tet**), KALQRPVAS (**CML2 tet**) and SSKALQRPV BCR/ABL peptides (**CML3 tet**) by flow cytometry. In parallel, HLA-A*0201 Flu (CML patient, panel **A**, last column) or CMV (CMV^+ healthy donor, panel **A**, last column) specific T cell responses were generated following the same protocol. Antigen specific T cells are expressed as a percentage of $CD3^+CD8^+$ T cells and are shown in the gate of each dot plot.

The stimulation of CML patient lymphocytes with BCR/ABL pulsed mDCs was unsuccessful in generating or expanding antigen specific T cells (**Figure 5.1**, panel **A**). The frequencies of BCR/ABL specific T cells detected with the three different CML/tetramers after antigen stimulation were reproducibly low in these patients and were not found to be statistically significant compared to the negative controls (mean from 0.05% to 0.07%, P>0.05, Wilcoxon signed rank test, **Figure 5.1** and **Figure 5.2**, panels **A**). In healthy donors, no GFKQSSKAL specific T cells were detected with the CML1 tetramer after stimulation with BCR/ABL pulsed mDCs (mean of 0.03%, **Figure 5.1**, panel **B**, BCR/ABL, CML1). Variable frequencies of antigen specific T cells were detected in the cultures from healthy donors with the CML2 and with the CML3 tetramers (mean of 0.10% and 0.14%, **Figure 5.1**, panel **B**). The frequencies of BCR/ABL specific T cells detected with these tetramers remained however low, even after three antigen stimulations and these were not found to be statistically significant compared to the negative controls (No peptide, P>0.05, Wilcoxon signed rank test). Thus, the stimulation of lymphocytes with the 17-mer BCR/ABL peptide-pulsed mDCs did not succeed in generating and expanding any HLA-A*0201 BCR/ABL specific $CD8^+$ T cells from the CML patients or the healthy donors assessed. Additionally, a substantial $CD8^-$ $Tetramer^+$ background was observed in some of these cultures, which in some cases reached the frequencies of the $CD8^+Tetramer^+$ T cells (**Figure 5.2**).

The highest frequencies of BCR/ABL specific T cells after stimulations were detected with the CML3 tetramer from healthy donor cultures (**Figure 5.1**, panel **B,** BCR/ABL, CML3). Although these frequencies remained low, these were found to be statistically significant compared to the frequencies detected in CML patients (P=0.0186, Mann-Whitney U test). Additionally, the SSKALQRPV peptide has been shown in a previous study to generate functional BCR/ABL specific T cells from HLA-A*0201 donors (Yotnda et al., 1998). This peptide was therefore selected and modified to contain the primary anchor residue for the HLA-A*0201 molecule (Leucine at position two). This modified peptide (SLKALQRPV, refer to as CML4) although slightly increases the peptide affinity for the HLA-A*0201 molecule, it may demonstrate a lower dissociation rate and subsequently HLA/peptide complexes may be presented longer to the T cells, thus increasing the peptide immunogenicity.

5.2 Assessment of wild-type and modified BCR/ABL peptides immunogenicity in the context of HLA-A*0201 molecules:

The efficiency of the modified BCR/ABL peptide (CML4) in generating antigen specific T cell responses were assessed from HLA-A*0201 CML patients and healthy donors and were compared to the wild-type BCR/ABL peptide (CML3) responses. Lymphocytes were stimulated with fully matured autologous mDCs and PBMCs (third stimulation) following the same protocol as described in section 5.1. In this case, APCs were pulsed with the 9-mer BCR/ABL wild type (CML3) or modified (CML4) peptides. HLA-A*0201 tetramers were generated for both CML3 and CML4 peptides and were used to monitor the frequencies of antigen specific T cell responses from these cultures. Tetramers were checked for correct HLA/peptide refolding as shown in chapter 2. BCR/ABL specific T cell responses to these antigens were assessed from nine CML patients (described in **Table 4.2**, TAA DC study*****) and six healthy donors. The frequencies of BCR/ABL specific T cells generated from CML patients and healthy donors were assessed after the last stimulation (day 21) with the CML3 and CML4 tetramers and are shown in **Figure 5.3**, panel **A** and **B** respectively. A representative HLA/tetramer staining obtained from a CML patient and a healthy donor is also shown in **Figure 5.4**, panel **A** and **B**.

The antigen specific T cell responses generated after the different stimulations were first assessed with the CML3 tetramers (wild-type peptide, squares). The stimulation of CML patients and healthy donors with the wild-type CML3 peptide did not succeed in expanding antigen specific T cells from both CML patients and healthy donors (**Figure 5.3** and **Figure 5.4**, CML3, CML3tet). The frequencies of BCR/ABL specific T cells detected after stimulations were low and not statistically significant compared to the frequency detected in the negative controls ($\leq$0.28% tetramer positive cells, $P>0.05$, Wilcoxon signed ranked test). Pulsing the APCs with the 9-mer or with the 17-mer BCR/ABL peptides (section 5.1) consistently failed to generate antigen specific T cell responses from these donors, as detected with the CML3 tetramer. The possible reasons for the lack of responses will be discussed later.

The potential enhancement of tumour specific T cell responses was then assessed by stimulating lymphocytes with mDCs pulsed with the modified CML4 peptide. Variable frequencies of CML3 tetramer positive T cells were detected after stimulation, from both CML patients and healthy donors (mean of 0.13% $\pm$0.16% and mean of 0.20% $\pm$0.13% respectively, **Figure 5.3** and **Figure 5.4**, CML4 peptide, CML3tet). These frequencies were

slightly higher in all donors compared to the frequencies detected after stimulations with the wild-type CML3 peptide, the differences were however not found to be statistically significant (P>0.05, Wilcoxon signed rank test). Thus, the modification of this peptide at an anchor residue position was not demonstrated to increase the magnitude of the BCR/ABL specific T cell responses from our cohort of donors.

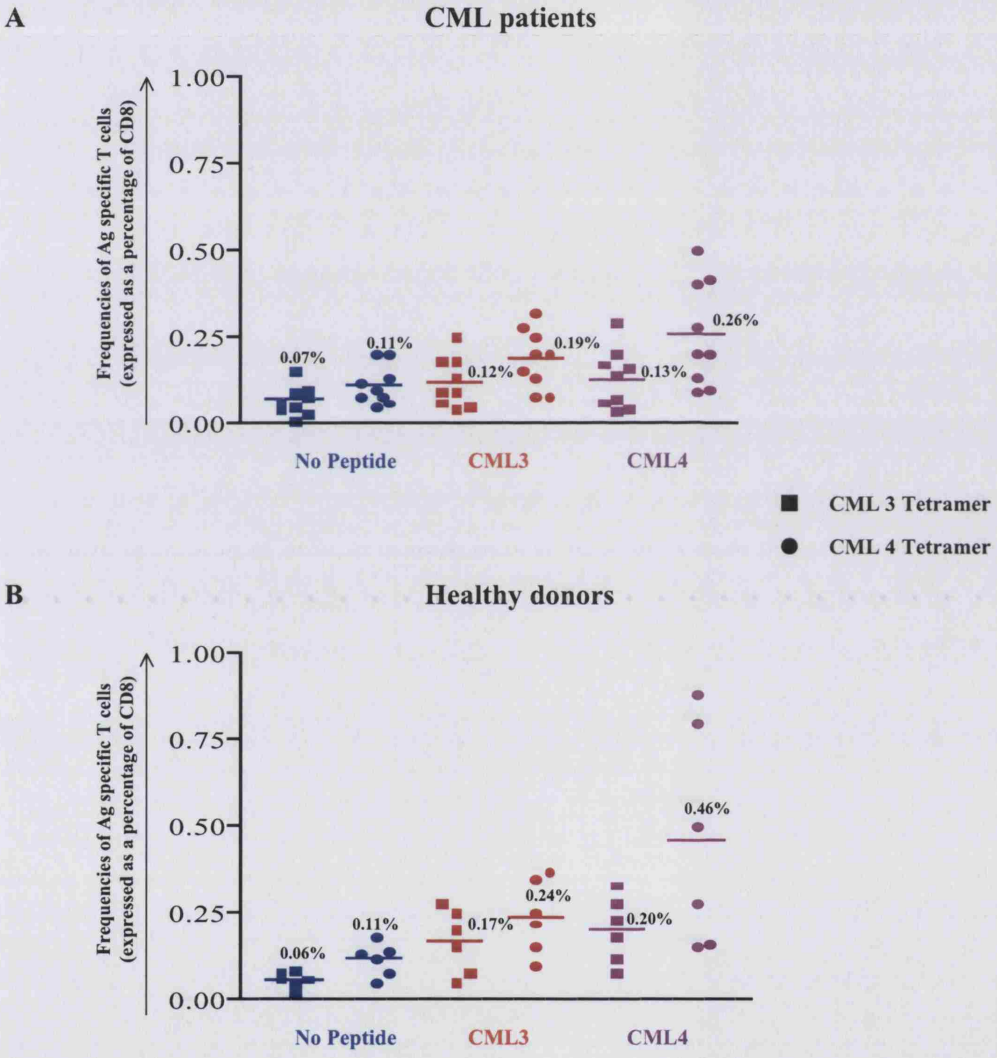


Figure 5.3: Peptide specific T cell responses to the HLA-A*0201 wild type and modified BCR/ABL peptide stimulation in CML patients and healthy donors:

Non adherent lymphocytes derived from nine HLA-A*0201 CML patient (panel **A**) and six HLA-A*0201 healthy donors (panel **B**) were stimulated either with autologous APCs un-pulsed (**No Peptide**), pulsed with 9-mer wild type (SSKALQRPV, **CML3**) or modified peptides (**SL**KALQRPV, **CML4**) as described in chapter 2. Activated T cells were harvested three days after the last stimulation (day 21) and stained with the **CML3** (SSKALQRPV, ■) and **CML4** HLA/tetramers (SLKALQRPV, ●). The frequencies of antigen specific T cells following stimulations were assessed by flow cytometry. BCR/ABL peptide specific T cells are expressed as a percentage of $CD3^{+}CD8^{+}$ T cells and the bar mean values are shown for each condition.

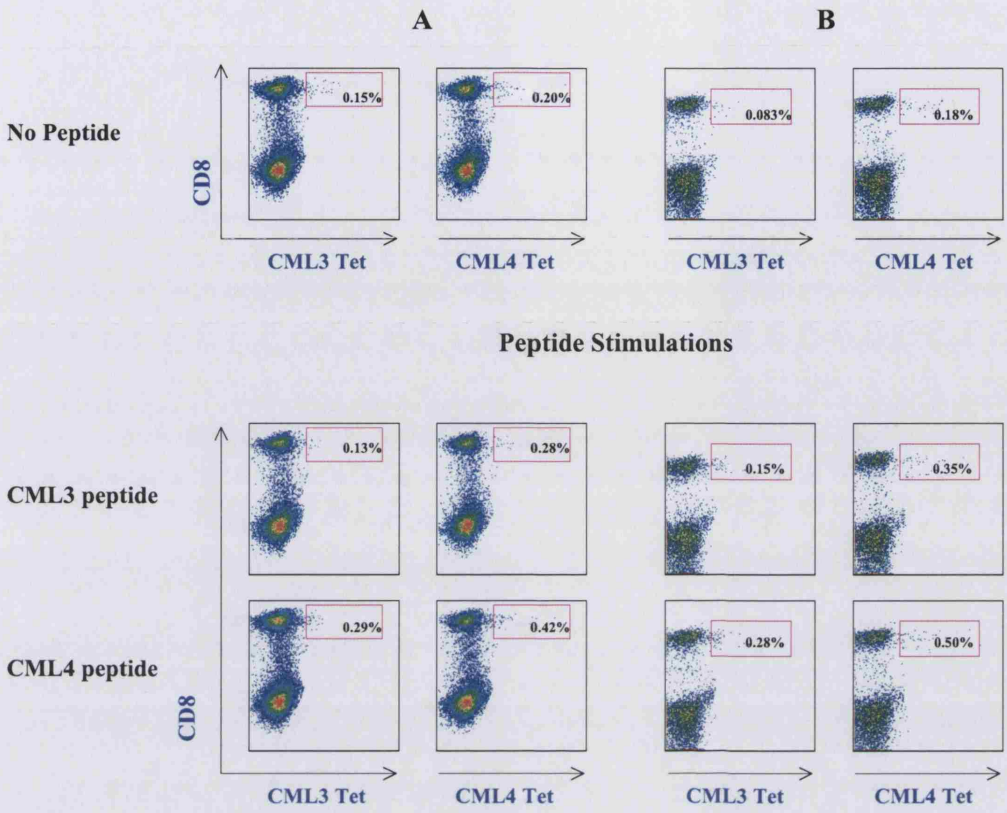


Figure 5.4: Representative HLA/tetramer staining of stimulated HLA-A*0201 wild-type (SSKALQRPV) and modified (SLKALQRPV) BCR/ABL peptide specific T cells:

The immunogenicity of BCR/ABL derived 9-mer wild-type (SSKALQRPV, CML3) and modified peptides (SLKALQRPV, CML4) was assessed from HLA-A*0201 CML patient (panel **A**, CML patient 31) and healthy donor (panel **B**). Lymphocytes were stimulated with autologous APCs un-pulsed (**No Peptide**) or pulsed with the different BCR/ABL peptides (**CML3** and **CML4**) following the same protocol as described in chapter 2. HLA-A*0201 tetramers refolded with the wild type (**CML3**) and modified (**CML4**) BCR/ABL peptides were generated and were used to assess the frequency of antigen specific T cells generated after three stimulations (day 21) by flow cytometry. BCR/ABL peptide specific T cells are expressed as a percentage of $CD3^{+}CD8^{+}$ T cells and are shown in the gate of each dot plots.

The antigen specific T cell responses following the different stimulations were also monitored with the HLA tetramers refolded with this modified peptide (CML4 tetramers). Although the CML4 peptide did not increase the magnitude of the BCR/ABL specific responses as detected with the CML3 tetramers, the numbers of antigen specific T cells detected with the CML4 tetramers were higher than the numbers detected with the CML3 tetramers, in both CML patients and healthy donors (**Figure 5.3** and **Figure 5.4**). The numbers of tetramer positive T cells detected with the CML4 tetramers varied a lot between the different healthy donors assessed, consequently these were not found to be statistically

different to the frequencies detected with the CML3 tetramers (P>0.05, Wilcoxon signed rank test). In CML patients however, the frequencies detected with the CML3 tetramers were consistently negligible, thus the difference in this case were found to be statistically significant (P<0.05, Wilcoxon signed rank test).

Since the numbers of tetramer positive T cells detected with the CML4 tetramer also increased in the negative controls, the responses to the wild-type CML3 peptide generated from both CML patients and healthy donors remained statistically not significant (P>0.05, Wilcoxon signed rank test). In contrast, the responses to the modified CML4 peptide were found to be significantly higher compare to the negative controls, when detected with the CML4 tetramers (P=0.0078 and P=0.0312 in CML patients and healthy donors respectively, Wilcoxon signed rank test, **Figure 5.3**, CML4 peptide, CML4 tet). These responses were however not found to be statistically different to the wild-type CML3 peptide responses (P=0.4258 and P=0.3125 respectively). Thus the modified CML4 peptide failed to increase the magnitude of the BCR/ABL specific T cell response when detected with either CML3 or CML4 HLA/tetramers.

It appears however that the CML4 tetramers have increased the detection sensitivity of antigen specific T cells by HLA/tetramer staining. The function of these CML4 peptide specific T cells remained to be assessed to verify the authenticity of these cells. In fact, it is possible that the CML4 peptide has a lower dissociation off-rate, which failed to induce antigen specific T cell signalling but may increase the antigen specific T cell binding. However the HLA/CML4 complexes may also recognize and bind T cells of different antigen specificities and/or of cross-reactive specificities, thus erroneously increasing the numbers of tetramer positive cells detected.

The cytotoxic activity of the antigen specific T cells stimulated with the wild-type (CML3) and the modified (CML4) BCR/ABL peptides was assessed three days after the last stimulation (day 21), in parallel with HLA/tetramer staining. Due to the limited number of lymphocytes expanded in some cultures, the cytotoxic activity of activated T cells could only be assessed from four CML patients and three healthy donors. BCR/ABL peptide activated T cells were tested for their specific recognition and killing of HLA-A*0201 b3a2 CML target cells in a ^{51}Cr cytotoxicity assay as described in chapter 2.

None of the BCR/ABL stimulated T cells demonstrated any cytotoxic activity against CML target cells above the background (HLA-unmatched and b2a2 CML cells, tested at a ratio of

10 to 1). The frequencies of BCR/ABL specific T cells detected from these donors after peptide stimulations were low and may have accounted for the lack of cytotoxic activity (<0.3% of tetramer positive cells detected with the CML3 tetramer, **Figure 5.3** and **Figure 5.4**).

In conclusion, both healthy donors and CML patients were shown to be capable of generating antigen specific T cell responses to viral antigens following the same protocol (**Figure 5.2**). However none of these donors demonstrated functional antigen specific T cell responses to the selected wild-type or modified BCR/ABL peptides. The lack of responses may be due to a number of factors, which will be further examined in the discussion section.

5.3 Assessment of Tumour associated antigens immunogenicity in the context of HLA-A*0201 molecules:

5.3.1: Generation of Tumour associated antigen responses in HLA-A*0201 CML patients and healthy donors:

In order to assess the relevance of the BCR/ABL tumour specific antigen for immunotherapeutic applications, a comparative analysis with other tumour antigens was undertaken. In the context of CML, no tumour associated antigens have been described in association with HLA-A*0301 and/or HLA-B*0801 molecules at the time of the study. Thus, it was not possible to perform a comparative analysis in the context of these alleles. As mention earlier a number of tumour antigens have been described in the context of the HLA-A*0201 molecules (WT1, PR1 and hTERT). However no antigens derived from the b3a2 BCR/ABL fusion protein region had been reported to be presented and capable of generating functional antigen specific T cells in the context of HLA-A*0201, thus a comparative analysis could not be performed for this allele.
The responses to the tumour associated antigens were however still assessed following the same stimulation protocol. This would provide confirmation that the stimulation protocol was able to stimulate tumour antigen specific T cell responses, at least in the context of the TAAs cited above. Additionally, this would also serve to demonstrate that CML patients are capable of expanding low frequent tumour antigen specific T cells and generating specific immune responses.

The tumour associated antigen specific responses to WT1, PR1 and hTERT peptides were generated from the same nine CML patients (described in **Table 4.2**, TAA DC study*****) and the six healthy donors in parallel with the wild-type CML3 and the modified CML4 BCR/ABL peptides responses. Fully matured autologous mDCs were pulsed with the different antigenic peptides and were assessed for their ability to generate antigen specific T cell responses following the same protocol as described in section 5.1. The responses were monitored three days after each stimulation by staining the activated T cells with the appropriate HLA/tetramers. The highest frequencies of TAA specific T cells generated from our donors were also detected after at least three antigen stimulations (day 21) and are shown in **Figure 5.5**. The responses to these tumour associated antigens were compared between CML patients and healthy donors (**Figure 5.5**, panel **A** and **B** respectively). The responses of these donors to the wild-type CML3 and to the modified CML4 BCR/ABL

peptides are also shown. As negative controls, lymphocytes were also incubated with un-pulsed mDCs (No peptide, blue dots). A representative HLA/tetramer staining obtained from a CML patient and a healthy donor is shown in **Figure 5.6**, panel **A** and **B**.

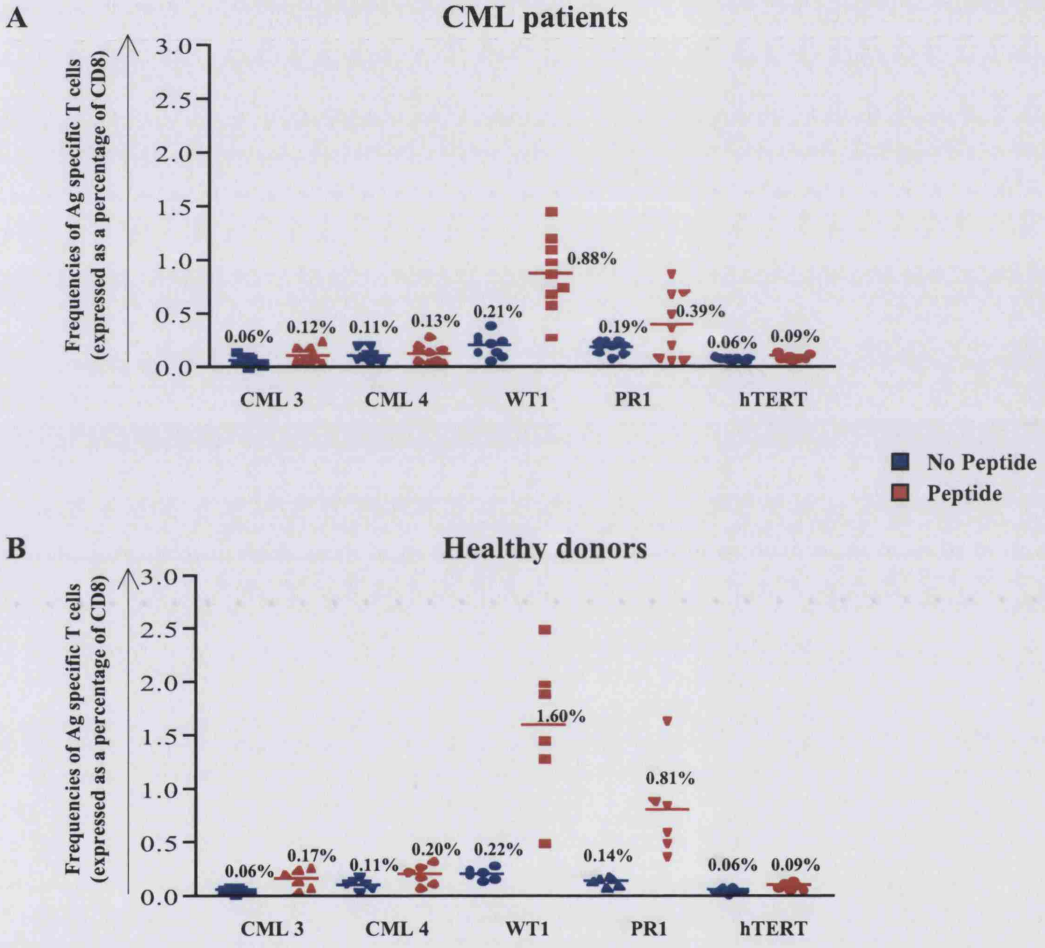


Figure 5.5: Tumour specific (CML3, CML4 BCR/ABL) and Tumour associated (WT1, PR1, hTERT) T cell responses stimulated in HLA-A*0201 CML patients and healthy donors:

Non adherent lymphocytes derived from nine HLA-A*0201 CML patients (panel **A**) and six HLA-A*0201 healthy donors (panel **B**) were stimulated following the same protocol as described in chapter 2. Autologous APCs were wither un-pulsed (■), pulsed (■) with the wild type (SSKALQRPV, **CML 3**) and modified (SLKALQRPV, **CML 4**) BCR/ABL peptides or with Wilms' tumour (**WT1**), proteinase 3 (**PR1**) and human telomerase (**hTERT**) associated antigenic peptides. Activated T cells were harvested three days after the last stimulation (day 21) and stained with the appropriate HLA/peptide tetramers. The frequencies of antigen specific T cells were assessed by flow cytometry. These are expressed as a percentage of $CD3^{+}CD8^{+}$ T cells and the bar mean values are shown for each condition.

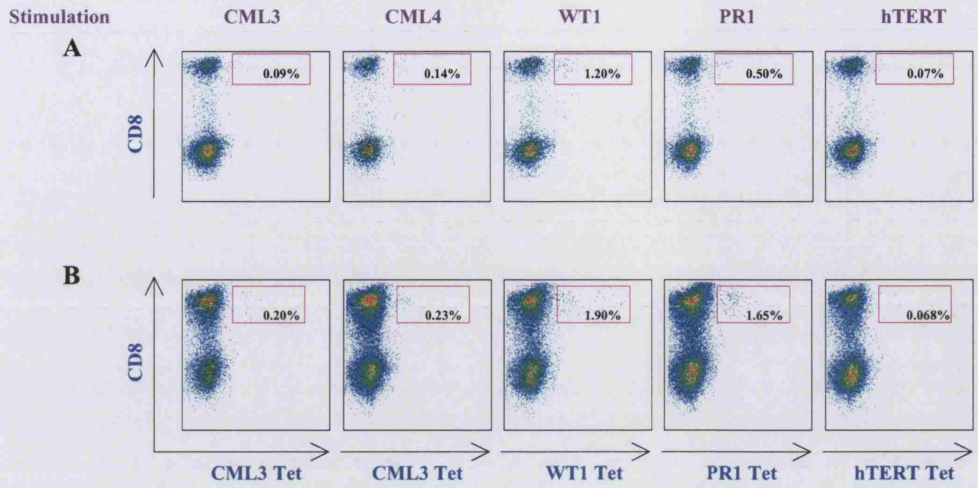


Figure 5.6: Representative HLA/tetramer staining of stimulated HLA-A*0201 Tumour specific (CML3, CML4) and Tumour Associated (WT1, PR1, hTERT) specific T cell responses:

The immunogenicity of the tumour specific BCR/ABL wild-type (SSKALQRPV, CML3) and modified peptides (SLKALQRPV, CML4) was compared with the immunogenicity of the tumour associated peptides from Wilms' tumour (RFMPNAPYL, WT1), proteinase 3 (VLQELNVTV, PR1) and human telomerase subunit (ILAKFLHWL, hTERT). Lymphocytes derived from a CML patient (panel A, CML patient 37) and from a healthy donor (panel B) were stimulated with peptide-pulsed APCs following the same protocol as described in chapter 2. HLA-A*0201 tetramers refolded with the different peptides were generated and were used to assess the frequency of antigen specific T cells generated after three stimulations (day 21) by flow cytometry. Antigen specific T cells are expressed as a percentage of $CD3^{+}CD8^{+}$ T cells and are shown in the gate of each dot plot.

The stimulation of lymphocytes with the WT1 and the PR1 peptides induced very variable antigen specific $CD8^{+}$ T cell responses from both CML patients (mean of 0.88%±0.6% and 0.39%±0.49% respectively) and healthy donors (mean of 1.60%±1.1% and 0.81%±0.84%, **Figure 5.5** and **Figure 5.6**). These responses were found to be statistically significant compared to the negative controls, at least in the context of the WT1 peptide in CML patients (P=0.0039, Wilcoxon signed rank test) and for both WT1 and PR1 peptides in healthy donors (P=0.0312 for both peptides, Wilcoxon signed rank test). None of the donors assessed generated antigen specific T cell responses to the hTERT peptide (mean of 0.09% ±0.06% in both CML patients and healthy donors, **Figure 5.5**, panel **A** and **B**). These data clearly demonstrate the competence of this stimulation protocol to expand low frequent PR1 and/or WT1 specific T cells from both CML patients and healthy donors.

The majority of patients included in this study received an alloHSCT and experienced at least a partial clinical response (6 out of 9 patients, **Table 4.2**, TAA DC study*****). The three remaining patients were treated with Imatinib mesylate or autoHSCT and to the exception of patient 34, failed to respond to the treatment. These patients demonstrated the lowest expansion of WT1 and PR1 specific T cells as measured with HLA/tetramers (≤0.68% and ≤0.09% respectively, **Figure 5.5**). Thus, these data suggest that the significant TAA specific T cell responses generated from some CML patients may be donor-derived.

The responses to TAA peptides were significantly higher than the responses generated to the wild-type CML3 or to the modified CML4 BCR/ABL peptides. The frequencies of WT1 specific T cells generated from both CML patients and healthy donors were found to be statistically significant compared to the frequencies of BCR/ABL peptide specific T cells detected in the stimulated cultures (P=0.0039 and P=0.0312 respectively, Wilcoxon ranked signed test, **Figure 5.5** and **Figure 5.6**). In the context of the PR1 peptide, only the responses generated in healthy patients were also found to be statistically significant compared to the BCR/ABL peptides responses (P=0.0312, Wilcoxon ranked test, **Figure 5.5** and **Figure 5.6**, panels **B**). The tumour associated WT1 and to some extent PR1 antigens appears to induce a greater expansion of antigen specific T cells than the selected HLA-A*0201 BCR/ABL peptides.

Higher numbers of both WT1 and PR1 specific T cells were generated from healthy donors than from CML patients (mean of 1.60%±1.1% and 0.81%±0.84% versus mean of 0.88%±0.6% and 0.39%±0.49% respectively, **Figure 5.5**). Only the responses to the WT1 peptide were however found to be statistically different (P=0.036, Mann-Whitney U test). Some of these patients were demonstrated to successfully generate antigen specific T cells in the context of viral antigens following the same protocol (section 5.1). Thus, as observed in the context of the BCR/ABL antigen (chapter 4), it is possible that patients are less capable to generate tumour specific T cell responses. This may be due to tolerance or anergic mechanisms, which will be discussed later.

5.3.2 Cytotoxic activity of antigen specific T cells stimulated with the tumour specific and the tumour associated peptides:

The cytotoxic activity of the antigen specific T cells generated after stimulation with peptides from the different tumour associated antigens or with the BCR/ABL specific CML3 and CML4 peptides was assessed three days after the last stimulation (day 21) in parallel to HLA/tetramers staining. Activated T cells were harvested and tested for the specific recognition and killing of peptide-pulsed T2 target cells in a ^{51}Cr cytotoxicity assay as described in chapter 2. Due to the low numbers of antigen specific T cells generated in some of these cultures, the specific cytotoxic activity could only be assessed from three CML patients and four healthy donors.

T2 cells are TAP deficient and thus lack endogenous peptide transport into the endoplasmic reticulum for HLA molecules loading. This results in a low cell surface expression of HLA-A*0201 molecules. As shown in chapter 3, the stabilisation of HLA molecules on the cell surface of T2 cells is dependent on the peptide affinity for the HLA-A*0201 molecule and on the peptide incubation time. Thus T2 cells were pulsed with the different antigenic peptides overnight and for an additional 8 hours (peak of HLA expression for these peptides, shown in chapter 3). Additionally, the cytotoxic activity of the generated antigen specific T cells was assessed using two peptides concentrations to pulse the T2 target cells as described in chapter 2.

Some PR1 and/or WT1 specific cytotoxic activity was observed when pulsing the T2 cells at the highest peptide concentration from two out of the three CML patients and three out of the four healthy donors assessed. These responses were found to be specific as the CTLs activities were above the activity detected against the control T2 target cells (un-pulsed or pulsed with the irrelevant Flu peptide). The percentage of specific killing and the corresponding frequencies of antigen specific T cells detected from these donors are shown in **Table 5.1**. A representative cytotoxic assay obtained from a CML patient and from a healthy donor is also shown in **Figure 5.7**, panel **A** and **B** respectively.

The highest cytotoxic activities against WT1 pulsed T2 target cells were observed from healthy donors (from 6.90% ±5.00% to 9.45% ±6.05% above the negative controls, **Table 5.1** and **Figure 5.7**, panel **B**). These healthy donors also generated some of the highest frequencies of WT1 specific T cells after antigen stimulation (from 1.45% to 1.99%, **Table 5.1** and **Figure 5.5**, panel **B**). Thus, the frequencies of antigen specific T cells generated

corresponded to some degree, to the specific cytotoxic activity detected. Unfortunately, the healthy donor who generated the highest frequencies of WT1 specific T cells had insufficient number of lymphocytes to test for specific cytotoxic activity and confirm these observations.

DONORS	*WT1 PEPTIDE*		*PR1 PEPTIDE*	
	% peptide specific killing*	**% tetramer positive cells****	**% peptide specific killing***	**% tetramer positive cells****
CML patient 37†	5.95%±2.85%	1.20%	2.50%±2.50%$^{+}$	0.50%
CML patient 38	1.50%±2.00%$^{+}$	1.10%	1.00%±0.75%	0.70%
Healthy donor 1†	9.45%±6.05%	1.90%	4.35%±1.85%	1.65%
Healthy donor 2	7.50%±4.90%	1.99%	3.15%±2.05%	0.85%
Healthy donor 3	6.90%±5.00%	1.45%	2.00%±1.50%	0.90%

Table 5.1: Peptide specific cytotoxic activity detected from WT1 and PR1 specific T cells generated from HLA-A*0201 CML patients and healthy donors:

PR1 and/or WT1 specific cytotoxic activity against peptide-pulsed T2 target cells (using the highest peptide concentration) was detected from the stimulated cultures derived from two out of three CML patients (patients 37 and 38) and three out of four healthy donors. The cytotoxic activity was measure in a ^{51}Cr release assay as described in chapter 2 and in **Figure 5.7**. *The percentage of peptide specific killing represents the ratio subtraction of the percentage of cytotoxic activity detected above the negative controls (T2 target cells un-pulsed or pulsed with the irrelevant Flu peptide) with the mean standard deviation between triplicate samples. **The percentage of antigen specific T cells detected from these cultures with the appropriate HLA/tetramers is also shown. $^{+}$No specific cytotoxic activity above the negative controls was detected from these samples. † The cytotoxic activity observed from these donors are also represented in **Figure 5.7**.

WT1 peptide specific cytotoxic activity was also observed from one out of the three CML patients assessed (5.95% ±2.85%, CML patient 37, **Table 5.1** and **Figure 5.7**, panel **A**). This patient generated 1.20% of WT1 specific T cells after stimulation as shown in **Figure 5.6**, panel **A**. Some patients who successfully expanded WT1 specific T cells to a frequency of 0.99% and 1.10% did not demonstrate specific cytotoxic activity against WT1 pulsed T2 cells, above that of the negative controls (un-pulsed T2 cells or pulsed with the irrelevant Flu peptide). The specific recognition and killing of peptide-pulsed target cells may require a minimum frequency of antigen specific T cells. Unfortunately the patient who generated the highest frequency of WT1 specific T cells could not be assessed due to the limited number of CTLs generated (1.45%, **Figure 5.5**, panel **A**).

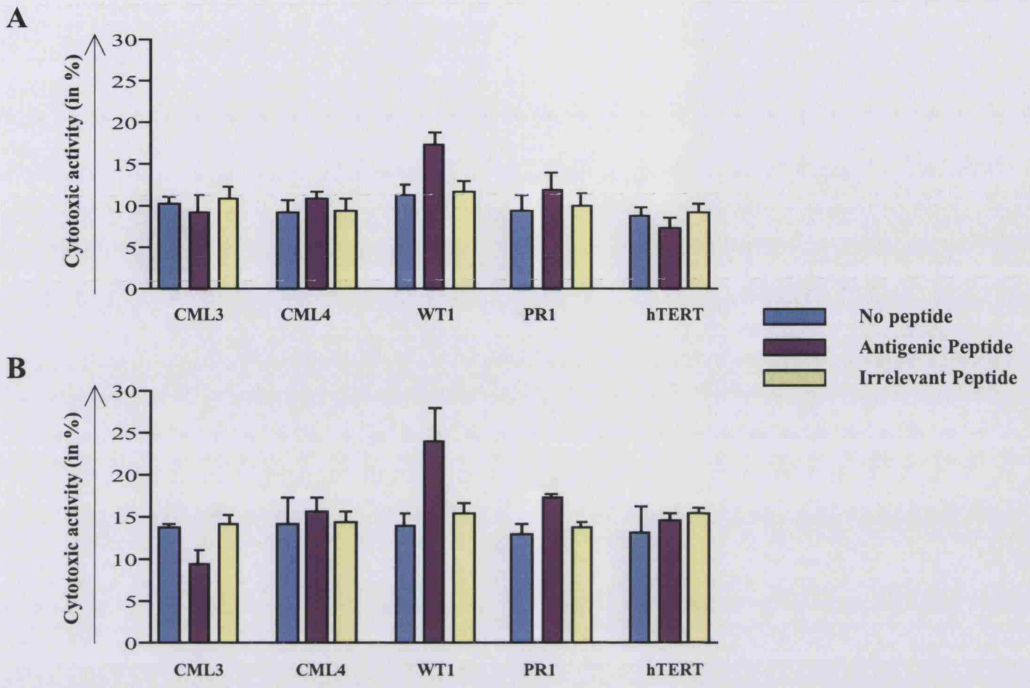


Figure 5.7: Cytotoxic activity of BCR/ABL and Tumour associated peptide specific T cells generated with mDCs:

PBMCs derived from HLA-A*0201 CML patient (panel **A**) and healthy donor (panel **B**) were stimulated with autologous APCs pulsed with the wild-type (**CML3**), the modified (**CML4**) BCR/ABL peptides or with the Wilms' tumour (**WT1**), Proteinase 3 (**PR1**) or human telomerase sub/unit (**hTERT**) tumour associated peptides following the protocol described in chapter 2 and shown in **Figure 5.6**, panel **A** and **B** respectively. Activated T cells were harvested three days after the last stimulation (day 21) and assessed for their specific cytotoxic activity against peptide-pulsed T2 cells by ^{51}Cr release. Peptide-pulsed T2 cells (in this graph 30 μg of peptide) were labelled and incubated for 4 hours at a ratio of 1:10 with effector cells. The peptide specificity of these CTLs was assessed in parallel against T2 target cells un-pulsed (No peptide) or pulsed with an irrelevant peptide (Flu peptide). The chromium released from target cells was measured as described in chapter 2 and the percentage of specific lysis was calculating using the following formula: 100× (cpm of experimental release-cpm of spontaneous release) / (cpm of maximum release-cpm of spontaneous release) where cpm represent the count per minutes. The error bars represent the standard deviation between triplicate samples.

In the context of the PR1 specific T cell responses, specific cytotoxicity was also observed from healthy donors. The healthy donors who generated the highest frequencies of PR1 specific T cells also demonstrated the highest cytotoxic activities against PR1 pulsed T2 target cells (from 2.00% ±1.50% to 4.35% ±1.85% above the negative controls, **Table 5.1** and **Figure 5.7**, panel **B**). It appears that in healthy donors, higher frequencies of PR1 specific T cells were required to generate the equivalent specific cytotoxic activity as for the

WT1 specific T cells (**Table 5.1**). Both WT1 and PR1 peptides demonstrated a similar binding affinity for the HLA-A*0201 molecule (as shown in chapter 3), so it is unlikely that the differences in specific cytotoxic activity in these donors was due to a quantitative difference of HLA/peptide complexes presented on the cell surface. Our data therefore would suggest that the WT1 peptide is more immunogenic than the PR1 peptide.

In CML patients, little or no PR1 specific cytotoxic activity was obtained above the negative controls (**Table 5.1** and **Figure 5.7**, panel **A**). A lower frequency of PR1 specific T cells was also detected in these patients (< 0.70% of tetramer positive cells). However the CML patient who generated the highest frequency of PR1 specific T cells could not be tested in this assay due to the insufficient number of lymphocytes that were expanded (0.88% shown in **Figure 5.5**, panel **A**).

As expected from the low frequencies of tetramer positive T cells generated in these donors, no hTERT specific cytotoxic activity was observed above the negative controls (un-pulsed T2 target cells or pulsed with the irrelevant Flu peptide, **Figure 5.7**). Additionally, none of the BCR/ABL peptide specific T cells generated from CML patients and healthy donors demonstrated specific cytotoxic activity against CML3 (wild-type) or CML4 (modified) peptide-pulsed T2 cells (**Figure 5.7**). The frequencies of wild-type CML3 or modified CML4 BCR/ABL peptides specific T cells generated from both CML patients and healthy donors were shown to be low (≤0.20%, **Figure 5.5**). This may explain the lack of ability in recognizing and inducing peptide-specific (**Figure 5.7**) or HLA-matched CML target cell cytotoxicity (section 5.2).

In order to confirm the relevance of the tumour associated specific T cells for immunotherapeutic applications, it would be important to also assess their activity against HLA-matched CML target cells. Unfortunately neither the effector cells nor the native CML target cells were available in sufficient numbers to perform these tests. However our data demonstrated the feasibility of this stimulation protocol in generating functional peptide-specific cytotoxic T cells for PR1 and/or WT1 antigens from both healthy donors and CML patients.

DISCUSSION

In this chapter we evaluated the feasibility of generating functional BCR/ABL specific T cells in the context of HLA-A*0201 from CML patients and healthy donors. Three BCR/ABL peptides candidates were selected for their predicted binding affinity for HLA-A*0201 and two of them (GFKQSSKAL and SSKALQRPV) have also been described as potential HLA-A*0201 associated BCR/ABL tumour specific peptides elsewhere (Rezvani et al., 2003; Yotnda et al., 1998). None of these peptides induced the expansion of antigen specific T cells from either CML patients or healthy donors. The majority of the patients included in this study were demonstrating at least a complete cytogenetic response, with little or no bcr/abl detection at a molecular level. In addition, both viral (CMV or Flu) and tumour associated (PR1 and/or WT1) specific T cells were successfully generated from these donors following the same protocol, thus demonstrating that the mDCs generated from these donors, including those from CML patients and their derived T cells were immune-competent. Thus, our data suggests that despite the importance of defining a natural HLA-A*0201 tumour specific epitope, the selected BCR/ABL peptides were not capable of generating antigen specific T cell responses and subsequently are not suitable for immunotherapeutic applications. It is worth mentioning that since the Yontda et al. report in 1998, no additional studies have demonstrated the generation of functional BCR/ABL (b3a2) specific T cells in the context of the HLA-A*0201 molecule (Yotnda et al., 1998).

The majority of these selected BCR/ABL peptides were shown to have a very low binding affinity for HLA-A*0201 (chapter 3). In an attempt to increase this binding affinity and subsequently the immunogenicity, the SSKALQRPV peptide was selected and modified to contain the primary HLA-A*0201 anchor residue (leucine) at position two. This SLKALQRPV CML4 peptide was shown to only slightly increase its binding affinity for the HLA-A*0201 molecule but it might still have a lower dissociation off-rate and thus be presented longer to T cells. The stimulation of lymphocytes derived from CML patients and healthy donors with this modified CML4 peptide failed however to expand antigen specific T cells and the responses were not statistically different to the wild-type CML3 peptide responses. Conversely, HLA tetramers refolded with this CML4 peptide detected higher frequencies of tetramer positive cells from the stimulated cultures compared to the CML3 tetramers. These CML4 tetramers appear to bind T cells of different antigen specificity as the tetramer positive cells did not recognize and target HLA-matched CML target cells. Thus, the modified CML4 peptide was also not demonstrated to increase the magnitude of

the antigen specific T cell responses from our cohort of donors and therefore its relevance as a target for immunotherapeutic treatment of CML is questionable.

The modification of weak tumour antigens have been demonstrated to significantly increase the antigen specific T cell responses without in some cases, altering the recognition and specific killing of wild-type tumour cells (Denkberg et al., 2002; Graff-Dubois et al., 2002; Keogh et al., 2001; Parkhurst et al., 1996; Scardino et al., 2002; Slansky et al., 2000; Tangri et al., 2001; Valmori et al., 1998; Vertuani et al., 2004). With the exception of two reports most of these studies have demonstrated a significant increase of the modified peptides binding affinity for the HLA molecule, which was associated with an increase in the peptides immunogenicity (Tangri et al., 2001; Vertuani et al., 2004). In our study, no significant increase of the HLA-A*0201 binding affinity was demonstrated for the modified CML4 peptide compared to the wild-type CML3 peptide. More importantly, the CML3 peptide itself has not been demonstrated to be presented on the surface of HLA-A*0201 CML cells, thus the modified CML4 peptide may also be irrelevant to the immune system. In both cases, CML3 and CML4 peptides failed to generate functional antigen specific T cell responses from our cohort of donors and therefore are not feasible targets for clinical applications.

In contrast, functional tumour associated antigens specific T cell responses were generated from both CML patients and healthy donors, at least in the context of PR1 and/or WT1 antigens. Other groups have demonstrated the successful priming of PR1 or WT1 specific T cell responses from healthy donors (Bellantuono et al., 2002; Gao et al., 2000; Molldrem et al., 1996; Molldrem et al., 1997; Molldrem et al., 1999; Oka et al., 2000). It is difficult to compare the magnitude of our responses with those described in these studies mainly because the stimulation protocols are quite different. Responses were induced with different APCs or were generated in an allo-reactive setting. Additionally, the specific cytotoxicity of the antigen specific T cells generated in this study was assessed from whole PBMC cultures. In contrast, the assessment of peptide or antigen specific cytotoxic activity in the previously published studies was performed from purified tetramer positive cells or from cloned CTL lines (Bellantuono et al., 2002; Gao et al., 2000; Molldrem et al., 1996; Molldrem et al., 1999). Higher frequencies of functional WT1 and PR1 specific T cells were generated from our cohort of healthy donors than from CML patients. This is consistent with the observations that the described responses to these antigens were generated only from healthy donors.

As reported elsewhere, patients derived tumour specific T cells may be tolerized and/or anergized *in-vivo* and thus not able to respond to antigenic stimulations *in-vitro* (Lee et al., 1999; Staveley-O'Carroll et al., 1998; Terabe and Berzofsky, 2004). The feasibility to expand these cells from healthy donors may also reflects the efficiency of the GvL responses to eradicate any residual tumour cells as observed in HSCT transplant setting. Higher numbers of TAA specific T cells were stimulated and expanded from patients who received an alloHSCT and these cells may have been donor-derived. In contrast to BCR/ABL specific responses, it is worth mentioning that the TAA responses generated from healthy donors may actually represent a memory or recall responses. As tumour associated antigens expression is not restricted to tumour cells, these TAA specific T cells can be primed in healthy donors *in-vivo*.

In contrast to the work by Vonderheide et al., no hTERT specific T cell responses from CML patients or healthy donors were successfully generated using this protocol (Vonderheide et al., 1999; Vonderheide et al., 2001). Again, it is difficult to compare with their published responses because their antigen specific T cell responses were generated from purified $CD8^+$ T cells using different APCs and performed with four successive antigen stimulations.

Our data clearly demonstrates that functional tumour associated antigen specific T cell responses, with the exception of the hTERT antigen, can be generated *in-vitro* from healthy donors and some CML patients. This was however not the case for tumour specific BCR/ABL antigens. Thus, in accordance with other groups, we believe that both WT1 and PR1 antigens may be more relevant as targets for immunotherapeutic applications to treat CML patients in the context of HLA-A*0201 molecules.

Although the stimulation protocol used in this study has been demonstrated to generate antigen specific T cell responses to viral and tumour associated antigens, it may not be optimum for priming and/or expanding weakly immunogenic BCR/ABL specific T cell responses. No responses were generated in the context of HLA-A*0201 molecules and a very limited number of healthy donors were demonstrated to expand functional BCR/ABL specific T cells in the context of HLA-A*0301 and HLA-B*0801 molecules (chapter 4). Thus we developed and assessed artificial systems that would provide controlled and optimised signals required for the successful generation of functional BCR/ABL specific T cells (chapter 6).

CHAPTER 6

Modulation of antigen-specific T cells responses by artificial antigen-presenting cells

INTRODUCTION

The stimulation of BCR/ABL specific T cells was shown in the previous chapters, to be difficult and in the majority of cases not successful. The current standard protocol available for the priming and/or expansion of low frequency and often low avidity tumour-specific T cells utilises monocyte-derived DCs as APCs. Unfortunately this technique has many drawbacks. The generation of DCs from every patient is not only laborious and time-consuming but due to the low frequency of DC precursors in the peripheral blood, it also requires a large quantity of blood donation and often limits the number of stimulations. The variability in both the quantity and the quality of DCs generated from patients also render the reproducibility of the technique very difficult. Additionally the culture conditions are less than ideal, often not meeting the GMP requirement for clinical applications. Alternative methods for the rapid generation and expansion of high number of antigen specific T cells have been developed with the new generation of "ready-to-use" artificial APCs (aAPCs). A number of aAPCs have been described, including both cellular and acellular technologies.

The human leukaemic cell line K562 was transfected with the 41BB Ligand and the human low affinity Fc γ receptor (CD32), the latter allowing the subsequent coating of the cells with anti-CD3 and anti-CD28 monoclonal antibodies (shown in **Figure 6.1, A**, (Maus et al., 2002; Thomas et al., 2002). These so-called K32 cells were demonstrated to activate and to rapidly expand both polyclonal and purified antigen specific T cells. One limitation of these K32 aAPCs is their lack of antigen specificity. Thus other cellular aAPC systems have been developed using Drosophila melanogaster insect cells or mouse friboblast cells. These cells were engineered to express HLA/peptide complexes along with costimulatory molecules B7.1 (CD80), ICAM-1 (CD54) and/or LFA-3 (CD58) (**Figure 6.1, B**, (Guelly et al., 2002; Latouche and Sadelain, 2000; Papanicolaou et al., 2003)). These aAPCs successfully generated high numbers of functional Flu, CMV or melanoma specific CTLs and were proven to be at least as efficient as the use of autologous DCs.

More recently, HLA-mismatched B cells were rendered HLA/peptide specific by coating HLA-A2$^-$ B cells with single chain streptavidin labelled anti-CD20 antibody fusion protein (B9E9scFvSA), which provides binding sites for biotinylated HLA-A2/peptide monomers (**Figure 6.1, C**, (Savage et al., 2004)). This antibody-targeted HLA/peptide complex-directed stimulation system exploits the APC functions of B cells to expand antigen specific CD8$^+$ T cells and has the advantage of preventing dominant allogeneic or xenogeneic responses. The ability of these aAPCs to stimulate antigen specific T cells was demonstrated for a number of antigens, including WT1 and melanoma derived peptides (Savage et al., 2004).

An alternative to cell-based aAPCs has also been developed, which consists in the engineering of acellular artificial systems. Indeed magnetic beads coated with anti-CD3, anti-CD28 and in some cases anti-CD40L were initially developed by Levine et al. for the rapid polyclonal expansion of T cells (Levine et al., 1997). Several groups have demonstrated the efficient *ex-vivo* restoration of the proliferative capacity and cytokine secretion profiles of various carcinoma patient T cells after stimulation with these beads (Hellstrom et al., 2001; Lum et al., 2001). However, although the long-term maintenance of purified CD4$^+$ T cells has been demonstrated, anti-CD3/anti-CD28 coated beads failed to support long-term survival of CD8$^+$ T cells (Laux et al., 2000; Levine et al., 1997).

More recently, streptavidin-coated microbeads (or microspheres) were rendered antigen-specific by immobilising biotinylated HLA/peptide complexes along with biotinylated anti-CD28 antibodies (**Figure 6.1, D** (Maus et al., 2003; Schilbach et al., 2005; Walter et al., 2003)). Costimulatory signals can also be provided by directly binding recombinant B7.1 (CD80) and ICAM-1 (CD54) proteins non-covalently to the negatively charged sulphate groups expressed on the polystyrene beads (**Figure 6.1, E** (Oosten et al., 2004)). Other acellular aAPC systems have been described by coating dimeric HLA/peptide-Immunoglobulin fusion proteins along with anti-CD28 antibodies onto magnetic beads (**Figure 6.1, F** (Oelke et al., 2003)). The flexibility of the Ig portion was shown to increase the TCR binding affinity for the HLA/peptide complexes presented this way. These acellular aAPCs were demonstrated of being capable of generating rapidly (in less than two weeks) a higher yield of functional CMV, Melanoma and mHAg specific CD8$^+$ T cells than that which could be achieved using conventional autologous DCs as APCs (Oelke et al., 2003; Oosten et al., 2004; Schilbach et al., 2005; Walter et al., 2003).

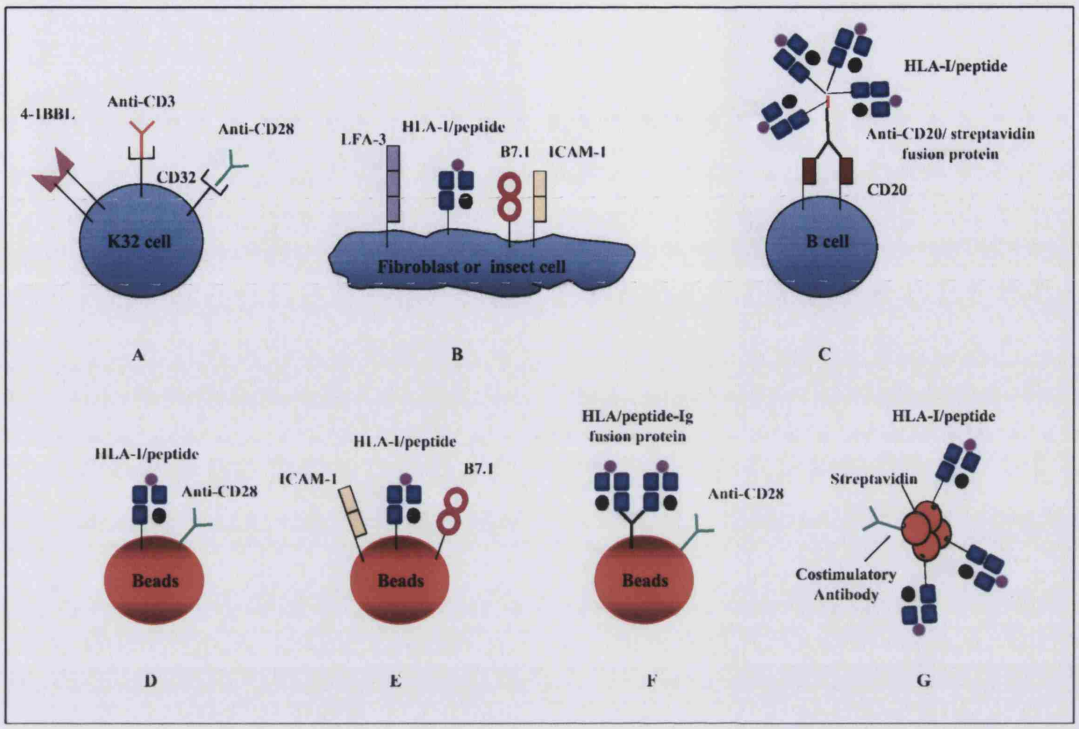


Figure 6.1: Representation of various artificial Antigen-presenting cell (aAPC) systems:

Examples of cellular and acellular aAPC systems are schematised. **A**: K562 cells were transfected with the costimulatory molecule 4-1BBL and the human low affinity Fc γ receptor CD32 antibody. These K32 cells were then coated with anti-CD3 and anti-CD28 antibodies and assessed for polyclonal or antigen specific T cells expansion capacity (Maus et al., 2002). **B**: Mouse fibroblast cells or insect cells (Melanogaster cells) were transfected with HLA class I molecules along with costimulatory molecules B7.1, ICAM-1 and/or LFA-3 (Guelly et al., 2002; Latouche and Sadelain, 2000). Antigenic peptides were provided exogenously by pulsing the aAPCs with the relevant peptide. More recently, fibroblast cells were additionally transfected with the antigenic peptide sequence, which was targeted to the endoplasmic reticulum for preferential loading onto HLA molecules (Papanicolaou et al., 2003). **C**: HLA-A2 negative B cells were targeted to present HLA-A*0201/peptide complexes by mobilising biotinylated HLA monomers onto streptavidin-labelled anti-CD20 antibody fusion protein (Savage et al., 2004). **D**: Acellular aAPCs were also developed by coating streptavidin-labelled microbeads with biotinylated HLA/peptide monomers along with biotinylated anti-CD28 costimulatory antibody (Maus et al., 2003; Schilbach et al., 2005; Walter et al., 2003). **E**: In some cases, the recombinant costimulatory molecules, such as B7.1 and ICAM-1 were non-covalently immobilised onto the beads (Oosten et al., 2004). **F**: HLA/peptide molecules have also been conjugated to an immunoglobulin heavy chain, producing a dimeric HLA/Immunoglobulin fusion protein. These fusion proteins were then immobilised onto beads along with anti-CD28 costimulatory antibody (Oelke et al., 2003). **G**: In this study, aAPC system was developed by cross-linking biotinylated HLA/peptide complexes with biotinylated costimulatory antibody onto a streptavidin core molecule.

The engagement of trimeric or tetrameric HLA/peptide complexes has been shown to be sufficient for triggering and transducing T cell receptor signals (Boniface et al., 1998). The infusion of MHC/peptide dimers or tetramers into animals was demonstrated to successfully suppress or activate antigen-specific T cells responses (Maile et al., 2001; O'Herrin et al., 2001).

In an attempt to stimulate and expand BCR/ABL specific T cells efficiently, an artificial APC system was developed, which consisted of cross-linking biotinylated HLA/peptide complexes with a biotinylated costimulatory antibody onto a streptavidin core molecule (shown in **Figure 6.1, G**). The feasibility of these modified tetramers to expand or from naïve individuals to prime, antigen-specific $CD8^+$ T cells was first assessed in a viral model, using the HLA-A*0201 restricted CMV immuno-dominant peptide (pp65, NLVPMVATV). After validating the experimental protocol conditions for the generation of CMV specific T cells with this model, the modified tetramers were tested for their capacity in generating anti-tumour CTLs in the context of HLA-A*0301 and HLA-B*0801 restricted peptides from the BCR/ABL tumour antigen.

RESULTS

6.1 Assessment of the specificity of HLA/peptide-costimulatory antibody complexes:

HLA/peptide-costimulatory antibody complexes were generated by cross-linking biotinylated HLA class I/peptide monomers and biotinylated co-stimulatory antibodies onto a streptavidin core molecule. These HLA/peptide-costimulatory antibody complexes will be referred to throughout this chapter as soluble Antigen Presenting Complexes (sAPCs). Costimulatory signals required for the activation of antigen specific T cells were provided by cross-linking anti-CD27 or anti-CD28 early costimulatory antibodies and anti-CD40L late T helper costimulatory antibody. The ratio of HLA/peptide complexes and costimulatory antibodies to cross-link on these sAPCs was established on the basis of maintaining the TCR ligand specificity. For this, sAPCs for HLA-A*0201/CMV peptide and anti-CD28 antibody were constructed at the different ratios of 3:1 (HLA/peptide: costimulatory antibody), 2:2 and 1:3. PBMCs derived from HLA-A*0201 CMV seropositive and seronegative donors were stained with the different sAPCs, as well as with HLA-A2/CMV conventional tetramers and soluble anti-CD28 antibody. The frequency of tetramer positive cells detected with these different tetramer complexes was assessed by flow cytometry and is shown in **Figure 6.2**.

The frequency of tetramer positive T cells detected with the sAPC at a 3:1 ratio was similar to the frequency detected with the conventional HLA/peptide tetramers. The increase of HLA/peptide-costimulatory antibody ratio from 3:1 to 1:3 however resulted in an increase in the frequency of CD8/sAPCs tetramer T positive cells detected from both the CMV seropositive donor and the CMV seronegative donor (from 0.26% to 5.04% and from 0.04% to 1.74%, **Figure 6.2**, panel **A** and **B** respectively). In addition, a high frequency of $CD8^-$ Tetramer positive cells background staining was detected from these donors with sAPCs which were comprised of the highest costimulatory antibody ratio of 1:3 (4.83% and 2.06%, **Figure 6.2**, lower right gate). However the single HLA/peptide complex present in this 1:3 sAPC was still able to display some degree of TCR specificity. In fact, the frequencies of tetramer positive T cells detected with this 1:3 sAPCs were lower when compared to the frequency of CD8/CD28 positive T cells detected with the anti-CD28 antibody, used at the same protein concentration (71.4% and 89.7%, **Figure 6.2**, right panel).

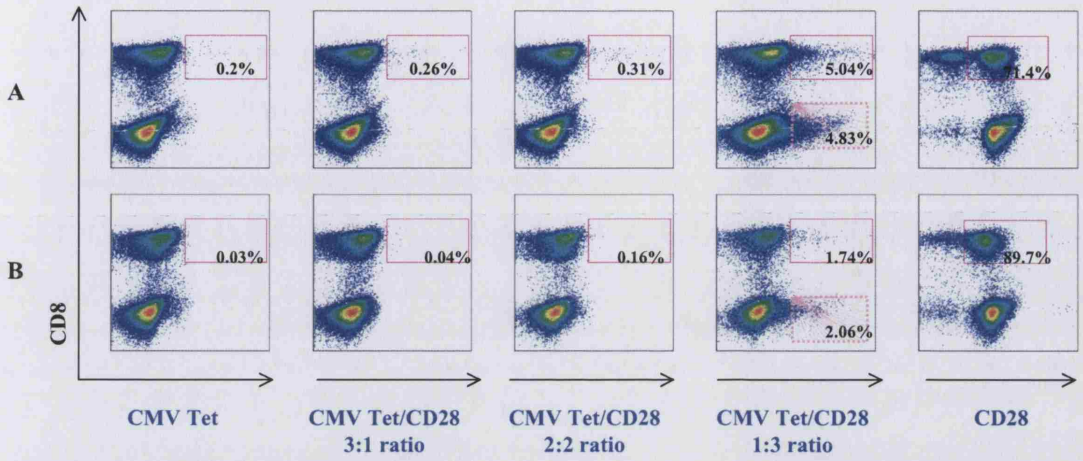


Figure 6.2: Establishment of HLA/peptide-costimulatory antibody ratio to cross-link in the sAPC complexes:

HLA-A*0201/CMV monomers were tetramerised with anti-CD28 antibodies at the ratio of 3:1 (3 HLA/peptide complexes for 1 costimlatory antibody), 2:2 or 1:3. PBMCs derived from HLA-A*0201 CMV seropositive (panel **A**) or CMV seronegative (panel **B**) healthy donors were stained with 1 μg of HLA-A*0201/CMV tetramer (CMV tet), each sAPC complexes or with anti-CD28 antibody (right panels) used at the same protein concentration than present in the sAPC 1:3 ratio. The frequencies of tetramer positive cells were assessed by Flow cytometry. Cells are gated on $CD3^+$ T cells and the percentage of $CD8^+$ $Tetramer^+$ cells are shown in each upper right gate. The frequency of $CD8^-$ $Tetramer^+$ background detected for some condition is also shown in the dashed lower right gate.

According to these data and in order to maintain the original TCR specificity, the ratio of 3 HLA/peptide molecules for 1 costimulatory antibody was chosen for the generation of sAPCs. The amount of HLA/peptide monomers to cross-link with costimulatory antibody was calculated according to their molecular weight as detailed in section 2.2.10 (Chapter 2). Accordingly, 1μg of HLA/peptide monomer was cross-linked with 0.8μg of costimulatory antibody onto a streptavidin core molecule. Each batch of sAPCs was constructed separately as described in chapter 2.

There was no evidence as to know the actual ratio of HLA/peptide molecules and costimulatory antibodies present in these soluble antigen presenting cell complexes. However, their successful cross-linking into the sAPCs was assessed in a modified ELISA assay as described in **Figure 2.13**, Chapter 2. ELISA plates were coated with goat anti-mouse IgG antibody and the bound sAPCs were then probed for the presence of HLA/peptide complexes with a rabbit anti-human β-2 microglobulin antibody. Conventional HLA/peptide tetramers and soluble costimulatory antibody controls were assessed in parallel. A representative ELISA experiment is shown in **Figure 6.3**. Both Fc regions of the costimulatory antibodies and HLA/peptide complexes were detected in all the sAPCs, above the background level of detection obtained with conventional HLA/peptide tetramers (light blue line) or soluble costimulatory antibodies (pink line). Thus, HLA/peptide monomers and costimulatory antibodies were successfully cross-linked in the soluble Antigen presenting complexes.

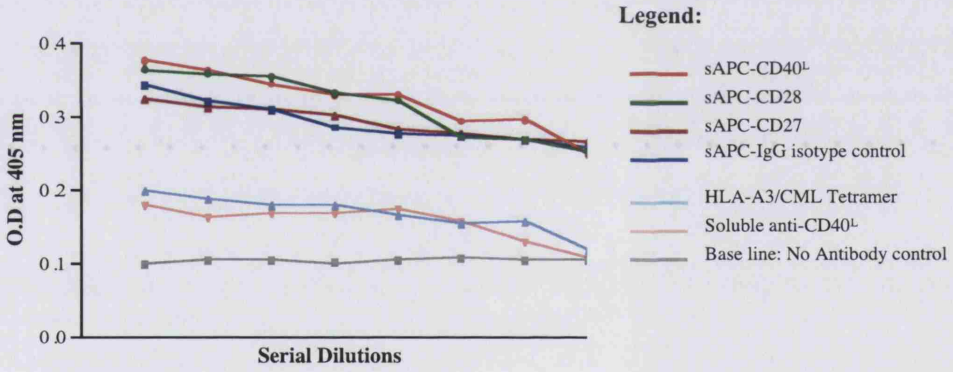


Figure 6.3: Assessment of HLA/peptide-costimulatory antibody cross-linking by modified Elisa:

The HLA/peptide-costimulatory antibody complexes (sAPCs) were tested for the presence of both refolded HLA class I molecules and costimulatory antibody (CD27, CD28, $CD40^L$ and IgG isotype control) in a modified ELISA assay (described in chapter 2). ELISA plates were coated with goat anti-mouse IgG antibody and bound complexes were then probed with a rabbit anti-human β-2 microglobulin. The ELISA sandwich reaction was then revealed with a goat anti-rabbit IgG coupled with alkaline phosphatase enzyme. The catalysis of p-Nitrophenyl phosphate (enzyme substrate) into a yellow product was measured at 405 nm in an ELISA reader plate. The base line reaction was obtained in the absence of primary antibody (goat anti-mouse IgG). Negative controls, including HLA/peptide tetramers and soluble costimulatory antibody, were assessed in parallel.

6.2 *In-vitro* model of anti-viral specific T cell modulation with sAPCs:

6.2.1 Optimisation of the stimulation protocol:

The capacity of sAPCs to activate and expand antigen specific T cells was first assessed in the context of an HLA-A*0201 restricted CMV memory response. In a preliminary experiment, PBMCs derived from HLA-A*0201 positive CMV seropositive donor were stimulated with HLA-CMV/anti-CD28 complexes (referred to as sAPC-CD28) as described in chapter 2. These sAPC-CD28 complexes present a limited number of costimulatory signals, thus stimulations were performed every 5 days. IL-7, IL-2 and IL-15 cytokines were added three days following each stimulation (chapter 2). Antigen-specific T cell modulation was assessed by measuring the frequencies of HLA-A2/CMV tetramer positive cells three days after each sAPC stimulation and a representative tetramer staining is shown in **Figure 6.4**.

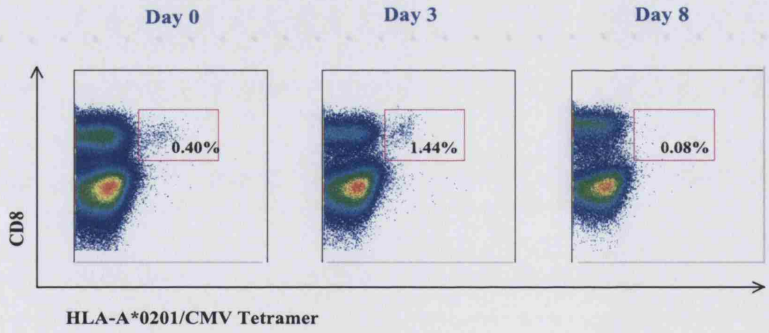


Figure 6.4: Optimisation of sAPCs stimulation protocol: Activation of CMV specific T cells:

PBMCs derived from an HLA-A*0201 CMV seropositive donor were stimulated every 5 days with HLA-A2/CMV-anti CD28 sAPCs (referred to as sAPCs-CD28). Lymphocytes were stained with HLA-A2/CMV Allophycocyanin tetramers and the frequency of CMV specific T cells was assessed by flow cytometry before stimulation (Day 0), three days after the first (day 3) and the second stimulation (day 8). Cells are gated on $CD3^+$ T cells and the percentage of $CD8^+$ $Tetramer^+$ cells are shown in each upper right gate.

The percentage of CMV specific $CD8^+$ T cells detected from this donor prior to stimulation was 0.40% (**Figure 6.4**, Day 0). The stimulation with sAPC-CD28 induced the expansion of CMV specific T cells to up to 1.44% of tetramer positive cells detected after the first stimulation (Day 3). However soon after the second stimulation, no CMV specific T cells were detected from the culture (0.08%, **Figure 6.4**, Day 8). Two consecutive stimulations with sAPCs seems to induce CMV specific T cells apoptosis.

One possibility is that these soluble antigen presenting complexes, in contrast to cellular antigen presenting cells are not eliminated by antigen specific T cells or through natural cell death and thus are constantly presenting HLA/peptide complexes to T cells. This may induce an antigen over-stimulation and consequently the elimination of the CMV specific T cell clones through activation-induced cell death. Such antigen exhaustion has been reported after multiple HLA class I tetramer injections in a mouse model, where the elimination of the tetramer reactive T cells was demonstrated to occur by anergy and/or activation-induced cell death (Maile et al., 2001). These soluble antigen presenting complexes may also be taken up by T cells and induce cell death. Another hypothesis is that these sAPCs remained cross-linked on the surface of the CMV specific T cells and subsequently these cells become targets and are eliminated. In order to circumvent these possible phenomenon, the sAPCs were removed from the culture medium three days following each stimulation, by washing the cells and re-plating them in fresh medium containing IL-7, IL-2 and IL-15 cytokines as described in chapter 2.

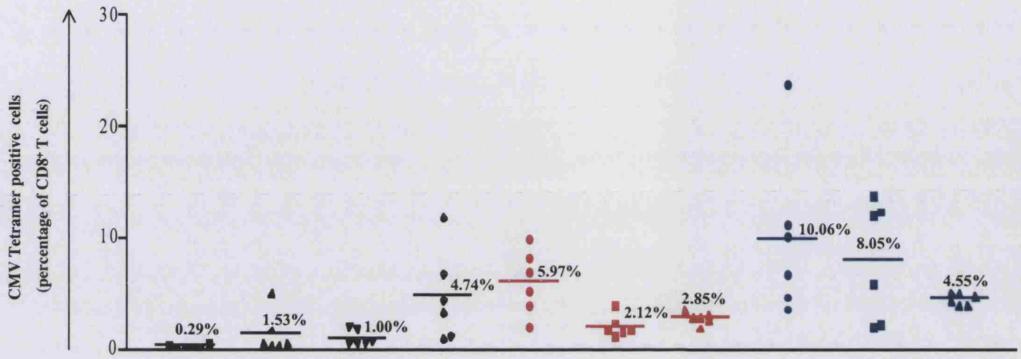
6.2.2 Activation of CMV specific T cells with sAPCs:

PBMCs derived from HLA-A*0201 CMV seropositive donors were stimulated with sAPCs providing both anti-CD27 and anti-CD28 costimulatory signals as described in Chapter 2. In order to investigate the optimal timing of signal delivery, lymphocytes were re-stimulated with either sAPC-CD27/28 (anti-CD27 and anti-CD28 costimulatory antibodies), sAPC-CD27/28/40^{L} or sAPC-CD40^{L} from the second and for the following stimulations. PBMCs were also stimulated with HLA-A2/CMV tetramers alone or in combination with soluble costimulatory antibodies. The total amount of HLA-A2/CMV molecules and co-stimulatory antibodies used for the stimulation were adjusted so that these were identical for each condition (described in Chapter 2). In order to assess the relative importance of the number of HLA/peptide molecules present in the stimulating complexes, PBMCs were also stimulated with sAPCs constructed with an irrelevant IgG isotype antibody control (3:1 ratio). Additional controls were performed by incubating lymphocytes without antigen or stimulating lymphocytes with peptide-pulsed irradiated autologous T cells blasts incubated at a ratio of 1:5.

CMV specific T cell activation and expansion upon the different types of stimulation was assessed by HLA/CMV tetramer staining three days after each stimulation. The highest frequencies of CMV specific T cells generated from six HLA-A*0201 CMV seropositve donors were observed after the fourth stimulation (day 20) and are shown in **Figure 6.5**, panel **A.** Representative HLA/tetramer dot plots are also shown in panel **B.**

The stimulation of PBMCs with the sAPC complexes generated a variable range of CMV specific T cells from 1.23% to 9.96% of total CD8^{+} T cells (**Figure 6.5**, panel **A**). The variability in the number of CMV specific T cells generated from these donors is likely to depend on a number of factors, including the differences in frequency of CMV specific T cells in the peripheral blood of these donors at the time of the blood harvest. These sAPCs induced a significant 6 to 20-fold increase in the number of CMV specific T cells compared to the frequency detected in the negative control (Medium, $P<0.05$, Wilcoxon signed ranked test). In addition, higher frequencies of CMV specific T cells were generated after stimulation with the different combination of sAPCs than with peptide-pulsed T cell blasts (mean values of 5.97%, 2.12% and 2.85% for sAPCs versus 1.53% for Peptide, **Figure 6.5**, panel **A**). Only the frequencies detected after stimulation with sAPC-CD28/CD27 were however found to be statistically different ($P=0.0312$, Wilcoxon signed ranked test).

A



1st Stimulation	Medium	Peptide	CMVTet/IgG	CMV Tet	sAPC-CD27/CD28			HLA/tetramer + soluble CD27/CD28		
2nd + Stimulation	Medium	Peptide	CMVTet/IgG	CMV Tet	CD27/28	CD27/28/40^{L}	CD40^{L}	CD27/28	CD27/28/40^{L}	CD40^{L}

B

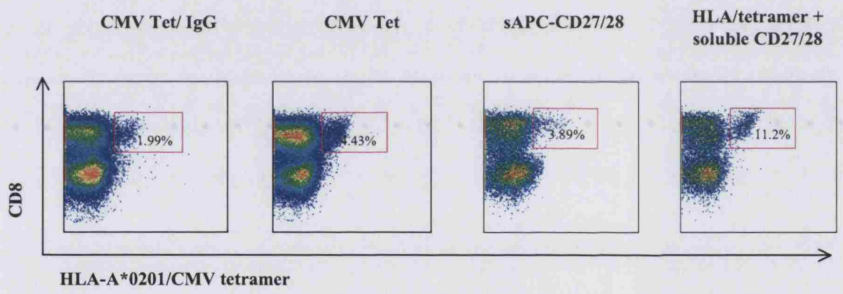


Figure 6.5: Activation of CMV specific T cells with sAPCs:

PBMCs derived from six HLA-A*0201 CMV seropositive donors were stimulated with aAPCs-CD27/28 (red) or with HLA-A2/CMV tetramers and soluble anti-CD27/28 costimulatory antibodies (blue). From the second and onwards stimulations, cells were re-stimulated either with aAPC-CD27/28, aAPC-CD27/28/40^{L} or aAPC-CD40^{L} (the same was applied for CMV tetramers and soluble costimulatory molecules). In parallel, PBMCs were incubated without antigen (**Medium**), or stimulated with CMV peptide pulsed-irradiated autologous T cell blasts at a ratio of 1:5 PBMCs (**Peptide**), HLA/CMV tetramers (**CMV Tet**), or with HLA/CMV-IgG tetramer (**CMV Tet/IgG**). Activated T cells were harvested three days after the fourth stimulation (day 20), stained with HLA-A2/CMV Allophycocyanin Tetramer and analysed by flow cytometry. Cells were gated on CD3^{+} T cells, and CMV specific T cells are expressed as a percentage of CD8^{+} T cells (panel **A**). The mean values are shown for each stimulation condition. The representative tetramer dot plots for some conditions are also shown in panel **B**.

The responses to sAPCs were then compared with the responses to HLA/tetramers plus soluble costimulatory antibodies. The stimulation of PBMCs with HLA-A2/CMV tetramers plus soluble costimulatory antibodies resulted in greater activation and expansion of CMV specific T cells from all the donors assessed (from 2.09% to 23.8%, **Figure 6.5**, panel **A**). The responses from the different donors were however very heterogeneous and thus the numbers of tetramer positive cells detected were not found to be statistically different to the numbers detected after stimulation with sAPCs ($P<0.05$, Wilcoxon signed ranked test).

As mention earlier, the amount of both HLA/peptide complexes and costimulatory antibodies were identical for all the different stimulation conditions. It is possible that the stimulation with soluble costimulatory antibodies induced a polyclonal T cell activation, which may have enhanced the activation of the CMV specific T cells. Another contributing factor may be that the presence of four HLA/peptide molecules increased the avidity of the T cell interaction with these tetramers, which in turn induced a stronger antigen specific T cell activation. In fact, the stimulation of PBMCs with HLA/CMV tetramers alone (CMV tet) demonstrated a significantly higher expansion of CMV specific T cells compared to the sAPC control, the CMV tet/IgG control (mean of 4.74% versus 1.00%, $P=0.0310$, Wilcoxon signed ranked test, **Figure 6.5**, panel **A** and also shown in panel **B**). Thus, the cross-linking of four versus three molecules of HLA/peptide monomers appears to induce higher levels of T cell signalling and subsequently higher expansions of antigen specific T cells.

The amount, as well as the timing of the costimulatory signals provided was then assessed. The highest percentages of tetramer positive T cells were detected after stimulation with sAPCs-CD27/CD28 or with HLA/tetramers plus soluble anti-CD27 and anti-CD28 antibodies (mean values of 5.97%±4.27% and 10.06%±14.74% respectively, **Figure 6.5**, panel **A** and **B**). The addition of anti-$CD40^L$ costimulatory signals from the second and for the following stimulations consistently resulted in a lower number of CMV specific T cells detected in the cultures. This was observed for both stimulation conditions with sAPCs and with HLA/tetramers plus soluble costimulatory antibodies (mean values of 2.12%±1.77% and 8.05%±5.65% respectively, **Figure 6.5**, panel **A**). Only the differences in numbers detected for the sAPC stimulation were found to be statistically significant ($P=0.0312$, Wilcoxon signed ranked test). A greater variation in the numbers detected after stimulation with HLA/peptide tetramers and soluble costimulatory antibodies was observed between the donors, thus the differences in this case were not found to be statistically significant ($P>0.05$, Wilcoxon signed ranked test).

In this sAPC system, a maximum of four ligands can be cross-linked onto the streptavidin core molecule. Thus, for comparison purposes between the different stimulation conditions, either the amount of HLA/peptide complexes presented or the amount of costimulatory molecules provided has to be maintained. As the number of HLA/peptide monomers presented was shown to significantly influence the expansion of antigen specific T cell, these were maintained between the different stimulation conditions. Accordingly, the addition of anti-$CD40^L$ antibody in the stimulation resulted by necessity in the decrease of anti-CD27 and anti-CD28 costimulatory signals. The dilution of the early anti-CD27 and/or anti-CD28 costimulatory signals appears to diminish the CMV specific T cell activation threshold and subsequently their expansion. The frequency of CMV specific T cells detected after stimulation with only sAPC-$CD40^L$ from the second stimulation was lower compared to the frequency detected after stimulation with sAPC-CD27/CD28 (mean values of 2.85%±0.95% versus 5.97%±4.27%, **Figure 6.5**, panel **A**). This was also observed for the HLA/tetramers plus soluble costimulatory antibodies condition (mean values of 4.54%±0.59% versus 10.06%±14.74%). The differences in frequencies were however not found to be statistically significant ($P>0.05$, Wilcoxon signed ranked test).

In conclusion, the nature, the amount and the timing of delivery of costimulatory signals provided on the sAPCs, as well as in the HLA/tetramer plus soluble costimulatory antibodies stimulation condition, appear to affect the activation signalling and the resulting frequency of antigen specific T cells detected from the stimulated cultures.

6.2.3 Generation of primary CMV specific responses with sAPCs:

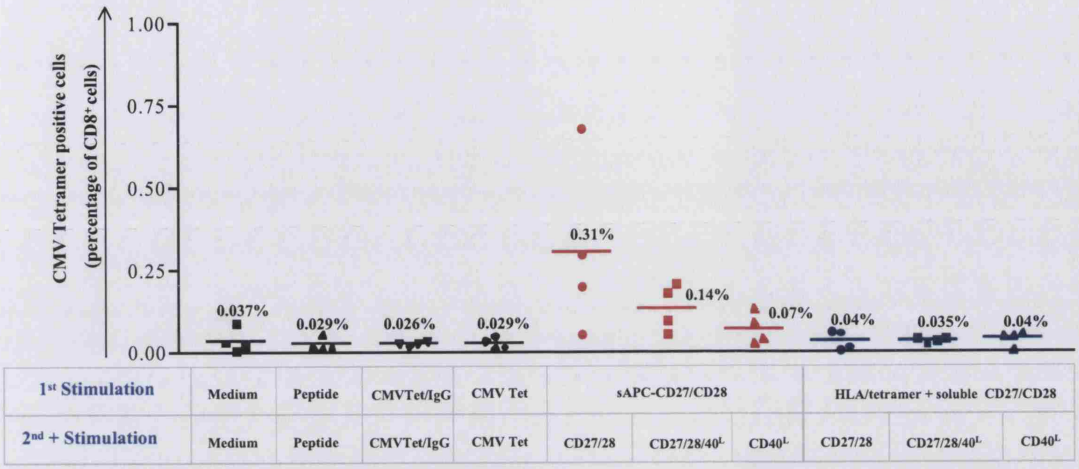
The efficiency of these soluble antigen-presenting complexes was further assessed in CMV seronegative donors. PBMCs derived from four HLA-A*0201 CMV seronegative donors were stimulated with sAPCs or with HLA/CMV tetramers and soluble costimulatory antibodies following the same protocol as described for the activation of CMV memory responses (section 6.2.2). In addition, the same controls including no antigen stimulation or stimulation with CMV/Tetramers, CMV/IgG Tetramers and peptide-pulsed autologous T cell blasts, were performed in parallel.

As expected, no CMV specific $CD8^+$ T cells were detected from the peripheral blood of these donors above the detection limit (0.01-0.04% of total $CD8^+$ T cells). The priming of CMV specific T cell responses with the various stimulation conditions was assessed three days after the fourth stimulation (day 20) by HLA/CMV tetramer staining and is shown in **Figure 6.6**, panel **A**. A representative HLA/tetramer dot plots are also shown in panel **B**.

The priming of PBMCs derived from CMV seronegative donors with sAPC-CD27/28 successfully induced the expansion of tetramer positive cells up to 0.68% after four antigen stimulations (**Figure 6.6**, panel **A** and **B**). These responses, however, were not found to be statistically significant compared to the negative control or to the stimulation with peptide-pulsed T cell blasts (Medium and Peptide, P=0.1250 and P=0.2500, Wilcoxon signed ranked test). This protocol was probably assessed from small numbers of donors and the responses generated were very heterogeneous among these donors (from 0.055% to 0.68%, mean value of 0.31%, **Figure 6.6**, panel **A**). Nevertheless, the stimulation with sAPC-CD27/CD28 was demonstrated to expand tetramer positive cells from at least one donor.

The CD27 and/or CD28 early costimulatory signals provided in the sAPC complexes appeared to be crucial for the priming of these responses as their dilution or replacement by anti-$CD40^L$ costimulatory antibodies (aAPC-CD27/28/40^L, aAPC-$CD40^L$) reduced or abrogated the expansion of these primary CMV specific T cells (mean values of 0.14%±0.08% and 0.07%±0.06% respectively, **Figure 6.6**, panel **A** and panel **B**).

A



B

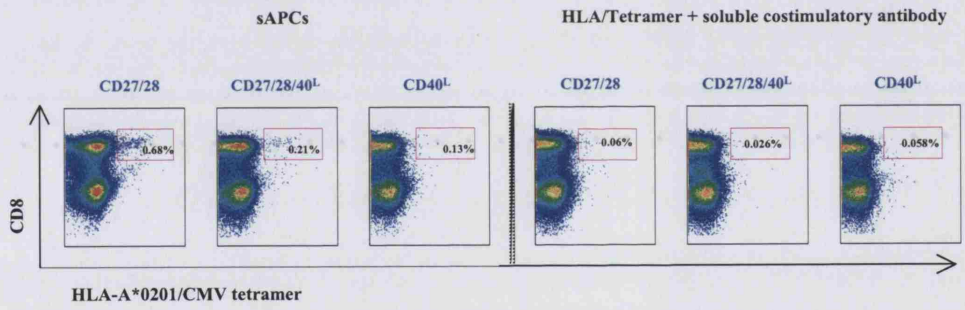


Figure 6.6: Priming CMV specific CD8$^+$ T cells with sAPCs:

PBMCs derived from four HLA-A*0201 CMV seronegative donors were stimulated with CMV peptide-pulsed irradiated autologous T cell blasts at a ratio of 1:5 (Peptide), HLA/CMV tetramers (CMV tet), HLA/CMV-IgG tetramers (CMV tet/IgG), sAPCs (red) or with HLA/CMV tetramers and soluble costimulatory antibodies (blue) following the protocol described for the CMV memory responses (section 6.2.1 and chapter 2). A negative control was performed in parallel by incubating PBMCs without antigen (Medium). The frequency of CMV specific T cells was assessed three days after the fourth stimulation (day 20). Activated T cells were stained with HLA-A2/CMV Allophycocyanin tetramers and analysed by flow cytometry. Cells were gated on CD3$^+$ T cells and CMV specific T cells are expressed as a percentage of CD8$^+$ T cells (panel **A**). The mean values are shown for each condition. Representative HLA/tetramer dot plots are also shown in panel **B**.

In contrast to the activation of memory T cells, stimulation with HLA/tetramers alone or in combination with soluble costimulatory antibodies failed to prime naïve CMV specific T cells (mean values from 0.035%±0.009% to 0.04%±0.03%, **Figure 6.6**, panel **A** and **B**). Thus the cross-linking of both HLA/peptide complexes and costimulatory molecules on sAPCs appears to be required for the efficient triggering of TCR activation and expansion of antigen specific T cells from CMV seronegative donors.

From these data, one hypothesis is that the sAPCs presenting high numbers of HLA/peptide complexes and costimulatory signals in a close proximity is required for the activation and subsequent proliferation of naïve antigen-specific T cells, but may have induced an antigen over-stimulation of memory antigen-specific T cells and subsequently their activation-induced cell death. In contrast, HLA/peptide tetramers plus soluble costimulatory antibodies may provide a lower signalling threshold, which is not sufficient for priming naïve T cells but enough to induce antigen-specific memory T cells proliferation.

To test this hypothesis, the primary and memory CMV specific responses upon the different stimulations were further characterised for activation marker expression (CD69), for proliferative responses and for antigen-specific T cell differentiation.

6.2.4 Analysis of the CD69 expression on activated CMV specific T cells:

The TCR triggering induced by HLA/peptide and costimulatory signals results in a signalling cascade that can be followed by the up-regulation of specific proteins, such as the cell surface expression of the activation marker CD69. The expression of CD69 on CMV specific T cells activated with sAPCs or HLA/CMV tetramers plus soluble costimulatory antibodies, was examined three days after the fourth stimulation in parallel with the HLA/CMV tetramer staining. The percentage of CMV tetramer positive cells expressing CD69 marker is shown in **Figure 6.7**, panel **A** and the representative histograms for three different stimulation conditions are shown in panel **B**. In the context of the primary CMV specific T cell responses, such analysis could only be performed for the two stimulation conditions, which induced the expansion of tetramer positive cells. The CD69 expression histograms for the naïve CMV specific T cells primed with sAPC-CD27/28 and sAPC-CD27/28/40^{L} are shown in **Figure 6.7**, panel **C**.

The stimulation of CMV specific T cells with sAPCs induced the up-regulation of CD69 cell surface expression on a high percentage of tetramer positive cells compared to the negative controls (from 42% to 82.9% of CMV specific T cells, **Figure 6.7**, panel **A** and **B**). Similarly, the majority of the CMV specific CD8^{+} T cells primed from a HLA-A*0201 CMV seronegative donor with sAPCs expressed cell surface CD69 marker (97% and 95.4%, **Figure 6.7**, panel **C**).
The expression of cell surface CD69 marker appears to be mediated by anti-CD27 and/or anti-CD28 costimulatory signals, since the dilution or absence of these signals resulted in a lower proportion of tetramer^{+} CD69^{+} T cells (**Figure 6.7**, panel **A**, sAPC-CD27/28/40^{L} and sAPC-CD40^{L}).

In contrast to the sAPCs, the stimulation with HLA/CMV tetramers alone (CMV tet or CMV tet/IgG) or with HLA/tetramers plus soluble costimulatory antibodies induced the expression of CD69 on a low percentage of CMV specific T cells (from 3.63% and 16.3% of CMV specific T cells, **Figure 6.7**, panel **A**). Additionally, after stimulations with autologous peptide-pulsed T cell blasts, only 23% of CMV specific T cells expressed CD69. It is important to mention that as CD69 is an early activation marker, its expression on these CMV specific T cells may have already been down-regulated. Nevertheless, these data demonstrate that the stimulation with the sAPC complexes induced different kinetics of CD69 expression on activated antigen specific T cells.

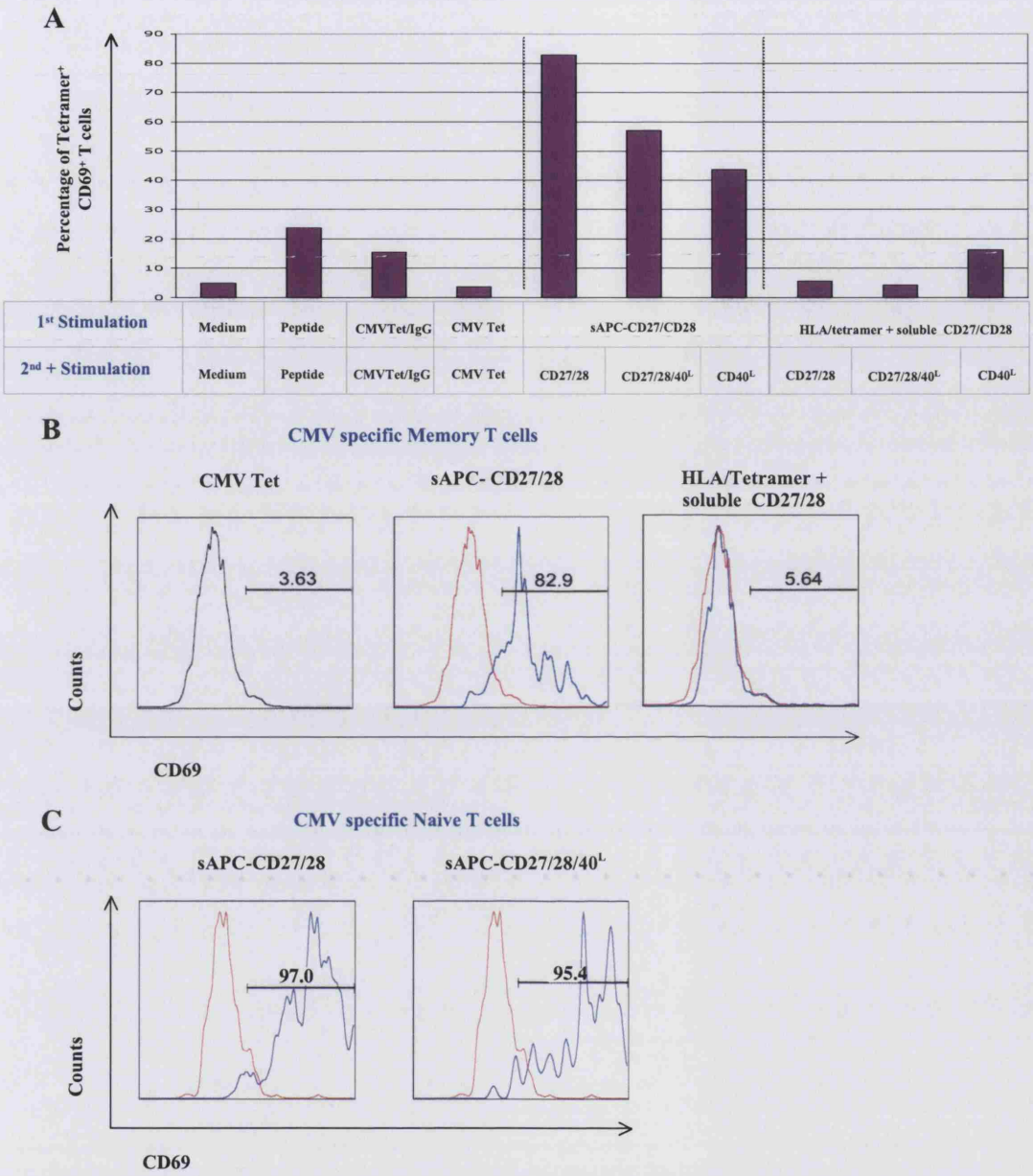


Figure 6.7: CD69 expression on CMV specific $CD8^+$ T cells activated with sAPCs or HLA/CMV tetramers plus soluble costimulatory antibodies:

PBMCs derived from a HLA-A*0201 CMV seropositive donor were stimulated with CMV peptide, HLA/CMV tetramers, HLA/CMV-IgG tetramers, sAPCs or with HLA-A2/CMV tetramers plus soluble costimulatory antibodies as described in chapter 2 and shown in **Figure 6.4**. A negative control was also performed by incubating PBMCs without antigen (Medium). Activated T cells were harvested three days after the fourth stimulations (day 21) and stained with HLA-A2/CMV Allophycocyanin Tetramers. Lymphocytes were acquired by flow cytometry and gated on $CD3^+$ $CD8^+$ CMV $tetramer^+$ cells. The cell surface expression of CD69 upon the different antigen stimulations is represented in panel **A**. The histograms for some conditions are shown in panel **B**, comparing the level of CD69 expression on CMV specific $CD8^+$ T cells after stimulation with CMV tetramer alone (red line) and with sAPCs or HLA/CMV tetramers plus soluble costimulatory antibodies (blue line). In the context of the primary CMV specific T cell response, the expression of CD69 was assessed from the HLA-A*0201 CMV seronegative donor who successfully expanded CMV specific T cells after stimulation with sAPC-CD27/28 or sAPC-CD27/28/40^L and are shown in panel **C**. The levels of CD69 expression on CMV specific $CD8^+$ T cells generated from this donor (blue lines) were compared with the levels detected after staining with an irrelevant IgG isotype control (red lines).

6.2.5 Analysis of CMV specific T cell proliferative responses upon stimulation:

The proliferation of primary and memory CMV specific T cell responses generated upon stimulation with sAPCs or with HLA/tetramers plus soluble costimulatory antibodies (described in section 6.2.2) were further characterised. PBMCs derived from HLA-A*0201 CMV seropositive and seronegative donors were labelled with CFSE prior to antigen stimulations and analysed for the dilution of fluorescence as described in chapter 2. CFSE is equally distributed between the progeny of dividing cells, which results in the halving of the fluorescence signal upon each cell division. The proliferative response of CMV specific T cells following the different antigenic stimulations was assessed by flow cytometry three days after the second (day 8) and the fourth stimulation (day 20). Lymphocytes were also incubated with the polyclonal PHA stimulus as a positive control of proliferation.

The antigen specific T cell proliferative responses were assessed from CMV seropositive donors first, and a representative CFSE staining profile is shown in **Figure 6.8**. The CMV specific $CD8^+$ T cell proliferative responses were compared between the stimulation with sAPCs and HLA/tetramers plus soluble costimulatory antibodies (**Figure 6.8**, panel **A** and **B** respectively).

After two antigen stimulations, CMV specific T cells demonstrated very little or no proliferative response, as shown by the high level of CFSE fluorescence detected for each stimulation condition (**Figure 6.8**, day 8). Only the CMV specific T cells stimulated with PHA demonstrated one or two cellular division (**Figure 6.8**, PHA).

After four stimulations, all CMV specific T cells from the positive control have divided and lost CFSE fluorescence (**Figure 6.8**, PHA, day 20). The stimulation with sAPCs induced variable CMV specific T cell proliferative responses depending on the nature of the costimulatory signals provided. The majority of CMV specific T cells stimulated with sAPC-CD27/28 or sAPC-CD27/28/40^L demonstrated a complete loss of CFSE fluorescence compared to the negative control (Medium). A significant number of cells have however undergone only four or five divisions after four consecutive antigen stimulations (**Figure 6.8**, panel **A**, day 20). Following stimulation with sAPC-$CD40^L$ all the CMV specific T cells demonstrate a complete loss of CFSE fluorescence (panel **A**, sAPC-$CD40^L$). Thus the stimulations of CMV specific T cells with sAPC-$CD40^L$ appear to induce a faster antigen specific proliferative response compared to the stimulations with aAPC-CD27/28 or with aAPC-CD27/28/40^L.

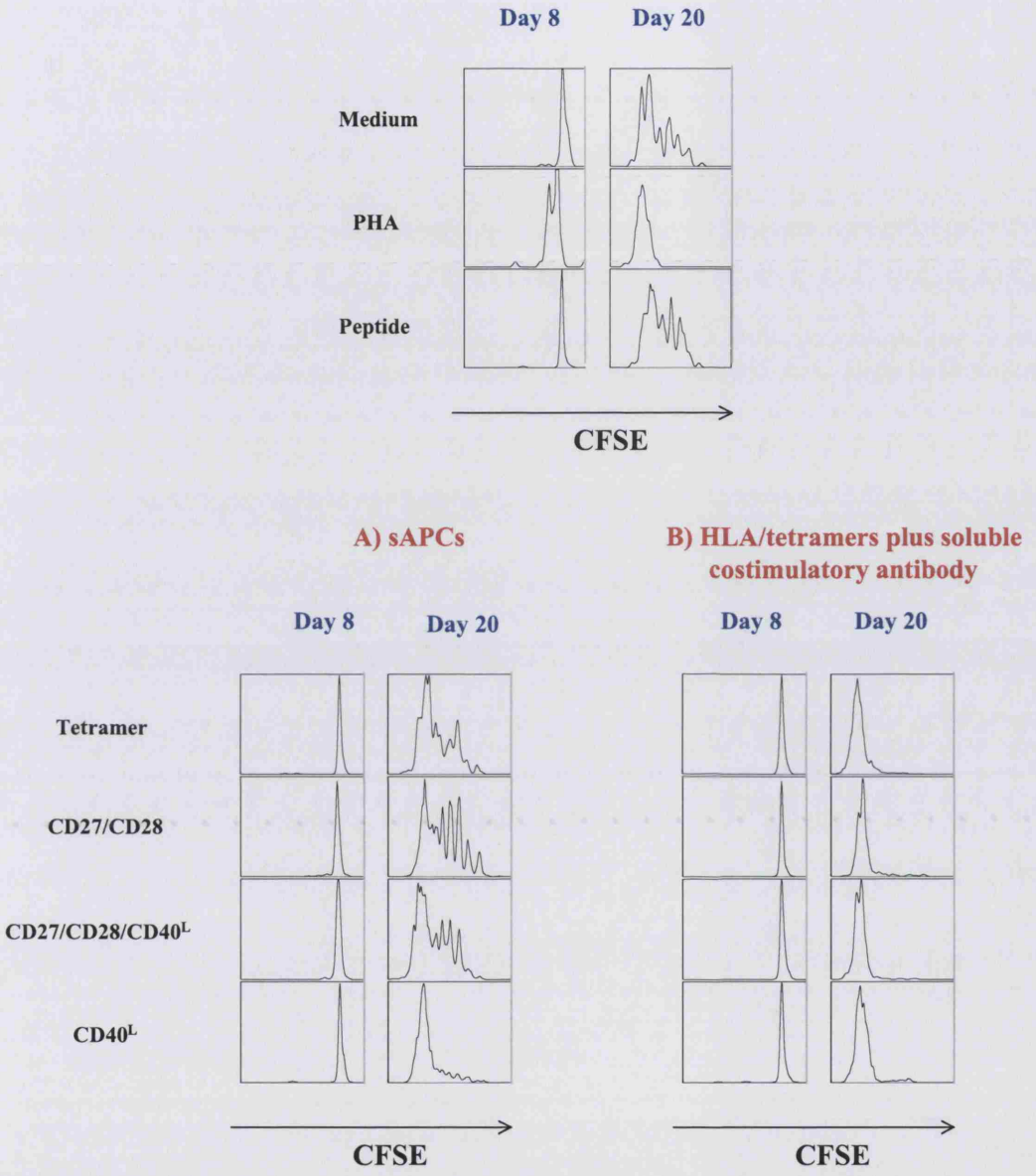


Figure 6.8: Proliferative response of CMV specific T cells stimulated with sAPCs or HLA/CMV tetramer plus soluble costimulatory antibodies:

PBMCs derived from an HLA-A*0201 CMV seropositive donor were labelled with CFSE and stimulated with CMV peptide, HLA/CMV tetramers, sAPCs (panel **A**), or with HLA-A2/CMV tetramers plus soluble costimulatory antibodies (panel **B**) as described in chapter 2 and shown in **Figure 6.4**. A negative control (Medium) and a positive control (polyclonal stimulus PHA) were also included. Activated T cells were harvested three days after the second and fourth stimulations, stained with HLA-A2/CMV Allophycocyanin Tetramer and acquired by flow cytometry. Cells are gated on $CD3^+$ $CD8^+$ CMV $tetramer^+$ cells, and analysed for the loss of CFSE fluorescence upon each cell division after two (**day 8**) and four (**day 20**) antigen stimulations.

In contrast to sAPCs, the stimulation of CMV specific T cells with HLA/CMV tetramers plus soluble costimulatory antibodies induced the complete loss of CFSE fluorescence after four stimulations regardless of the costimulatory signals provided (**Figure 6.8**, panel **B**, day 20).

These data suggest that the stimulation of CMV specific T cells with HLA/tetramers plus soluble costimulatory antibodies induced a faster proliferative response compared to sAPCs stimulation. HLA/tetramers, which present four HLA/peptide complexes may demonstrate a higher affinity for the TCR, which resulted in a stronger antigen specific T cell activation and subsequently proliferation. Lymphocytes incubated with conventional HLA/tetramers in fact also demonstrated a faster proliferative response compared to sAPC-IgG control (**Figure 6.8**, Tetramer panel **A** and **B**).

Thus it appears that the higher frequency of CMV specific T cells detected from the cultures stimulated with HLA/tetramers plus soluble costimulatory antibodies was consequent to a faster proliferation of antigen specific T cells rather than the hypothesised triggered activation-induced cell death by sAPCs.

The proliferative responses of the CMV specific T cells primed from naïve donors were then assessed. Due to the low frequencies of tetramer positive T cells generated from some of these donors, the proliferative responses could not be analysed for each antigen stimulation conditions. The priming of CMV specific T cells with sAPC-CD27/28 from one donor generated however 0.68% of tetramer positive T cells (shown in **Figure 6.6**, panel **B**). The proliferative responses of these primed antigen specific T cells were analysed after the second (day 8) and the fourth (day 20) stimulations with sAPCs-CD27/28 in parallel to HLA/tetramer staining and are shown in **Figure 6.9**. These responses were compared with the proliferative response of CMV specific memory T cells (**Figure 6.9**, panel **A** and **B** respectively).

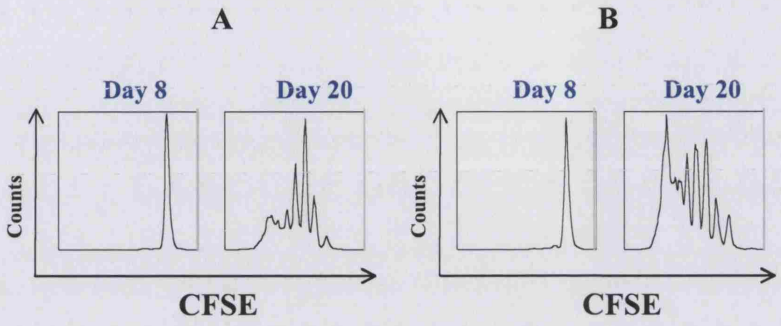


Figure 6.9: Comparison between primary and memory CMV proliferative responses following sAPC-CD27/28 stimulations:

PBMCs derived from HLA-A*0201 CMV seronegative (**A**) and seropositive (**B**) donor were labelled with CFSE and stimulated with sAPC-CD27/28 as described in chapter 2. Activated T cells were harvested three days after the second (**day 8**) and fourth (**day 20**) stimulations and stained with HLA/CMV Allophycocyanin tetramers. Cells were gated on $CD3^{+}CD8^{+}$ CMV $tetramer^{+}$ cells and analysed for the loss of CFSE fluorescence following each cellular division.

A high level of CFSE fluorescence was reproducibly detected from the CMV specific T cells following the second antigen stimulation. This was observed for both the naïve donor and the antigen-experienced donor (**Figure 6.9**, panel **A** and **B**, day 8). After four stimulations, the majority of CMV specific T cells generated from the CMV seropositive donor have proliferated and demonstrated a complete loss of CFSE fluorescence (**Figure 6.9**, panel **B**, day 20). Following the same cycle of stimulation, CMV specific T cells generated from the seronegative donor have however only undergone two or three cell divisions and have maintained a high level of CFSE fluorescence (panel **A**, day 20). The primary CMV specific T cell response demonstrated a slower proliferative response compared to the secondary responses, following the same antigen stimulation. It is known that a higher threshold of activation signals is required for the successful generation of primary immune responses. Thus, a higher number of stimulations as well as a higher amount of sAPCs may be necessary for a similar antigen specific T cells expansion from both naïve and antigen-experienced donors.

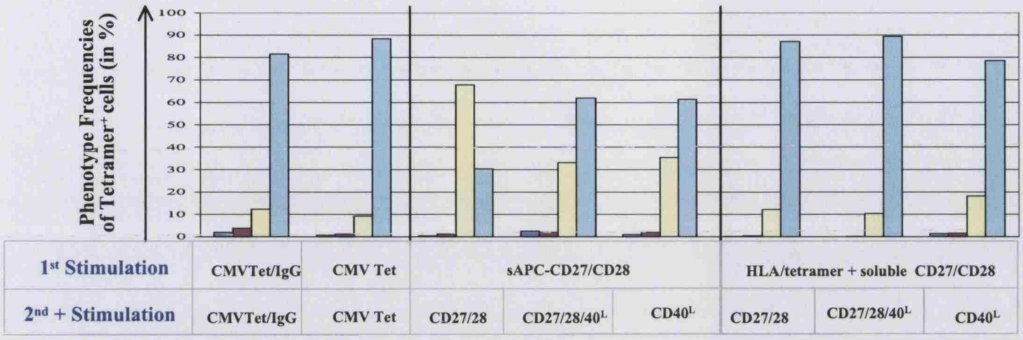
6.2.6: Analysis of phenotypic marker expression on activated CMV specific T cells:

The differentiation of antigen specific T cells following stimulations with sAPCs or with HLA/tetramers and soluble costimulatory antibodies was characterised. Due to the limited number of CMV specific T cells primed from naïve donors, the phenotypic analysis could only be performed in the context of CMV memory responses. CMV specific T cells were analysed for the expression of CD27 and CD45RO cell surface phenotypic markers following the different stimulations (as described in section 6.2.2). The frequencies of Naïve ($CD27^+CD45RO^-$), Memory ($CD27^+CD45RO^+$), Effector ($CD27^-CD45RO^+$) and Terminal Effector ($CD27^-CD45RO^-$) CMV specific $CD8^+$ T cells were assessed after the fourth stimulation in parallel with HLA/tetramer staining. A representative staining is shown in **Figure 6.10**, panel **A**. The dot plots for some stimulation conditions are also shown in **Figure 6.10**, panel **B**.

The stimulation of CMV specific T cells with sAPCs induced the differentiation of antigen specific T cells into both effector and terminal effector cells (frequency of 32% to 68% $CD27^-$ $CD45RO^+$ cells and 30% to 62% $CD27^-$ $CD45RO^-$ cells respectively, **Figure 6.10**, panel **A**). The co-stimulation with anti-CD27 and anti-CD28 antibodies appears to induce the preferential differentiation and expansion of CMV specific T cells into an effector phenotype (sAPC-CD27/28). The addition of anti-$CD40^L$ antibodies from the second and onward stimulations induced the CMV specific T cells to further differentiate into terminal effectors (**Figure 6.10**, panel **A**, sAPCs-CD27/28/40^L and sAPCs-$CD40^L$). The activation with sAPCs-$CD40^L$ was previously demonstrated to induce a faster CMV specific T cell proliferative response and their differentiation into terminal effector T cells. In addition, the frequency of tetramer positive cells detected after stimulation with sAPCs-$CD40^L$ was shown to be lower compared to the other stimulation conditions (section 6.2.2). Thus, these data suggest that $CD40^L$ signalling induced the activation-induced cell death of CMV specific T cells.

Similarly, stimulation with the sAPCs constructed with an irrelevant IgG isotype control induced the differentiation of the low percentage of CMV specific T cells into terminal effectors (81.5%, CMVtet/IgG). In this case however, the lack of costimulation signalling has probably triggered the apoptosis of these terminal differentiated antigen specific T cells.

A



Legend

- CD27+CD45RO- Naïve T cells
- CD27+CD45RO+ Memory T cells
- CD27-CD45RO+ Effector T cells
- CD27-CD45RO- Terminal Effector T cells

B

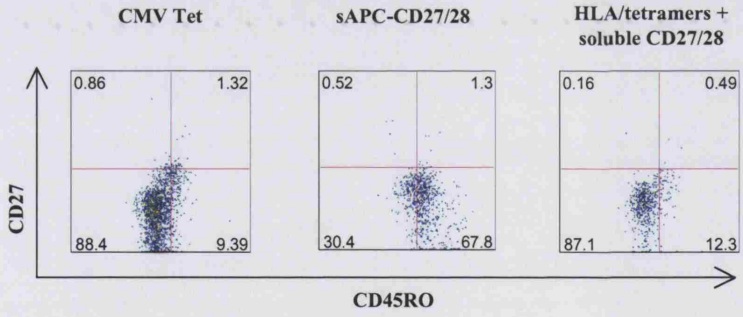


Figure 6.10: Phenotypic characteristic of CMV specific $CD8^+$ T cells activated with sAPCs or HLA/CMV tetramers and soluble costimulatory antibodies:

The phenotypic characteristic of CMV specific T cells stimulated with autologous peptide-pulsed T cells blast at 1:5 ratio (Peptide), HLA/CMV tetramers (CMV Tet), HLA/CMV-IgG tetramers (CMV Tet/IgG), sAPCs or HLA/CMV tetramers and soluble costimulatory antibodies as described in chapter 2 and section 6.2.2 is represented. Activated T cells were harvested three days after the fourth stimulations (day 20) and stained with HLA/CMV Allophycocyanin tetramer, CD27 and CD45RO cell surface markers. Lymphocytes were acquired by flow cytometry and gated on $CD3^+$ $CD8^+$ CMV $tetramer^+$ cells. The percentages of naïve ($CD27^+CD45RO^-$), memory ($CD27^+CD45RO^+$), effector ($CD27^-CD45RO^+$) and terminal effector ($CD27^-CD45RO^-$) CMV specific $CD8^+$ T cells are shown in panel **A** and representative dot plots are also shown in panel **B**.

The stimulation of CMV specific T cells with HLA/tetramers alone or in combination with soluble costimulatory antibodies failed to expand and/or maintained the memory compartment and resulted predominantly in their differentiation into terminal effector T cells (from 78.6% to 87.1% of tetramer positive cells, **Figure 6.10**, panel **A** and **B**). In this case no difference in the phenotype frequencies of CMV specific T cells were observed between the different costimulatory signals provided.

In conclusion, HLA/tetramers and soluble costimulatory antibodies induce a faster antigen specific T cell proliferative response, which resulted in the generation of a high frequency of terminal effector CMV specific T cells from antigen-experienced individuals (section 6.2.2). In contrast, the stimulation of CMV specific T cells with sAPCs was shown to result in a slower antigen specific T cell proliferative response within both effector and terminal effector compartments. It appears therefore that the lower frequency of CMV specific T cells generated with sAPCs from the CMV seropositive donors was consequent of a lower activation threshold. It is possible that these sAPC complexes have a lower affinity for the TCR compared to conventional HLA/tetramers. However, the cross-linking of HLA/peptide molecules with costimulatory antibodies was required for the successful priming of CMV specific T cells from naïve donors.

6.3 *In-vitro* modulation of BCR/ABL tumour specific T cell responses:

The capacity of sAPCs to prime and/or expand BCR/ABL specific T cells was next assessed in the context of HLA-A*0301 and HLA-B*0801 molecules. HLA/BCR-ABL peptide monomers were cross-linked with anti-CD27, anti-CD28 or anti-CD40^{L} costimulatory antibodies at a ratio of 3:1 (HLA: Antibody) and assessed for their successful cross-linking in a modified ELISA as described in chapter 2 and shown in section 6.1. PBMCs derived from HLA-A*0301 or HLA-B*0801 donors were stimulated with various combinations of sAPCs or HLA/BCR-ABL tetramers and soluble costimulatory antibodies following the protocol optimised for the CMV specific T cell responses (described in section 6.2.2 and chapter 2). In addition, controls including stimulation with no antigen, HLA/tetramers-IgG, HLA/tetramers and peptide-pulsed T cell blasts were performed in parallel. The ability of these sAPCs to generate BCR/ABL specific T cell responses was evaluated from both healthy donors and CML patients.

6.3.1 Stimulation of BCR/ABL specific T cells with sAPCs from healthy donors:

PBMCs derived from five HLA-A*0301 and four HLA-B*0801 healthy donors were stimulated with sAPCs, in combination with anti-CD27, anti-CD28 or with both anti-CD27 and anti-CD28 costimulatory antibodies respectively as described in chapter 2. Lymphocytes were re-stimulated following the same conditions, with or without the addition of anti-CD40^{L} costimulatory antibody from the second and subsequent stimulations (the same was applied for HLA/tetramers and soluble costimulatory antibodies). The BCR/ABL specific T cell responses generated from these donors were assessed by HLA/tetramer staining three days after the fourth stimulation (day 20) and are shown in **Figure 6.11**. The frequency of tetramer positive T cells generated from HLA-A*0301 and HLA-B*0801 healthy donors are represented in panel **A** and **B** respectively. In addition, representative HLA/tetramers staining obtained from a HLA-A*0301 (**Figure 6.12**) and a HLA-B*0801 donor (**Figure 6.13**) are also shown.

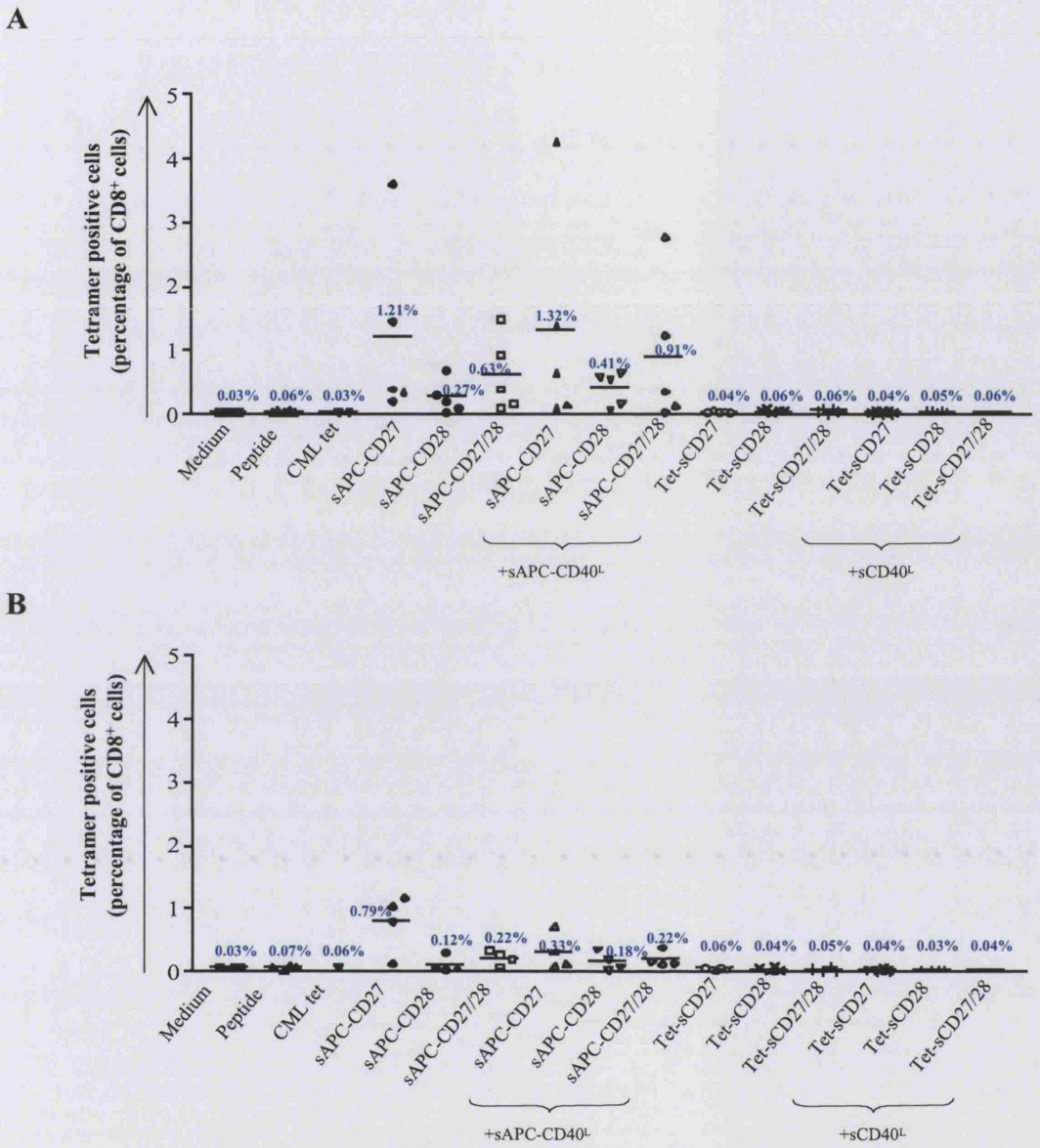


Figure 6.11: Stimulation of BCR/ABL specific T cells from healthy donors with sAPCs:

PBMCs derived from five HLA-A*0301 (panel A) and four HLA-B*0801 healthy donors (panel B) were stimulated with sAPCs or HLA/tetramers and soluble costimulatory antibodies (Tet-s) providing anti-CD27, anti-CD28, or both anti-CD27 and anti-CD28 costimulatory antibodies as described in chapter 2. From the second and subsequent stimulations cells were restimulated with the same conditions, with or without the addition of anti-$CD40^L$ costimulatory antibodies. Cells were also incubated without antigen (Medium), stimulated with peptide-pulsed irradiated T cell blasts incubated at a ratio of 1:5 PBMCs (Peptide), or HLA/tetramers (CML tet). The frequency of BCR/ABL specific T cells was assessed three days after the fourth stimulation (Day 20). The frequency of tetramer positive cells is expressed as a percentage of $CD3^+CD8^+$ T cells. The mean frequency values are shown for each condition.

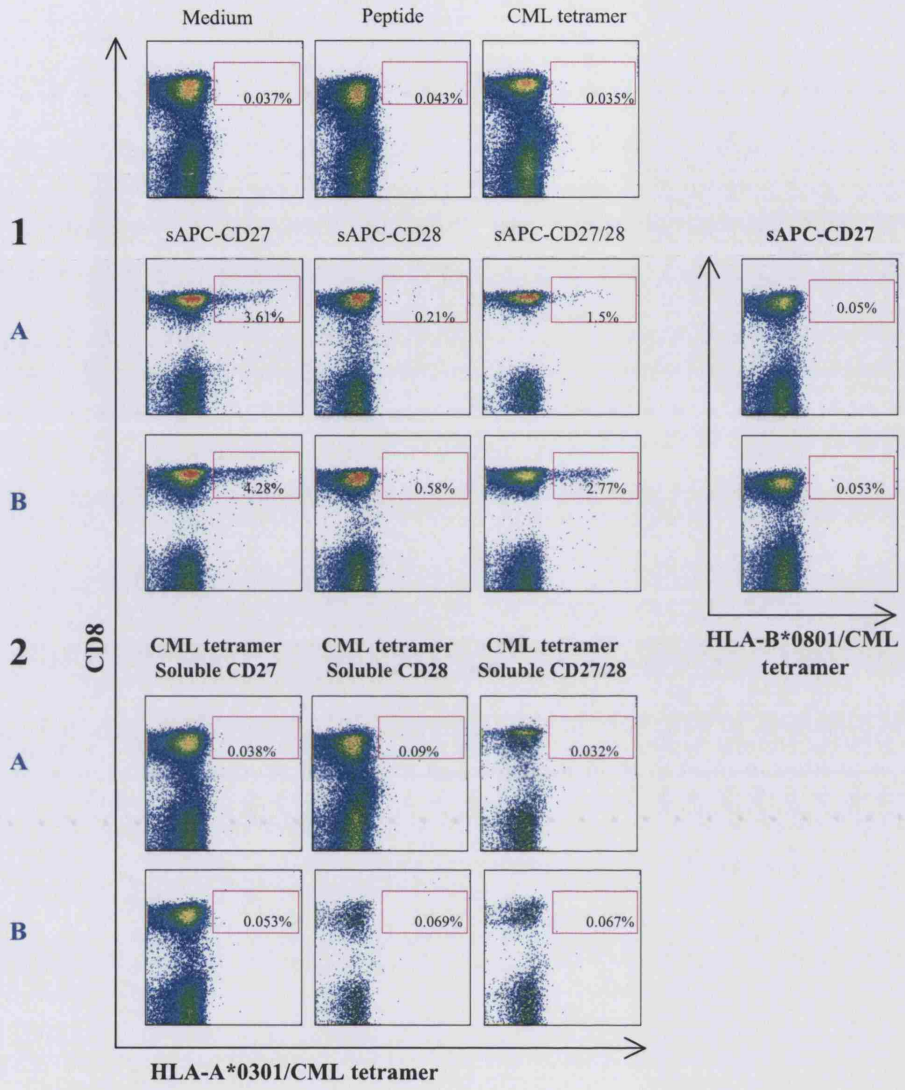


Figure 6.12: Representative HLA/tetramer dot plots of BCR/ABL specific T cells generated from an HLA-A*0301 healthy donor following stimulation with sAPCs:

PBMCs derived from an HLA-A*0301 healthy donor were stimulated with sAPCs (**1**) or HLA/CML tetramers and soluble costimulatory antibodies (**2**), providing anti-CD27, anti-CD28 or both anti-CD27 and anti-CD28 costimulatory antibodies. Cells were re-stimulated every 5 days without (**A**) or with (**B**) the addition of anti-$CD40^{L}$ costimulatory antibody as described in chapter 2. In parallel, PBMCs were incubated without antigen (**Medium**), or stimulated with either autologous peptide pulsed-irradiated T cell blasts incubated at a ratio of 1:5 PBMCs (**Peptide**), or with HLA/CML tetramers (**CML tetramer**). Activated T cells were harvested three days after the fourth stimulation and stained with HLA-A3/CML or HLA-B8/CML Allophycocyanin Tetramers and analysed by flow cytometry. Cells were gated on $CD3^{+}$ T cells and the frequency of tetramer positive cells are expressed as a percentage of $CD8^{+}$ T cells, shown in each dot plot gate.

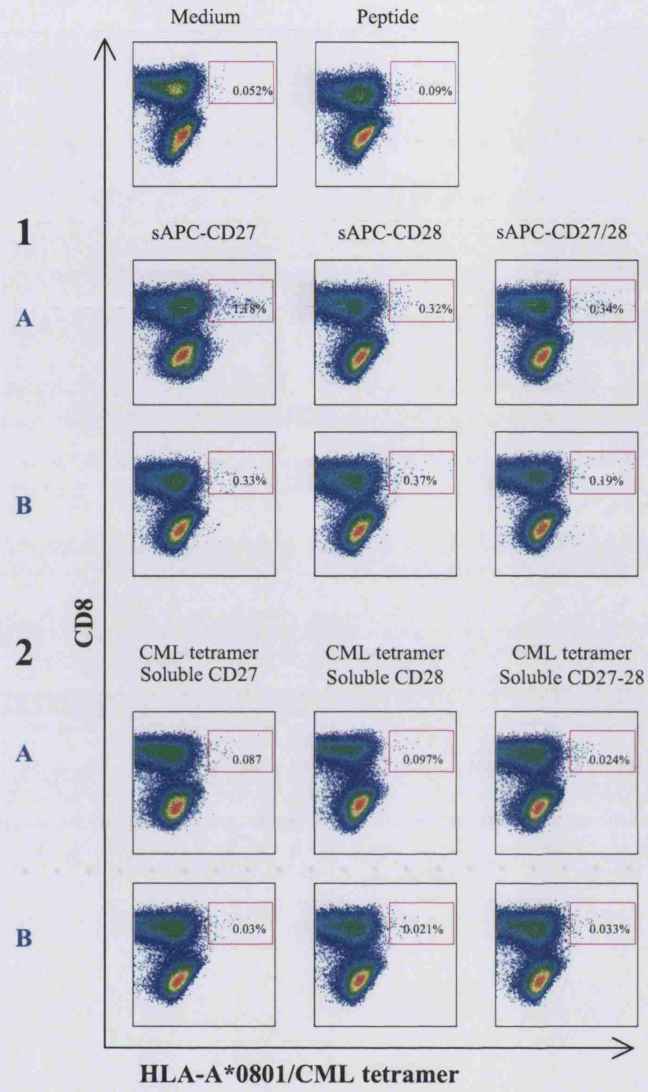


Figure 6.13: Representative HLA/tetramer dot plots of BCR/ABL specific T cells generated from an HLA-B*0801 healthy donor following stimulation with sAPCs:

PBMCs derived from an HLA-B*0801 healthy donor were stimulated with sAPCs (**1**) or HLA/CML tetramers and soluble costimulatory antibodies (**2**), following the protocol described previously (chapter 2). Cells were re-stimulated without (**A**) or with (**B**) the addition of anti-CD40^{L} costimulatory antibody from the second and subsequent stimulations. Cells were also incubated without antigen (**Medium**) or stimulated with autologous peptide pulsed-irradiated T cell blasts incubated at a ratio of 1:5 PBMCs (**Peptide**). Activated T cells were harvested three days after the fourth stimulation and stained with HLA-B8/CML Allophycocyanin tetramer and analysed by flow cytometry. Cells were gated on CD3^{+} T cells and tetramer positive cells are expressed as a percentage of CD8^{+} T cells, shown in each dot plot gate.

The stimulation of PBMCs with sAPCs complexes successfully generated BCR/ABL specific T cells from some donors. The frequencies of tetramer positive cells detected from HLA-A*0301 healthy donors after stimulation with sAPCs varied between 0.032% and 4.28% (**Figure 6.11**, panel **A** and **Figure 6.12**, panel **I**). In the context of HLA-B*0801 molecules, the frequencies of tetramer positive T cells detected after stimulations with these sAPCs complexes were lower and varied between 0.034% and 1.18% (**Figure 6.11**, panel **B** and **Figure 6.13**, panel **I**). The variability in the number of tetramer positive T cells detected from the different donors may reflect the differences in the nature and frequency of antigen specific T cells in each individual T cell repertoire. The frequency of BCR/ABL specific T cells detected from the peripheral blood of healthy donors was shown to be low and ranged from 0.01% to 0.12% (chapter 3).

No tetramer positive T cells were detected in the negative control (Medium) or after stimulations with either peptide-pulsed autologous PHA blasts (Peptide) or HLA/tetramers alone (CML tet). This was observed in the context of HLA-A*0301 and HLA-B*0801 healthy donors (≤ mean of 0.07%, **Figure 6.11**, panel **A** and **B** respectively).

Similar to the CMV specific T cell responses, the nature of the costimulatory signals provided in the sAPCs complexes appears to affect the levels of priming and/or expansion of BCR/ABL specific T cells. The stimulation with sAPC-CD27 generated the highest percentages of tetramer positive cells from HLA-A*0301 and HLA-B*0801 healthy donors (mean values of 1.21%±2.4% and 0.79%±0.66% respectively, **Figure 6.11** panel **A** and **B**). The early anti-CD27 costimulatory signal was found to be crucial for the successful expansion of BCR/ABL specific T cells, as its absence (sAPC-CD28) or dilution (sAPC-CD27/28) reproducibly abrogated or reduced the frequency of tetramer positive cells detected (mean values from 0.12%±0.2% to 0.61%±0.89%, **Figure 6.11**). The stimulation with sAPC-CD28 alone was not sufficient to prime or expand BCR/ABL specific T cells from these donors (from 0.032% to 0.7%, **Figure 6.11**; **Figure 6.12** and **Figure 6.13** panel **I**).

The addition of anti-$CD40^L$ costimulatory antibody from the second and subsequent stimulations with sAPCs further increased or at least maintained the frequency of tetramer positive T cells detected from these cultures. This was especially observed from HLA-A*0301 healthy donors (mean values from 0.41%±0.25% to 1.32%±2.96%; **Figure 6.11**, panel **A** and **Figure 6.12**, panel **I**). In the context of HLA-B*0801 healthy donors, however,

the addition of anti-CD40^L costimulatory antibody did not result in the expansion of BCR/ABL specific T cells (mean values between 0.18±0.19% and 0.33%±0.4% **Figure 6.11**, panel **B** and **Figure 6.13**, panel **I**). The frequency of tetramer positive T cells detected from some HLA-B*0801 healthy donors after stimulations in the presence of anti-CD40^L costimulatory antibody were even lower than after stimulations with sAPC-CD27 alone (from 0.11% to 0.73% versus 0.13% to 1.18%, **Figure 6.11**, panel **B** and **Figure 6.13**, panel **I**).

In contrast to sAPCs, the stimulation with HLA/tetramers and soluble costimulatory antibodies was not efficient in generating BCR/ABL specific T cells. Low numbers of tetramer positive cells were detected from both HLA-A*0301 and HLA-B*0801 healthy donors following four stimulations with various combination of HLA/tetramers and soluble costimulatory antibodies (mean values from 0.03%±0.01% to 0.06%±0.03%, **Figure 6.11**; **Figure 6.12** and **Figure 6.13**, panel **II**). Thus the cross-linking of HLA/peptide complexes with costimulatory antibodies was required to prime and expand BCR/ABL specific T cells from some of the healthy donors. According to the observations made in the context of the CMV specific T cell responses generated using a similar stimulation protocol, these data suggest that BCR/ABL specific T cells expanded from some of the healthy donors may originate from naïve precursor T cells.

In an attempt to assess the specificity of the tetramer positive cells detected in these cultures, activated BCR/ABL specific T cells were also stained with an irrelevant HLA/peptide tetramer. An example of such HLA/tetramer control staining is shown in the context of HLA-A*0301 molecules in **Figure 6.12**, panel **I**, right column. As no appropriate negative peptide control was available (HLA-A*0301 restricted tumour derived peptide described for CML), activated T cells were stained with the HLA-B*0801/BCR-ABL tetramers. No tetramer positive cells were detected with the irrelevant HLA-B8/BCR-ABL tetramers from the cultures stimulated with sAPC-CD27 or sAPC-CD27/40^L (≤0.053%, **Figure 6.12**, panel **I**, right column). This suggests that the detection of cells with the HLA-A*0301/BCR-ABL tetramers was specific.

Additionally, the specific binding of the antigen specific T cells to the HLA/peptide tetramers interferes with the subsequent binding of anti-CD3 antibody (Hoffmann et al., 2000). The CD3 mean fluorescence "down-regulation" of the HLA-A*0301/BCR-ABL tetramer positive T cells was assessed and a representative staining is shown in **Figure 6.14**.

The CD3 mean fluorescence of the tetramer positive cells was slightly lower compared to the mean fluorescence of the tetramer negative cells (red line and blue line respectively, **Figure 6.14**, panel **B**). This provides some indications that the HLA/peptide tetramers do bind to the TCR of the labelled cells and that the HLA-A*0301/BCR-ABL tetramer positive cells detected are specific.

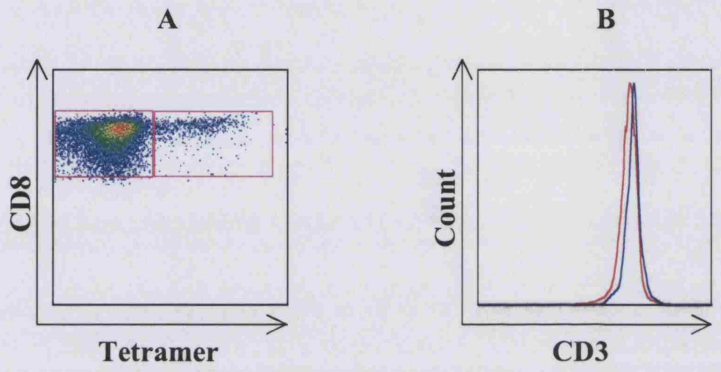


Figure 6.14: Assessment of CD3 mean fluorescence of tetramer positive T cells:

The specificity of HLA-A3/CML tetramer positive cells generated with sAPCs stimulations was assessed for their CD3 mean fluorescence. The tetramer positive and negative $CD8^+$ T cells were gated from the HLA/tetramer dot plot (**A**) and analysed for CD3 mean fluorescence (**B**). The binding of antigen specific $CD8^+$ T cells to HLA/peptide tetramers interfere with the subsequent binding of anti-CD3 antibody, which results in a lower CD3 mean fluorescence of $CD8^+$ $tetramer^+$ (red) than $CD8^+$ $tetramer^-$ (blue) T cell populations.

6.3.2 Time course analysis of BCR/ABL specific T cell responses generated with sAPCs from an HLA-A*0301 healthy donor:

A time course analysis of the antigen specific T cell responses was carried out from the healthy donor who generated the highest frequency of BCR/ABL specific T cells (HLA-A*0301 donor shown in **Figure 6.12**). The frequency of tetramer positive cells detected from this donor was monitored three days following each stimulation and is shown in **Figure 6.15**.

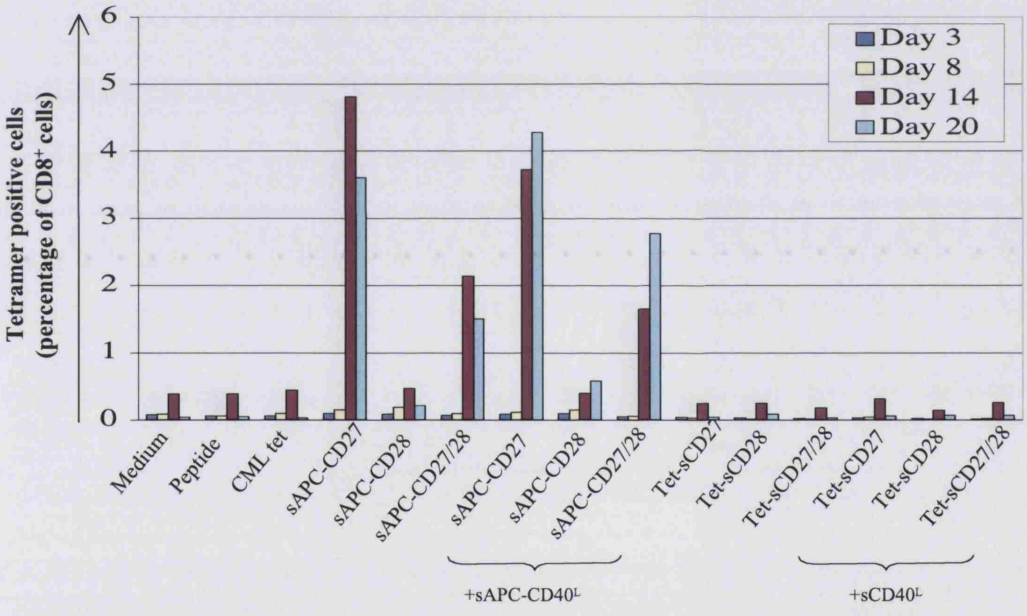


Figure 6.15: Time course analysis of HLA-A*0301 BCR/ABL specific $CD8^+$T cell responses generated from a healthy donor with sAPCs:

PBMCs derived from a HLA-A*0301 healthy donor were stimulated with various combinations of sAPCs or HLA/tetramers and soluble costimulatory antibodies (Tet-s) as described in chapter 2 and shown in Figure 6.11. Cells were also incubated without antigen (Medium), stimulated with peptide-pulsed irradiated T cell blasts incubated at a ratio of 1:5 PBMCs (**Peptide**), or HLA/tetramers (**CML tet**). The frequency of BCR/ABL specific T cells were monitored three days after the first (Day 3), second (Day 8), third (Day 14) and fourth (Day 20) stimulation. Cells were gated on $CD3^+$ T cells and the frequencies of tetramer positive cells are expressed as a percentage of $CD3^+CD8^+$ T cells.

BCR/ABL specific T cells were reproducibly detected from this donor only after stimulations with sAPCs. No or few tetramer positive cells were detected in the negative control (Medium) or following stimulations with either autologous peptide-pulsed T cell blasts (Peptide) or HLA/peptide tetramers alone and in combination with soluble costimulatory antibodies (Tet-s; ≤0.44%, **Figure 6.15**).

Three consecutive stimulations with sAPCs were necessary to prime and/or expand BCR/ABL specific T cells from this donor (day 14, **Figure 6.15**). The highest percentage of tetramer positive T cells generated from this donor was reproducibly detected after stimulation with sAPC-CD27 (4.81%, day 14). In addition, as previously observed the signalling via this early anti-CD27 antibody was required to generate and detect BCR/ABL specific T cells. The replacement or dilution of anti-CD27 antibody by anti-CD28 and/or anti-$CD40^L$ antibodies was again found to abrogate or reduced the expansion of tetramer positive cells from this donor (from 0.4% to 3.72% day 14, **Figure 6.15**).

Following the fourth and last stimulation the signalling via anti-CD27 costimulatory antibody remains crucial; however the addition of anti-$CD40^L$ costimulatory antibody induced a further expansion of BCR/ABL specific T cells (**Figure 6.15**, day 20).
The highest percentage of BCR/ABL specific T cells after four stimulations were detected from the cultures stimulated with sAPCs in combination with anti-CD40L antibody (sAPC-CD27/$CD40^L$, 4.28%, day 20). In addition, the frequency of tetramer positive T cells between the third and the fourth stimulation increased only for the conditions, which included anti-$CD40^L$ costimulatory signals from the second stimulation (from 0.4% and 3.72% to 0.58% and 4.28%, day 20, **Figure 6.15**).

Thus, these data suggest that at least from this healthy donor the nature, the amount as well as the timing of costimulatory signals provided is independently important for the successful generation of BCR/ABL specific T cells.

6.3.3 Stimulation of BCR/ABL specific T cells with sAPCs from CML patients:

Since the sAPCs seemed able to prime BCR/ABL specific T cells from healthy individuals, their ability to generate BCR/ABL specific T cell responses from CML patients was next assessed. Due to the limited amount of blood and subsequently PBMCs from these patients, it was only possible to test a few conditions from three HLA-A*0301 and two HLA-B*0801 CML patients (CML patients 8, 10, 12 and 16, 19 shown in **Table 4.1**, chapter 4). Patient derived PBMCs were stimulated following the same protocol and including the same controls as described in chapter 2 and section 6.4.1. In relation to the responses generated from healthy donors and because of the constraints on cell numbers, patient derived PBMCs were stimulated with sAPCs providing at least the anti-CD27 costimulatory signal. BCR/ABL specific T cells were detected after four stimulations from some patients and are shown in **Figure 6.16**. A representative HLA/tetramer staining is also shown in **Figure 6.17**.

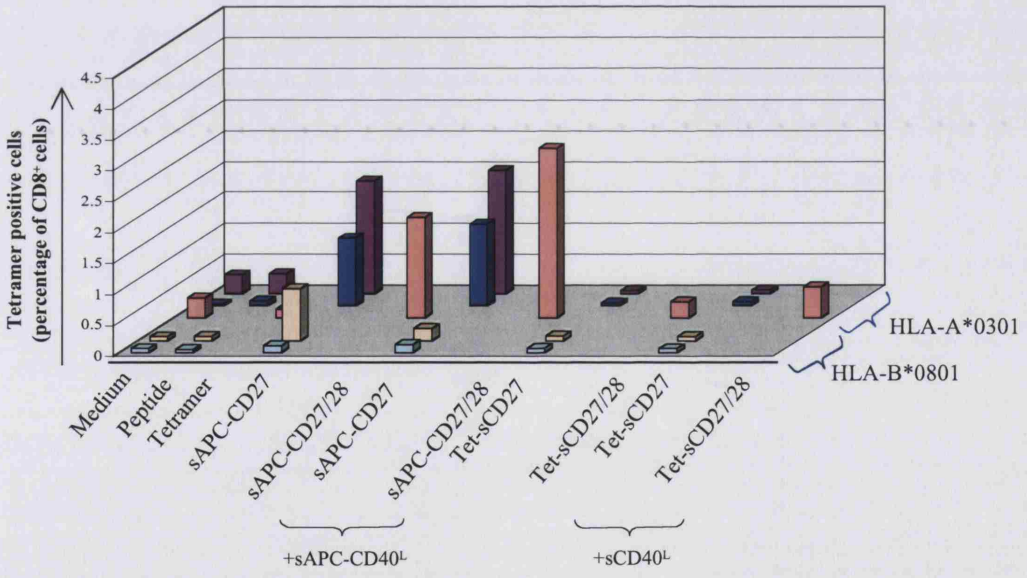


Figure 6.16: Stimulation of BCR/ABL specific T cells from HLA-A*0301 CML patients with sAPCs:

PBMCs derived from two HLA-B*0801 and three HLA-A*0301 CML patients were stimulated with sAPCs or with HLA/CML tetramers and soluble costimulatory antibodies as described in chapter 2. From the second and subsequent stimulations, cells were re-stimulated with the same conditions with or without the addition of anti-$CD40^L$ costimulatory antibodies. In parallel, PBMCs were incubated without antigen (Medium), or stimulated with either autologous peptide-pulsed irradiated T cell blasts (Peptide), or with HLA/CML tetramers according to the number of PBMCs available from the patients. Activated cells were harvested three days after the fourth stimulation (day 20) and stained with the appropriate HLA/CML tetramers. The frequencies of tetramer positive cells are expressed as a percentage of $CD3^+CD8^+$ T cells.

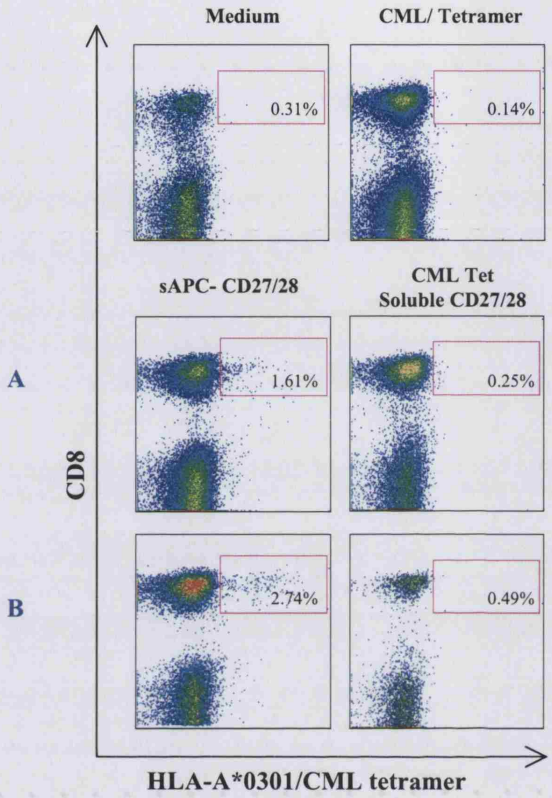


Figure 6.17: Representative HLA/tetramer dot plots of BCR/ABL specific T cells generated from an HLA-A*0301 CML patient following stimulation with sAPCs:

PBMCs derived from an HLA-A*0301 b3a2^{+} CML patient were stimulated with sAPC-CD27/28 or HLA/CML tetramers and soluble anti-CD27 and anti-CD28 costimulatory antibodies. Cells were re-stimulated without (**A**) or with (**B**) the addition of anti-CD40^{L} costimulatory antibodies. In parallel, PBMCs were incubated without antigen (**Medium**) or stimulated with HLA/CML tetramers (**CML tetramer**). Activated T cells were harvested three days after the fourth stimulation and stained with HLA-A3/CML Allophycocyanin Tetramer and analysed by flow cytometry. The frequencies of tetramer positive cells are expressed as a percentage of CD3^{+}CD8^{+} T cells and are shown in each dot plot gate.

In a similar way to the responses generated in healthy donors, BCR/ABL specific T cells were successfully stimulated and expanded from CML patients only after stimulation with sAPCs. The frequency of tetramer positive cells detected after stimulation with sAPCs varied between 0.11% and 2.74% compared to the negative control (Medium, ≤0.3%, **Figure 6.16**).

BCR/ABL specific T cell responses were detected from all the HLA-A*0301 CML patients assessed (from 1.08% to 2.74%, **Figure 6.16** and **Figure 6.17**). In the context of HLA-B*0801 however, tetramer positive T cells were detected after stimulation with sAPC-CD27 from only one out of the two patients assessed (0.85%, **Figure 6.16**). All the patients assessed in this study were in complete cytogenetic remission, with low bcr/abl transcript detection at the molecular level. Thus, these data suggest that HLA-A*0301 CML patients are more capable of expanding BCR/ABL specific T cells than HLA-B*0801 patients.

The addition of anti-$CD40^L$ costimulatory antibody from the second stimulation was also found to further increase the expansion of tetramer positive cells from the HLA-A*0301 CML patients (from 1.08% and 1.81% to 1.31% and 2.74% **Figure 6.16** and **Figure 6.17**). Thus, the BCR/ABL specific T cell responses primed from both healthy donors and CML patients were affected by the nature and the timing of costimulatory molecules signalling, at least in the context of HLA-A*0301 molecule.

The stimulation with HLA/tetramers alone or in combination with soluble costimulatory antibodies also failed to stimulate and expand BCR/ABL specific T cells from CML patients (from 0.037% to 0.25%, Figure 6.15 and Figure 6.16). Thus, as observed in the context of healthy donors and according to the CMV specific T cell responses, these data suggest that the BCR/ABL specific T cells primed from CML patients also derive from naïve T cell precursor.

Although these experiments remain preliminary and a higher number of both CML patients and healthy donors would need to be assessed, the ability of these sAPCs to prime and expand BCR/ABL specific T cells from at least HLA-A*0301 healthy donors and CML patients is very encouraging.

6.3.4 Cytotoxic activity of BCR/ABL specific T cells stimulated with sAPCs:

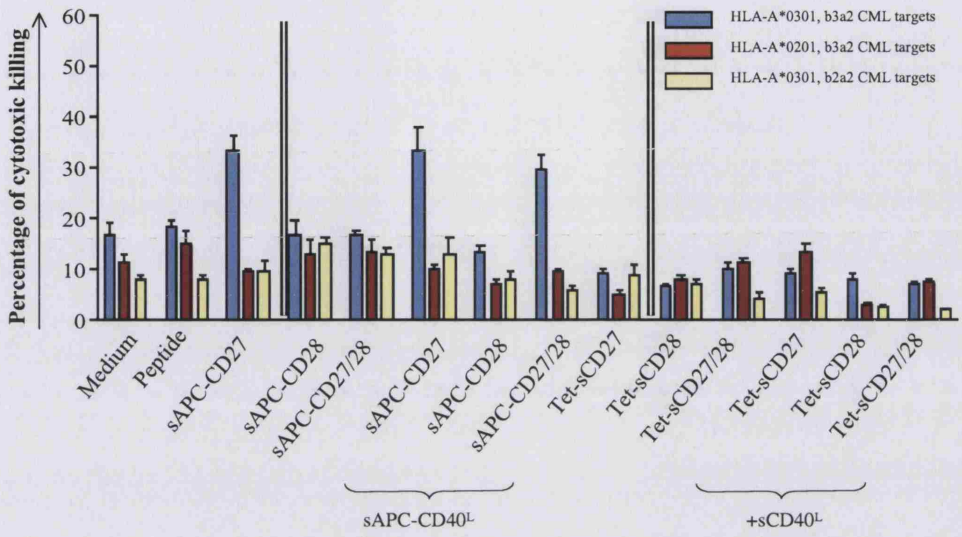
The BCR/ABL specific T cells generated following stimulations with sAPCs were tested for their specific cytotoxic activity against HLA-matched CML target cells. Antigen activated T cells were harvested three days after the fourth stimulation (day 20) and incubated with ^{51}Chromium labelled CML target cells at a ratio of 1:20. This ratio was increased based on the specific cytotoxic responses obtained in the previous chapter assessed at a ratio of 1:10. Both the HLA and the peptide specificity of these CTLs were tested in parallel by assessing the cytotoxic activity against HLA-unmatched CML cell target cells and HLA-matched but antigen mismatched b2a2 target cells. The cytotoxic activity of the BCR/ABL specific T cells was determined by measuring the chromium release by the CML target cells as described in chapter 2.

The cytotoxic activity was assessed from three HLA-A*0301 and two HLA-B*0801 healthy donors as well as from two HLA-A*0301 and one HLA-B*0801 CML patient. None of the HLA-B*0801 donors demonstrated a specific cytotoxic activity against HLA-matched CML target cells above the background (HLA-unmatched target cells). The frequency of tetramer positive cells generated from these donors was shown to be low and these do not appear to recognized primary CML cells.

In contrast, in the context of the HLA-A*0301 molecule, one healthy donor and one CML patient demonstrated antigen specific cytotoxic activity as shown in **Figure 6.18**, panel **A** and **B** respectively.

The cytotoxic responses generated from the healthy donor were first assessed (**Figure 6.18**, panel **A**). Only T cells stimulated with sAPC-CD27, sAPC-CD27/40^{L} and sAPC-CD27/28/40^{L} demonstrated specific cytotoxic activity against HLA-matched CML target cells above the controls (from 12.8% to 18.2% above HLA-unmatched or b2a2 CML target cells). These stimulation conditions had also been previously shown to expand the highest frequency of tetramer positive cells (from 2.77% to 4.28%, shown in **Figure 6.12**, panel **I**). Although the frequency of tetramer positive cells varied between the stimulation conditions, these BCR/ABL specific T cells demonstrated a similar percentage of specific cytotoxic activity against HLA-matched CML target cells. In addition, the BCR/ABL specific T cells successfully expanded following sAPC-CD27/28 stimulation (1.5% tetramer positive cells) did not recognize and kill CML target cells above the background. Thus, it appears that the costimulatory signals provided for the activation of antigen specific T cells influence the specific cytotoxic function of these cells.

A



B

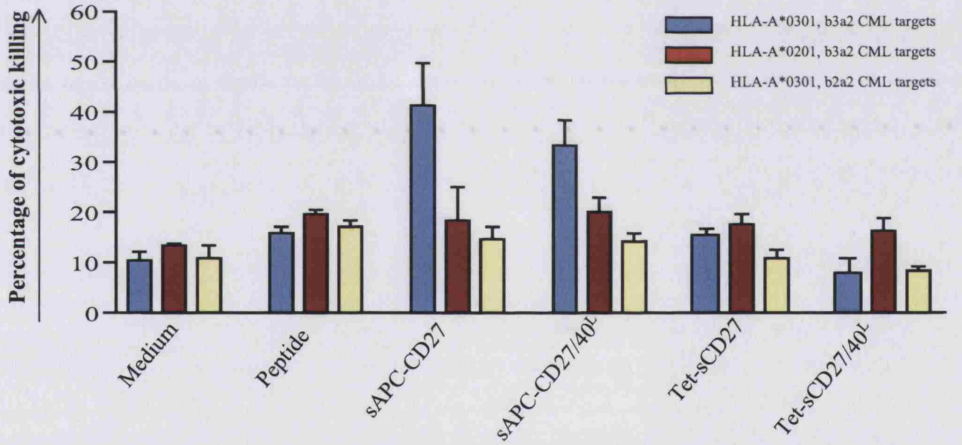


Figure 6.18: Cytotoxic activity of BCR/ABL specific $CD8^+$ T cells generated with sAPCs:

PBMCs derived from HLA-A*0301 healthy donor (panel **A**) and CML patient (panel **B**) were stimulated with the various combinations of sAPCs or HLA/CML tetramers and soluble costimulatory antibodies (as described in chapter 2) and were assessed for their cytotoxic activity by Chromium release assay. Controls cells (Medium, Peptide) were also included in the assay. Activated T cells were harvested three days after the fourth stimulation (day 20) and incubated with HLA-matched $b3a2^+$ or $b2a2^+$ CML target cells and HLA-unmatched $b3a2^+$ CML target cells. The specific cytotoxic activity was measured after 4 hours incubation of target cells with effector T cells at a ratio 1:20 respectively. The percentage of specific lysis was calculated using the following formula: 100× (cpm experimental release - cpm spontaneous release) / (cpm maximal release-cpm spontaneous release) where cpm represents the count per minutes. The error bars represent the standard deviation between triplicate samples.

None of the T cells stimulated with either peptide-pulsed autologous PHA blast cells or HLA/tetramers alone and in combination with soluble costimulatory antibodies demonstrated specific cytotoxic activity above the background (HLA unmatched or b2a2 targets). This was expected, as the frequency of tetramer positive T cells detected from these cultures was low.

One out of two HLA-A*0301 CML patients tested also demonstrated specific cytotoxic activity against HLA matched b3a2 CML target cells (**Figure 6.18**, panel **B**). The BCR/ABL specific T cells detected after stimulations with sAPC-CD27 and sAPC-CD27/40^{L} (shown in **Figure 6.17**) specifically recognized and killed HLA-matched CML target cells (8.5% and 5% above the background respectively, **Figure 6.18**, panel **B**). No specific cytotoxic activity above the background was observed from the cultures left un-stimulated (Medium) or stimulated with peptide-pulsed PHA blast cells (Peptide) or HLA/tetramers and soluble costimulatory antibodies (Tet-s). A higher percentage of specific cytotoxic activities were generated from the healthy donor than from the patient, however higher frequencies of tetramer positive cells were also detected from this healthy donor.

In conclusion, these experiments demonstrate the feasibility of generating functional BCR/ABL specific T cells in the context of HLA-A*0301 from at least one healthy donor and one CML patient.

DISCUSSION

In this chapter we studied the potential modulation of antigen specific $CD8^+$ T cell responses using modified HLA tetramers as soluble antigen-presenting complexes (sAPCs). These sAPCs are devoid of natural antigen processing and presentation machinery and thus their application is limited to known HLA-restricted antigen specific responses. Their simplicity is however very attractive as they are easy to generate, and the number as well as the nature of molecules presented to the T cells can be completely controlled.

The modulation of antigen specific T cell responses was first validated in the context of HLA-A*0201 restricted CMV immuno-dominant peptide. These sAPCs were demonstrated to be more robust than natural APCs such as DCs. As they persistently present the antigen, we demonstrated that their removal from the cultures was required to avoid antigen specific T cells exhaustion. The limited number of molecules that can be presented on these modified tetramers was an initial concern for its overall avidity of interaction with T cells. The comparison between presenting three or four HLA/peptide molecules to the T cell clearly demonstrated a higher expansion of CMV specific memory T cells as well as their differentiation into terminal effector cells when stimulating with four HLA/peptide molecules per complex. HLA/peptide tetramers and soluble costimulatory antibodies were much more efficient for the expansion of antigen specific T cells and induced from 50 to 200 fold expansion of CMV specific $CD8^+$ T cells after four stimulations. The priming of CMV specific T cells from naïve individuals was however successful only after stimulation with sAPCs. The cross linking of HLA/peptide and costimulatory antibody on these sAPCs was in fact shown to be mandatory for the priming of naïve T cell responses. It has been demonstrated that in contrast to antigen specific memory T cells, the immunological synapses of naïve T cells are not yet constituted (Tuosto et al., 2001). Thus it is possible that naïve T cells require to received both HLA/peptide and costimulatory signals in close proximity to initiate the TCR activation cascade. One way of testing this hypothesis would be to initially prime CMV specific T cells with sAPCs and then further expand these cells with HLA/tetramers and soluble costimulatory antibodies. Unfortunately it was not possible to perform these experiments as I was reaching the end of my PhD.

The BCR/ABL specific T cell responses assessed from healthy donors and CML patients were successfully generated only after stimulations with sAPCs, but not HLA/tetramers and soluble costimulatory antibodies. Accordingly, these tumour specific responses appeared to resemble to a primary immune response. This was expected from healthy donors as they

have not encountered the antigen and subsequently primed an immune response *in-vivo*. However the BCR/ABL specific T cells successfully expanded from some CML patients also appeared to derive from naïve T cell precursors in comparison to the CMV responses model. Thus these data suggest that the patients assessed, which were in complete cytogenetic remission lacked or had deleted BCR/ABL specific precursor T cells. Although these experiments remain preliminary, the sAPCs appeared to be capable of generating functional antigen specific T cells from both an HLA-A*0301 healthy donor and a CML patient and constitute an encouraging alternative method to prime tumour specific T cell responses. No cytotoxic BCR/ABL specific T cells were however generated from any of our HLA-B*0801 donors. As observed in the previous chapter, the HLA-A*0301 restricted BCR/ABL derived antigen appears to be more immunogenic than the HLA-B*0801 restricted one.

For both CMV and BCR/ABL responses, the nature, amount and timing of the costimulation(s) provided influenced the magnitude and the differentiation of the antigen specific T cells generated. The stimulation of CMV specific responses providing anti-CD27 antibody and/or anti-CD28 efficiently induced a significant expansion of antigen specific $CD8^+$ T cells. The signalling via anti-CD27 costimulatory molecules was shown to be mandatory for the efficient priming of both CMV and BCR/ABL specific T cell responses and confirms the major role of CD27 costimulation for the activation and proliferation of human naïve T cells preferentially, particularly with respect to tumour responses (Couderc et al., 1998; Hintzen et al., 1995; Lorenz et al., 1999). In our experiments, the co-stimulation of BCR/ABL responses with sAPC-CD28 antibody alone was not sufficient to generate detectable tetramer positive T cells. However it has been previously demonstrated that high levels of CD28 signalling were required for the expansion of tumour specific T cells (Latouche and Sadelain, 2000). Additionally, anti-$CD40^L$ signalling was shown to play a major role as a late activation signal and to bypass the need for helper T cells (Bennett et al., 1998; Schoenberger et al., 1998). Its signalling function has been shown in our experiments and by others to be crucial for the generation of tumour specific T cell responses (Couderc et al., 1998; Diehl et al., 1999; French et al., 1999).

The dilution of the initial costimulatory signal by the addition of a second costimulatory signal was also demonstrated to affect the magnitude of both CMV and BCR/ABL specific responses. Antigen stimulations with sAPC-CD27 alone induced the expansion of higher number of CMV or BCR/ABL specific T cells than stimulations with sAPC-CD27/28 or sAPC-$CD27/40^L$.

Finally, the overall antigen-specific T cell responses were demonstrated to be dependent on the timing of the costimulation. In the context of HLA-A*0301 restricted BCR/ABL responses, early activation signals provided by anti-CD27 antibody were clearly required for the initiation of BCR/ABL specific $CD8^+$ T cell responses, however the maintenance and expansion of these antigen specific T cells beyond the third stimulation was dependent on late anti-$CD40^L$ costimulation. Anti-$CD40^L$ antibody was also shown to induce a faster proliferation and differentiation of CMV specific T cells into terminal effectors.

In this study, the modulation of antigen specific T cell responses were tested using anti-CD27, anti-CD28 and/or anti $CD40^L$ costimulatory molecules, however other costimulatory molecules may also enhanced these responses and need to be assessed in the future. For example the expression of the adhesions molecules LFA-3 (CD58) and/or ICAM-1 (CD54) in conjunction with CD80 (anti-CD28) on cellular or acellular artificial APCs has been demonstrated to significantly increase the fold expansion of functional antigen-specific T cells in the context of CMV, melanoma and mHAgs (Guelly et al., 2002; Latouche and Sadelain, 2000; Oosten et al., 2004; Papanicolaou et al., 2003). These adhesion molecules were shown to induce the cellular membrane rearrangement and to initiate the formation of the immunological synapse between the T cells and the APCs. In the context of the soluble antigen-presenting complexes assessed here, the signalling via these adhesion molecules may not be critical. Another costimulatory molecule expressed on activated T cells the 4-1BB Ligand was shown to induce a strong proliferation and amplification of cytotoxic T cell functions (Cannons et al., 2001; Shuford et al., 1997). 4-1BB signalling was specifically demonstrated to protect T cell from activation-induced cell death and to maintain antigen specific T cells survival and proliferation over 70 days of culture (Hurtado et al., 1997; Maus et al., 2002). Such signalling may be beneficial for the long-term survival of CMV memory specific T cells stimulated with sAPCs.

Finally, the data presented here demonstrates the ability of sAPCs to modulate antigen specific $CD8^+$ T cell responses in the context of CMV and in two cases of BCR/ABL. These techniques could be easily applied to a variety of HLA/peptide complexes. The use of HLA/peptide tetramers has been demonstrated *in vivo* in a mouse model, the clinical application of such vaccination in human remains however unclear (Maile et al., 2001). The presence of Streptavidin in these sAPCs complexes would be of a primary concern as it has been shown that streptavidin (or avidin) is immunogenic. In addition, the Fc regions of the costimulatory antibodies cross-linked on these sAPCs could induce the undesirable complement-dependent killing of the targeted cells. Nevertheless these sAPCs are proving to be efficient to prime and/or expand a high number of antigen specific T cells *ex-vivo*, which could be safely re-infused into patients as a specific T-cell adoptive immunotherapy.

CHAPTER 7

Conclusion

The BCR/ABL p210 fusion protein has long been considered an ideal target antigen for the development of immunotherapeutic strategies in CML due to its central role in malignant transformation and to its unique novel amino acid sequence solely expressed in leukaemic cells. In fact, the BCR/ABL breakpoint protein represents a rare truly tumour-specific antigen. The data presented in this PhD thesis illustrate the problems associated with generating BCR/ABL specific $CD8^+$ T cell responses *in-vitro* for the development of immunotherapeutic treatment of CML patients.

Some HLA types including HLA-A*0301 and HLA-B*0801 have been associated with a reduced incidence of CML (Posthuma et al., 1999). This has given rise to the hypothesis that BCR/ABL derived peptides can be processed and presented in the context of these particular HLA molecules with subsequent generation of protective specific immune response. The feasibility of detecting and stimulating BCR/ABL specific T cell responses were therefore first assessed in the context of HLA-A*0301 and HLA-B*0801 molecules.

As shown in chapter 3, two 9-mer peptides derived from the BCR/ABL b3a2 junctional region, KQSSKALQR and GFKQSSKAL, were eluted from HLA-A*0301 and HLA-B*0801 leukaemic cell respectively. This data demonstrates that BCR/ABL peptides can be naturally processed and presented in the context of these HLA molecules on the cell surface of HLA-transfected K562 cells and on leukaemic cells derived from CML patients.

HLA tetramer complexes, refolded with the appropriate BCR/ABL peptide, were then generated and used to monitor the frequency of BCR/ABL specific T cells in the peripheral blood of CML patients. The frequencies of BCR/ABL specific T cells were low and in some patients even absent. In our cohort of CML patients, higher frequencies of tetramer positive T cells were detected from patients who were at least in partial clinical remission compared to those remaining in active phase of the disease. In the latter patients, the large tumour burden may have affected their ability to generate antigen-specific T cell responses *in-vivo*.
In chapter 4, a number of *in-vitro* stimulation protocols were then assessed for their ability to expand these tetramer positive cells. Our results demonstrated that conventional stimulation protocols, including the use of T cells and DCs as APCs, were not sufficiently

efficient to stimulate and expand BCR/ABL specific T cells from CML patients. However, CMV or Flu specific T cell responses were successfully generated *in-vitro* from these patients following identical stimulation protocols. In view of the low frequency of tetramer positive T cells detected in patients and the low avidity of BCR/ABL peptides as demonstrated in chapter 3, the successful generation of BCR/ABL specific T cells may require additional and stronger co-stimulation. It is also possible that the large tumour burden and the defective leukaemic DC functions described in CML patients (Dong et al., 2003) may have induced BCR/ABL specific T cell tolerance *in-vivo*, thus rendering their expansion highly difficult *in-vitro*. Additionally, given the assumption that HLA-A*0301 and HLA-B*0801 alleles are protective against developing CML, it may be considered therefore that individuals who bear these HLA types yet still develop CML may belong to one of the worst groups from whom to stimulate BCR/ABL specific T cell responses *in-vitro*.

In healthy individuals, the basal frequency of BCR/ABL specific T cells detected in the peripheral blood was negligible. This is to be anticipated due to the fact that the BCR/ABL fusion protein expression is restricted to leukaemic cells and should therefore not be present in normal cells. In contrast to CML patients, the stimulation of PBMCs derived from healthy donors induced a statistically significant expansion of BCR/ABL specific T cells. However the levels of these responses remained low and in the majority of cases these antigen-specific T cells did not recognize or kill HLA-matched CML cells.

The use of the longer 17-mer BCR/ABL peptide to stimulate antigen-specific T cell responses was demonstrated to induce a higher frequency of tetramer positive T cell expansion when compared to the use of the 9-mer BCR/ABL peptides. Thus, this longer peptide may contain other HLA-class I and/or HLA-class II epitope, which indirectly enhanced the stimulation and expansion of HLA-A*0301 and HLA-B*0801 restricted BCR/ABL specific $CD8^+$ T cell responses. Moreover this longer peptide has been demonstrated to efficiently generate HLA-class II restricted BCR/ABL specific T cell responses in healthy donors (Bertazzoli et al., 2000; Bosch et al., 1996; Osman et al., 1999; ten Bosch et al., 1995; Yasukawa et al., 1998). Although anti-tumour responses were initially thought to be mainly mediated by $CD8^+$ cytotoxic T cells, tumour-specific $CD4^+$ T cells have since been demonstrated to both contribute to and sustain these responses, including that seen in the GvL response (Claret et al., 1997; Dodi et al., 2002; Drobyski et al., 1993; Fenton et al., 1998; Hung et al., 1998; Jiang and Barrett, 1997; Marzo et al., 2000; Pawelec et al., 2001). With the advances in the generation of HLA class II tetramers, it

should soon become possible to assess BCR/ABL specific $CD4^+$ T cell responses both qualitatively and quantitatively and subsequently extend immunotherapies to include both CD8 and CD4 positive T cells.

The generation of BCR/ABL specific T cell responses has also been attempted using plasmacytoid DCs as APCs. The lymphoid origin of pDCs suggests that these cells may not derive from Philadelphia positive myeloid progenitors and may therefore function more efficiently as APCs than myeloid DCs. As shown in chapter 4, significantly reduced numbers of pDCs were detected in the peripheral blood of CML patients assessed at diagnosis compared to healthy donors. The reduced frequency of pDCs in these patients may render them unable to efficiently generate antigen-specific T cell responses *in-vivo* and makes their study *in-vitro* difficult. Responses to peptide-pulsed pDCs could only be assessed from healthy donors and yet in this group, two consecutive stimulations were insufficient in generating BCR/ABL specific cytotoxic T cell responses. The relevance of pDCs in mediating anti-tumour responses remained to be confirmed, especially from CML patients, as these cells may represent the only DCs subset, which is not affected by the malignant transformation. Moreover, the frequency of pDCs was shown to be restored in patients responding to Imatinib Mesylate. It is possible that the reduction of the myeloid malignant clones by this therapy has indirectly resulted in the normalisation of circulating pDC numbers in these patients. Additionally Mothys' group have recently confirmed that both the frequency and the function of pDCs were restored in patients following Imatinib Mesylate treatment (Mohty et al., 2004). Their data would therefore suggest a more direct effect of Imatinib Mesylate on the function of pDCs.

In conclusion, it was not possible to generate functional BCR/ABL specific T cells from either CML patients or healthy donors using these different conventional stimulation protocols. The priming and/or expansion of tumour specific T cells may require a higher level of co-stimulation than those provided by conventional APCs. The standard protocol for the generation of immune responses utilises mDCs, as these cells are capable of both priming naïve T cells and boosting memory T cell responses. However DCs have also been shown to induce antigen-specific T cell tolerance, especially in the context of tumour antigen (Brown et al., 2003; den Boer et al., 2001; Steinman et al., 2003). In these studies, the suppressive function of DCs has been attributed to the lack of longevity of antigen presentation and/or the nature of costimulatory molecule expressed. It is also possible that

the HLA/BCR-ABL peptide complexes presented on peptide-pulsed DCs are diluted out among other HLA/peptide complexes.

Thus, in order to control both the numbers and the nature of HLA/peptide complexes and costimulatory molecules presented to T cells, artificial soluble antigen presenting complexes (sAPCs) were developed and consisted of cross-linking biotinylated HLA/peptide complexes with a biotinylated costimulatory antibody onto a streptavidin core molecule.

In chapter 6, the stimulation of PBMCs derived from healthy donors or CML patients with sAPCs was demonstrated to induce a significant expansion of BCR/ABL specific T cells in some donors. In the context of HLA-A*0301, these antigen-specific T cells were also demonstrated in two cases to be cytotoxic to leukaemic cells in an HLA-restricted manner. The successful activation of these antigen specific T cells was shown to be dependent on costimulation with anti-CD27 antibody (sAPC-CD27). In addition, stimulation with sAPC-$CD40^{L}$ was shown to enhance the proliferation of BCR/ABL specific T cells. These data support and confirm the crucial roles of CD27 and $CD40^{L}$ costimulatory signalling for the activation of tumour specific T cells (Couderc et al., 1998; Lorenz et al., 1999).

The numbers of HLA/peptide molecules presented on the sAPCs was demonstrated, in the CMV activation model, to affect the magnitude of the antigen-specific T cell responses. HLA/tetramer plus soluble costimulatory antibodies were in fact shown to induce a greater proliferation of CMV specific memory T cells compared to the sAPCs, possibly due to the higher avidity of HLA/tetramers for T cell interactions. However, close proximity of HLA/peptide complexes and costimulatory molecules in the sAPCs appeared to be required for the priming of CMV specific naïve responses. From these observations, it is possible that the activation threshold of BCR/ABL specific T cells could be enhanced by increasing the number of HLA/peptide molecules presented on the sAPCs. In our system, however, this number was limited to four molecules and this would suggest the development of pentameric sAPCs or the use of streptavidin-coated beads onto which one could cross-link these molecules.

The data presented here also showed that higher expansions of BCR/ABL specific T cells were achieved in the context of HLA-A*0301 in contrast to HLA-B*0801. In addition, specific cytotoxic activity against HLA-matched CML target cells was observed only in HLA-A*0301 donors. As both HLA-A*0301 and HLA-B*0801 restricted BCR/ABL

peptides were demonstrated to be presented on the cell surface of leukaemic cells, this observation would suggest that the HLA-B*0801 associated BCR/ABL peptide may have a lower affinity for the HLA molecule and thus may not be engaging with its corresponding ligand long enough to sufficiently activate T cells. Similarly, it is also possible that there are no T cells specific for the HLA-B*0801/BCR-ABL complexes in the repertoire of these donors.

The identification of BCR/ABL epitopes and their efficiency in stimulating antigen-specific T cell responses was also extended to the most common Caucasian HLA molecule, namely HLA-A*0201. As shown in chapter 3, no peptides from the BCR/ABL b3a2 junctional region were eluted from the surface of HLA-A*0201 transfected K562 cells or HLA-A*0201 CML cells. In addition, screening for antigen-specific T cells was performed using HLA/tetramers refolded with three peptides derived from the BCR/ABL b3a2 region, selected for their prediction to bind HLA-A*0201 molecules. No BCR/ABL specific T cells were detected in the peripheral blood of HLA-A*0201 CML patients or healthy donors. Thus it would appear that there are no 9-mer peptides from the BCR/ABL b3a2 fusion protein capable of being processed and presented in the context of HLA-A*0201. If this is the case, one would not expect T cells specific for HLA-A*0201/BCR-ABL complexes to be present *in-vivo*. Accordingly as shown in chapter 5, it was not possible to generate antigen-specific T cell responses *in-vitro* using these peptides. The HLA-A*0201 associated BCR/ABL peptides assessed in this study are therefore not useful for consideration in immunotherapeutic applications.

The three potential HLA-A*0201 associated BCR/ABL peptides were also demonstrated experimentally to be very low HLA binders. Thus it is quite possible that these peptides do not stabilize HLA-A*0201 cell surface molecules sufficiently to prime and/or expand antigen-specific T cells. In order to increase the peptide HLA binding affinity, the SSKALQRPV peptide was modified to contain an HLA-A*0201 dominant anchor residue, i.e. leucine, at position two. This modified peptide did not significantly increase its HLA binding affinity nor the magnitude of the antigen-specific T cell response compared to the wild-type peptide. Although a higher frequency of tetramer positive cells was detected with the modified peptide, these T cells did not recognize HLA-A*0201 CML cells or peptide-pulsed T2 cells and brings into question the antigen specificity of these T cells.

In comparison, other tumour associated antigens presented in the context of HLA-A*0201, namely WT1 and PR1, were shown in chapter 5 to successfully generate functional antigen-specific T cell responses from healthy donors and some CML patients. Both WT1 and PR1 peptides were demonstrated to bind to HLA-A*0201 with a higher affinity than the BCR/ABL peptides. The improved responses seen with these peptides may therefore be due to their enhanced presentation to T cells. These observations support the idea that WT1 and PR1 are more efficient targets as tumour antigens than BCR/ABL antigens for the application of immunotherapy in CML.

In respect of clinical considerations, the data presented in this PhD study suggest the following:

- Patient-derived BCR/ABL specific T cells may be tolerized *in-vivo* and consequently not capable of responding to *in-vitro* stimulations. Statistically significant expansion of BCR/ABL specific T cells was only achievable from healthy donors.

- Patients' treatment may affect their ability to generate BCR/ABL specific T cell responses *in-vitro*. For example, patients who received alloHSCT were shown to be more likely responding to *in-vitro* stimulations compared to those treated solely with Imatinib mesylate. In the former group, the expanded antigen-specific T cells may be all donor-derived. This would agree with the previous observations that BCR/ABL specific T cells can be generated from healthy donors only. In addition, some of these responses may be reflective in part of the clinical GvL effect that is known to be mediated in patients following alloHSCT and DLI.

- Patients' clinical response to treatment also seems to affect their capacity to generate tumour specific T cell responses *in-vitro*. Higher frequencies of tetramer positive T cells were expanded from patients who experienced at least a complete cytogenetic remission than patients who remained in active phase of the disease. This was observed both in patients receiving HSCT or Imatinib mesylate treatment. The reduction of the tumour burden may therefore promote the development of antigen specific T cell responses in these patients.

In conclusion from the data presented in this PhD, HSCT still appears to remain the treatment of preference for CML patients, favouring the development of donor-derived anti-leukaemic immune responses. In order to minimise any potential GvHD, more specific DLI could be developed by stimulating donor-derived T cells *ex-vivo* and adoptively transferring the successfully expanded tumour specific T cells. With respect to BCR/ABL antigen, such stimulation would require the use of sAPCs or a more developed artificial APC system. In addition BCR/ABL specific T cell responses were, in this study, only demonstrable in the context of HLA-A*0301. As an alternative strategy, donors could be vaccinated with BCR/ABL peptides (including both HLA class I and HLA class II epitopes) prior to the transplant procedure ensuring the infusion of antigen-specific T cells in the graft. Such donors may be more capable of generating BCR/ABL specific CD8 and CD4 T cell responses *in-vivo* than CML patients. This may also overcome the necessity for additional DLI post HSCT, thus reducing the risk of triggering GvHD in patients.

There is however still a number of patients who do not have an available HLA-matched donor and could therefore not benefit from such therapy. In this case combinatorial therapies, such as the one recently described by Bocchias' group and the one by Clark et.al in progress, could improve patient clinical remission. In their study, patients treated for at least 12 months with Imatinib mesylate or interferon alpha were given BCR/ABL peptide vaccinations and shown to improve cytogenetic responses after six vaccinations (Bocchia et al., 2005). BCR/ABL specific CD4 T cell proliferative responses, and to a lesser extent CD8 T cell INFγ production were demonstrated from the majority of patients assessed. Thus, the reduction of tumour burden prior to peptide vaccination resulted in better clinical responses compared to previous vaccination trials, which only included patients in late disease phase (Cathcart et al., 2004; Pinilla-Ibarz et al., 2000). In addition, the combination of the effective Imatinib mesylate debulking therapy with immunotherapy could overcome any Imatinib resistance and/or loss of clinical responses observed in treated patients (Branford et al., 2002; Gorre et al., 2001; Mahon et al., 2000).

In view of the poor capacity of BCR/ABL antigen to generate functional antigen-specific T cells and also to its limited HLA allele population coverage, the development of multi-peptides vaccination, including other tumour associated antigens, could be more applicable for a larger number of patients. Additionally such vaccination strategy may prove to be more efficacious in generating anti-tumour responses to at least one antigen.

In conclusion, although the generation of BCR/ABL specific T cell responses have proven to be difficult, the advances in understanding the requirement for the successful activation of tumour specific T cells and the promising success of recent clinical trials would indicate that it may still be worth the fight.....

APPENDIX 1: Ethical approval

1.1 Application form

ROYAL FREE HOSPITAL AND MEDICAL SCHOOL ETHICS COMMITTEE

APPLICATION FORM Word 95

EC REF.....(leave blank)

SHORT TITLE OF PROJECT

(or your reference number)
Monitoring of leukaemia antigen specific T cells in CML patients pre and post BMT: focusing on translocation (t9;22) resulting in peptide specific antigen in CML patients.

FULL TITLE

Detection of leukaemia antigen specific cytotoxic T lymphocytes (CTL) for bcr-abl fusion protein in CML patient. Assessment of CTL levels pre and post bone marrow transplantation or stem cell transplantation using HLA-class I tetramers for HLA-A*0301/HLA-B*0801

CONTACT NAME & ADDRESS
(to be used in all correspondence relating to this application)

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Anthony Nolan Research Institute
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Email : idod@rfhsm.ac.uk
Fax: 0207-284-8331

When completing this form on a word processor you may expand any section but please ensure that you retain the page breaks so that each section starts at the top of a new page. Handwritten applications will not be accepted.

APPLICATION FORM Checklist

Please indicate if the following have been enclosed by placing a circle around Yes/No/Not applicable options

Seventeen copies of each of the following:

Application form	(Yes)	No	Not applicable
Research subject consent form	(Yes)	No	Not applicable
Research subject information sheet	(Yes)	No	Not applicable

One copy of each of the following:

Protocol	Yes	No	(Not applicable)
Advertisement for research subjects	Yes	No	(Not applicable)
GP/Consultant information sheet or letter	(Yes)	No	Not applicable
Letter of invitation to research subjects	Yes	No	(Not applicable)
Questionnaire*	Yes	No	(Not applicable)
Copy of statement of indemnity	Yes	No	(Not applicable)
Copy of CTX/CTC/DDX	Yes	No	(Not applicable)
Annex A**	Yes	No	(Not applicable)
Joint Trust & Medical School form for research project registration	(Yes)	No	Not applicable

***For questionnaire based research only two copies of the application form together with two copies of the questionnaire need be submitted**

****Required if the study includes the use of ionising or radioactive substances or X-Rays.**

SECTION 1 Details of Applicants

1. Responsible Consultant(s)

Name Professor Alejandro Madrigal

Department Anthony Nolan Research Institute

Name Dr Mike Potter

Department Haematology, Royal Free Hospital

2. Name(s), qualification(s) and status of research worker(s) directly involved

Name Dr Anthony Dodi Status Senior Research Scientist

Qualifications MSc., PhD Signature.....

Name Sylvie Rusakiewicz Status PhD Student

Qualifications BSc (Hons) Signature.....

3. Are other departments involved? YES/NO

If yes, which: Haematology, RFHSM.

Signatures of Head(s) of collaborating department(s) denoting approval

.Professor G. Prentice Department Haematology

.....

4. Who is sponsoring the study:
Anthony Nolan Bone Marrow Trust

SECTION 2 Details of Project

5. Aims and objectives of project

We aim to monitor the frequency, and occurrence of BCR-ABL peptide specific cytotoxic T lymphocytes and investigate the subsequent expansion of these bcr-abl specific CD8+ T cells in CML patients pre and post bone marrow transplantation or stem cell transplantation. We will attempt to relate increases or decreases in bcr-abl positive T cells populations with the increase or clearance of philadelphia positive (Ph+) cells in these patients following transplant and during periods of relapse and subsequent remission following DLI. Patients status will be assessed by performing RT-PCR monitoring for Ph+ chromosome.
We will be able to follow the flux in the responding T cell population during episodes of CML disease and correlate the effects on disease progression with corresponding changes in the CD8 positive bcr-abl specific T cell population. This type of study has now become possible through the use of soluble HLA molecule constructs (tetramers).

6. Scientific background of study *(Approx 250 words)*

Chronic myelogenous leukemia (CML) is the result of monoclonal expansion of transformed pluripotent stem cells and is characterized in more than 95% of patient by the presence of philadelphia chromosome (Ph). Ph is the result of translocation between the chromosomes 9 and 22 (q 34; q11), which leads to the expression of a chimeric p210 bcr-abl fusion protein, abnormally present in leukemic cells.

HLA-matched sibling bone marrow transplantation is the only existing treatment leading to the elimination of Ph positive clone cells; however relapse of the disease occurs. This can however be reversed by the infusion of more donor lymphocytes (DLI) this however introduces the possibility of increased risk from GVHD, the mechanism operating in DLI is unclear. A clearer understanding as to which lymphoid populations are responsible for eliminating the relapsing leukaemia cells will lead to more efficient and improved strategies for patient treatment.
The junctional region of bcr-abl represent a potential leukemic specific antigen which can be recognized by cytotoxic T cells. Indeed, as it is abnormally present only in leukemic cells, the resulting peptides will be presented on cell surface by HLA molecule and therefore make the bcr-abl translocation an ideal target for immunotherapy.

Therefore the in vitro detection and subsequent amplification of specific bcr-abl T cell population in CML patients pre and post bone marrow transplantation will allow us to obtain a better understanding of what is happening to these specific T cells throughout the course of the disease and during treatment, when a specific immune response to the leukemic cells is clearly manifest.

Our group in collaboration with others has now obtained evidence for the presentation by leukaemic cells of BCR-ABL specific peptides. Tetramers have been constructed with these peptides and we would consider monitoring patients to define what is occurring in these cell populations during the course of CML and during treatment.

SECTION 2 (continued) Details of Project

7. Brief outline of project *(Approx 250 words)*

A 20ml blood sample will be taken from the patients prior to BMT conditioning chemotherapy and from the donor at the final medical before bone marrow harvest. PBMC's will be separated from the blood and stored for use in functional assays. Blood samples will be taken from the patients at a number of different time points during routine checks. Clinical information about the patient, including GvHD and CMV status, will be gathered post-transplant.

Donor samples will be analysed to determine the presence of, and increases or decreases in the level of bcr-abl specific CD8+ T cells by staining the PBMC with bcr-abl specific HLA class I tertramers. Where possible, patients will be followed up for 2 year post transplant and samples for screening will be collected when patients return for outpatient appointments. PBMC will be isolated from whole blood and then stained with HLA class I tetramers specific for the bcr-abl antigen, stained cells will then analysed by the use of FACS.

Total RNA will be extracted from the blood and used for screening for Ph chromosome by performing a RT-PCR . The data obtained will be correlated with episodes of GVHD/ GVL following DLI.

8. Study design *(e.g. cohort, case control, epidemiological analysis)*

This is a study to monitor the frequency and occurrence of bcr-abl antigen specific CD8+ T cells in bone marrow transplant patients pre and post BMT.

SECTION 2 (continued) Details of Project

9. Size of the study. **Will the study involve:**

(a) Human Subjects? (Yes) *No*

i) How many patients will be recruited?
40

ii) How many controls will be needed?
20

iii) What is the primary end point?
12 to 24 months post-transplant.

iv) How was the size of the study determined?
Empirically

v) What is the statistical power of the study?
Undefined

(b) Patient Records? (Yes) *No*

i) How many records will be examined?
40

ii) How many control records will be examined?
20

iii) What is the primary end point?
12 to 24 months post-transplant.

iv) How was the size of the study determined?
Empirically

v) What is the statistical power of the study?
Undefined

10. Independent Review

Has the study been externally independently reviewed (Yes) *No*

If *yes* state which body/individual has undertaken the review
If *no* you must obtain the signature of a person who has reviewed the study and who can guarantee its potential usefulness, either on increasing scientific knowledge or enhancing therapeutic power.
NB The person must be wholly independent of the study (i.e. not the head of department responsible) and must have the requisite experience and status to perform the task

Name: Date.....
Signature

Status.: Head of Department of Immunology

SECTION 3
Recruitment of Subjects

11. How will the subjects in the study be:

i) selected?

i) We will preferentially select patients resident in the UK to allow for ease of follow up. We will also select patients in whom HLA tissue typing has been carried out at a molecular level through the Anthony Nolan Bone Marrow Trust. This is to ensure that we can match patients with available tetramers.
ii) Donors will be obtained through the Anthony Nolan Bone Marrow Trust where tissue typing will be carried out at a molecular level.

ii) recruited?

Full informed consent will have been obtained from both donor and recipient before entering into this study. Both the donor and recipient will be seen separately.

iii) what inclusion criteria will be used?

i) Prospective unrelated donor for HLA-matched patient
ii)Sibling donors
iii) Written informed consent from both patient and donor.

iv) what exclusion criteria will be used?

None

How will the control subjects be:
(Type N/A if no controls)

i) selected?

i) age matched
ii) healthy

ii) recruited?
Healthy age matched donors on the Anthony Nolan Bone Marrow Trust Register will be used.

iii) what inclusion criteria will be used?

Healthy individuals who have given their written informed consent.

iv) what exclusion criteria will be used?
i) No previous autoimmune disease
ii) No HIV infection
iii) No previous transplant

12. Will there be payment to research subjects of any sort? *Yes* (*No*)

If yes, how much per subject and for what?

SECTION 4 Consent

13. Is *written* consent to be obtained? *Yes* (*No*)

If yes, please attach a copy of the consent form to be used
If no written consent is to be obtained, please justify

14. How long will the subject have to decide whether to take part in the study?

Donors will be asked at the pre-harvest medical and patients will be asked prior to transplant. They will be given as long as they need to decide.
If fewer than 24 hours please justify

15. Will the subject be give a written information sheet or letter? (*Yes*) *No*

If yes please attach a copy to this application form
If no, please justify:

16. Have any special arrangements been made for subjects for whom English is not a first language? (*Yes*) *No*
N/A

If yes, give details:
An interpreter is present for the pre-transplant consultation as part of standard clinical practice. This service will be available to donors if required.

If no, please justify:

17. Will any of the subjects be from one of the following vulnerable groups?

Children under 18
People with learning difficulties
Unconscious or seriously ill
Psychiatric patients
Elderly patients
Other vulnerable groups *Yes* (*No*)
If yes, please justify

It will be important for the study to assess the level of bcr-abl specific cells found in CML patients of all ages post bone marrow transplantation.
What special arrangements have been made to deal with the issue of consent for the subjects above?
Written consent will be obtained from the parent or guardian of the subject.

SECTION 5 Details of intervention

18. Substances to be given to the subjects (Special diets, drugs, isotopic tracers etc. State dose, and mode and frequency of administration)

None

19. Does the study involve the use of a new medicinal product or medical device or an existing product outside the terms of its product licence? *Yes* (*No*)

If yes has a pharmaceutical or other commercial company arranged this trial and obtained a Clinical Trial Certificate or Clinical Trial Exemption? *Yes* *No*

Or have you obtained approval of the licensing authority by means of a DDX? *Yes* *No*

DDX or CTX Number.....(Please attach a copy)

20. Will any ionising or radioactive substances or X-Rays be administered? *Yes* (*No*)

If yes please fill in Annex A

21. Please list those procedures in the study to which subjects will be exposed indicating those which will be part of normal care and those that will be additional (e.g. taking more samples than would otherwise be necessary). Please also indicate where treatment is withheld as a result of taking part in the project.

No tests are required that are not routinely performed. No treatment will be withheld at any point in this study. Additional blood samples required for this study will be taken during normal checking procedures at various time points post-transplant, up to 24 months. Samples required from donors will be taken at the pre-transplant medical. No additional venepunctures will be required.

SECTION 6 Risks and ethical problems

22. Are there any potential hazards? *Yes* (*No*)

If yes, please give details, and give the likelihood of occurrence. Also give details of precautions taken to meet them, and arrangements to deal with adverse events.

23. Is this study likely to cause discomfort or distress? *Yes* (*No*)

If yes, please justify and give details

24. Are there any particular ethical problems or considerations that you consider to be important or difficult with the proposed study? *Yes* (*No*)

If yes, please give details

25. Will information be given to the patient's General Practitioner? *Yes* (*No*)

Please note: permission should always be sought from research subjects before doing this

If yes, please enclose a copy of the information sheet for the GP

If no, please justify

Information will be given to the consultant.

26. If the study is on hospital patients, will consent of all Consultants whose patients are involved in this research be sought? (*Yes*) *No*

If no, please justify

SECTION 7 Indemnity and Confidentiality

27. **Is this NHS sponsored research to which HSG (96) 48 applies?** *Yes* (*No*)
or
Is this research sponsored by the medical school for which indemnity is provided by an insurance policy taken out by the medical school? (*Yes*) *No*
or
Is this pharmaceutical company sponsored research, the company providing indemnity to the most recent ABPI guidelines? *Yes* (*No*)
If yes please enclose a copy of the indemnity form

or
Is indemnity being provided in some other way *Yes* (*No*)

If yes please specify

---

28. **In the case of equipment or medical devices will indemnity be provided by the manufacturer to the level specified in ABHI (Association of British Health-Care Industries) guidelines.** *Yes* *No*

If no please justify ***N/A***

---

29. **Will the study data be retrieved from computer?** *Yes* (*No*)

Will the study date be held on computer? (*Yes*) *No*

If, yes, will the Data Protection Act (1984) be followed? (*Yes*) *No*

---

30. **Will the study include Audio/video recording?** *Yes* (*No*)

If yes (1) has specific consent been obtained? *Yes* *No*

(2) what will happen to the recordings at the end of the study?

---

31. **Will medical records be examined by research workers outside the employment of the NHS?** (*Yes*) *No*

If yes, please ensure that this is made clear to the patient on the information sheet.
This is included in the participant information sheet.

PLEASE ENSURE THAT YOU COMPLETE THE CHECKLIST AND ENCLOSE ALL RELEVANT DOCUMENTS

DECLARATION

The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

I agree to supply interim reports on an annual basis and a final report, and to advise the ethics committee of any adverse or unexpected events that may occur during this project.

The research for which I am seeking ethical approval will be undertaken in no more than four LREC's geographical boundaries (applications involving five or more centres should be sent to a multi-centre research ethics committee - see health service guidelines HAG(97)23).

Signature ofResponsible Consultant.....

Date.....

FOR ETHICS COMMITTEE USE

First Meeting.....

Applicant interviewed

Approved without changes

Approved after revision

Rejected

Signature of Chairman..... **Date of Approval**.....

ANNEX A Radiation

This section to be completed if the study involved the use of ionising or radioactive substances

1) Radioactive substances

a) Details of substances to be administered:

b) Estimated total effective dose (mSv)

c) ARSAC Licence:

1) Name of holder

2) Reference number:

3) Date issued:

d) Signature of Radiation Protection Advisor (RPA)

.....

Date.....

2) X-Rays, CT etc.

a) Details of all radiographic procedures carried out in this study:

Investigation	Organ	Frequency

2) Total Estimated Effective Dose (mSv):

3) Signature of Radiation Protection Advisor

.....

Date.....

1.2 GP/(Consultant) Information sheet

Title of Project

Monitoring of BCR-ABL antigen specific T cells in Bone marrow transplant patients before and after transplant.

We at the Anthony Nolan Research Institute and Haematology Dept of the RFH are currently embarking on a project to investigate the reconstitution of the immune system after bone marrow transplantation. As you will be aware, the immune system is severely depleted after pre-transplant conditioning. Research has shown that specific cells in the normal immune system are responsible for combating many virus infections but also possibly in combating emergent leukaemia cells. These cells are of specific importance to transplant patients post grafting. The precise details of how fast these cells regenerate and how effective they are in fighting residual leukaemic cells in Bone marrow transplant patients still remains unclear. This project uses newly developed techniques which will provide valuable information about the levels and activity of these cells in patients before and after bone-marrow transplantation.
To help us carry out this work we require 20mls of blood. These samples would be taken at the same time as the regular checks occurring at the hospital and will not require additional vene-puncture.

TRANSPLANT RECIPIENTS- blood is required prior to transplant and then subsequently at the same times when blood is being taken for routine monitoring of CMV status, this is usually once weekly during the early phase post transplant. Monitoring will continue at outpatient appointments for up to 9 months following transplant.

DONORS- blood is required once at the pre-donation health check.

The cells extracted from these samples will only be used for the research project that the patient has agreed to take part in.

The proposed study will use class I soluble HLA molecules in the form of tetramers. These will be used to specifically detect the presence of circulating BCR-ABL antigen specific T cells in the peripheral blood of patient before and after BMT and during episodes of GVH.
Relapse can be a significant problem in immunosuppressed patients following BMT, and before their new haemopoetic system has been able to fully develop to provide the appropriate level of immune response to counteract the occurrence of residual leukaemic cells through the regrowth of pre exisiting leukaemic progenitors that have escaped conditioning. Monitoring the immune potential of a patient to mount a specific immune response to residual leukaemic cells and during relapse, will allow us to obtain a better understanding of what is happening to the levels of anti leukaemia specific T cells in patients who relapse, during DLI, and during episodes of GVHD post BMT. This will be compared with levels of these cells in patients pre transplant. The acquisition of such data will allow us to correlate the ability of patients to resolve relapse as a result of DLI with the concurrent expansion or development of leukaemia specific CD8+ T cells in their peripheral circulation. The analysis of such information has implications for improved therapy or clinical intervention at specific time points resulting in improved treatment of patients relapsing post BMT.

The role of anti leukaemia specific T cells in the resolution of relapse following DLI is well documented. However specific details about the dynamics of expanded leukaemia specific populations the relative relevance of CD4+ T cells to the action of CD8+ antigen specific cells and whether CD8 population expansions are productive or unable to act specifically to remove leukaemia positive cells in BMT patients are all questions which need answering. In addition the contribution of chimerism to the cellular dynamics of antigen specific T cells in BMT patients is also an important question that needs to be investigated.

1.3 PARTICIPANT INFORMATION SHEET

Title of Project
Monitoring of BCR-ABL antigen specific T cells in CML patients before and after Bone marrow transplant.

We at the Anthony Nolan Research Institute are currently embarking on a project to investigate the presence of naturally occurring anti leukaemic white cells present in the immune system before and after bone marrow transplantation. As you will be aware, the immune system is severely depleted after pre-transplant conditioning. Research has shown that specific cells in the normal immune system are responsible for combating many virus infections and also possibly malignant cells such as leukaemic cells. These cells are of specific importance to transplant patients with leukaemia. The precise details of how fast these cells regenerate and how effective they are in fighting residual leukaemic cells in Bone marrow transplant patients still remains unclear. Also unclear is why these cells fail to function properly before transplant thus allowing leukaemia to develop. This project uses newly developed techniques which will provide valuable information about the levels and activity of these cells in patients before and after bone-marrow transplantation.

To help us carry out this work we require 20mls of blood (which is approximately an egg cup full). These samples would be taken at the same time as the regular checks you will have at the hospital and will not require you to have any extra venepunctures.

TRANSPLANT RECIPIENTS- blood is required prior to transplant and at the same times when blood is being taken for routine monitoring of CMV status, this is usually once weekly during the early phase post transplant, you will then be monitored at your outpatient appointments for up to 1 year following transplant.

DONORS- blood is required once at your pre-donation health check.

The cells extracted from these samples will only be used for the research project you have agreed to take part in.

If you are willing to take part in this study and agree to provide the samples requested, your medical records will also be examined by researchers from The Anthony Nolan Bone Marrow Trust's Research Institute, who are outside the employment of the NHS. All information will remain confidential.

1.4 PARTICIPANT CONSENT FORM

TITLE OF PROJECT

Monitoring of BCR-ABL antigen specific T cells in CML patients pre and post Bone marrow transplant and during DLI therapy

The patient should complete the whole of this sheet themselves.

Have you read the Patient Information Sheets? **YES / NO**

Have you had an opportunity to ask questions and discuss this study? **YES / NO**

Have you received satisfactory answers to all your questions? **YES / NO**

Have you received enough information about the study? **YES / NO**

Who have you spoken to? Dr / Mr / Mrs_____

Do you understand that you are free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting your future medical care? **YES / NO**

Do you agree to enter your this study and in doing so give permission for the researchers to have access to your medical notes? **YES / NO**

Do you give permission for us to notify your GP that you have entered this study? **YES / NO**

Do you agree to enter in this study? **YES / NO**

Patient's name _____
(in block letters)

Signature _____ Date |__|__|__|__|

Dr's signature _____ Date |__|__|__|__|

Dr's name _____
(in block letters)

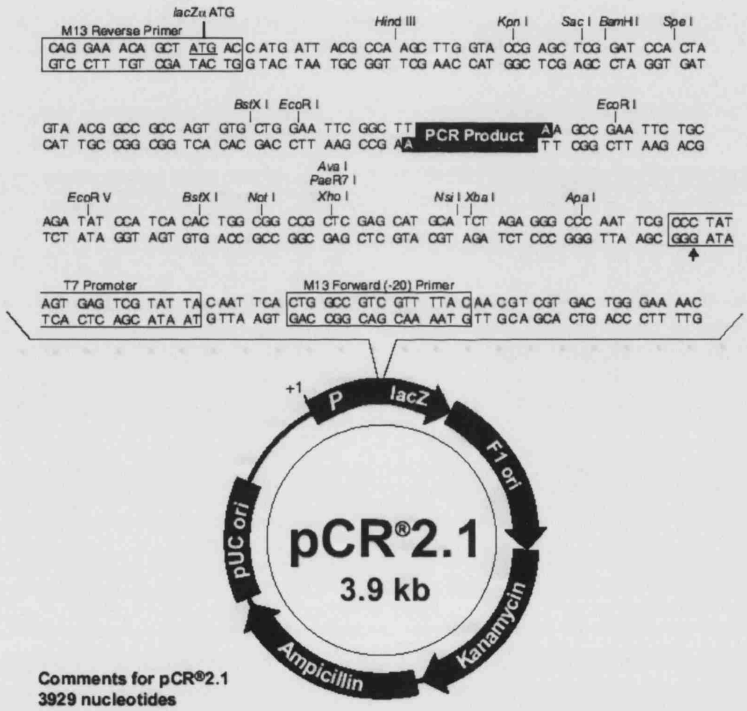
File one copy in patients notes, one copy in CRF and one copy to patient.

APPENDIX 2: Cloning Vector maps

2.1 pCR 2.1 Vector

Map and Features of pCR®2.1

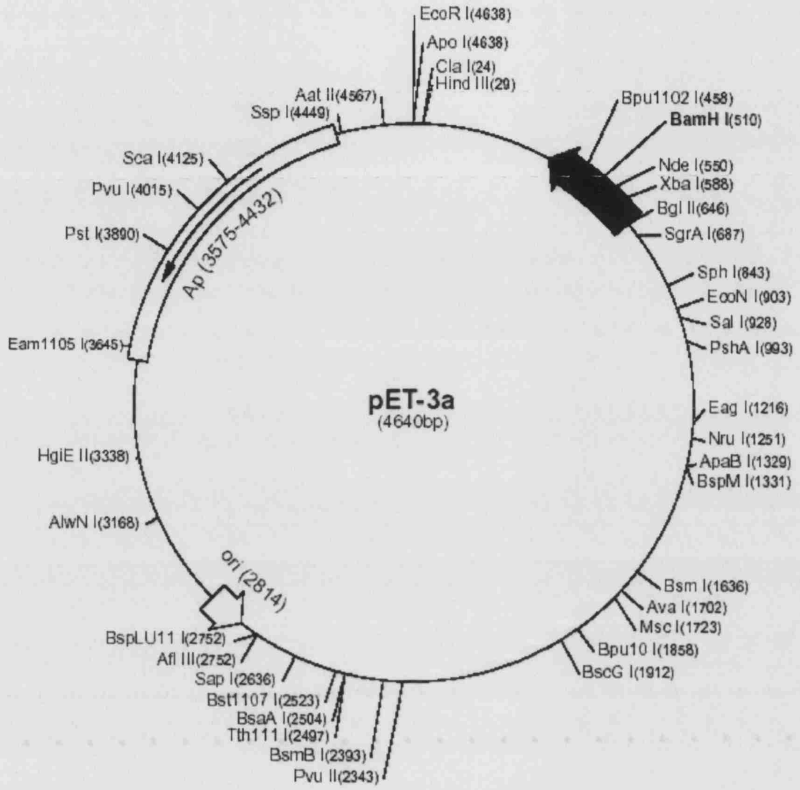
Map of pCR®2.1 The map of the linearized vector, pCR®2.1, is shown below. The arrow indicates the start of transcription for the T7 RNA polymerase. **The complete sequence of pCR®2.1 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (page 18).**

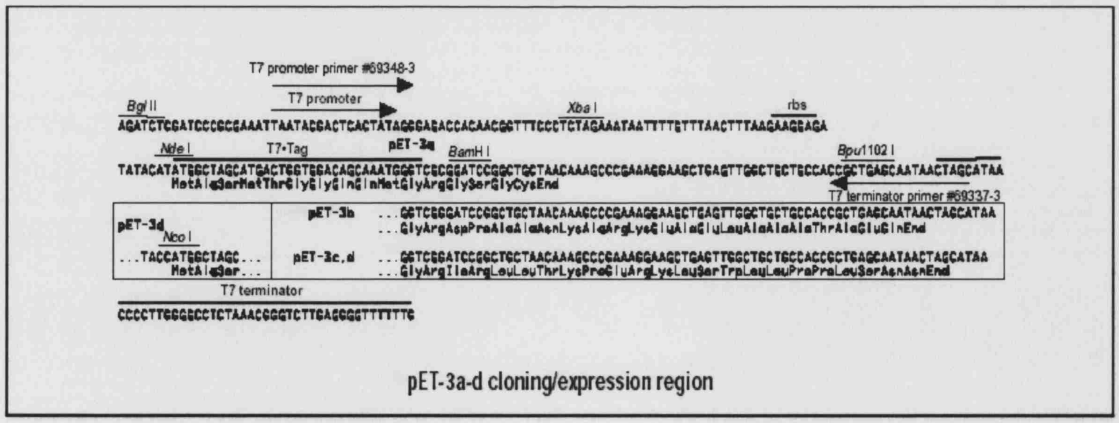


Comments for pCR®2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

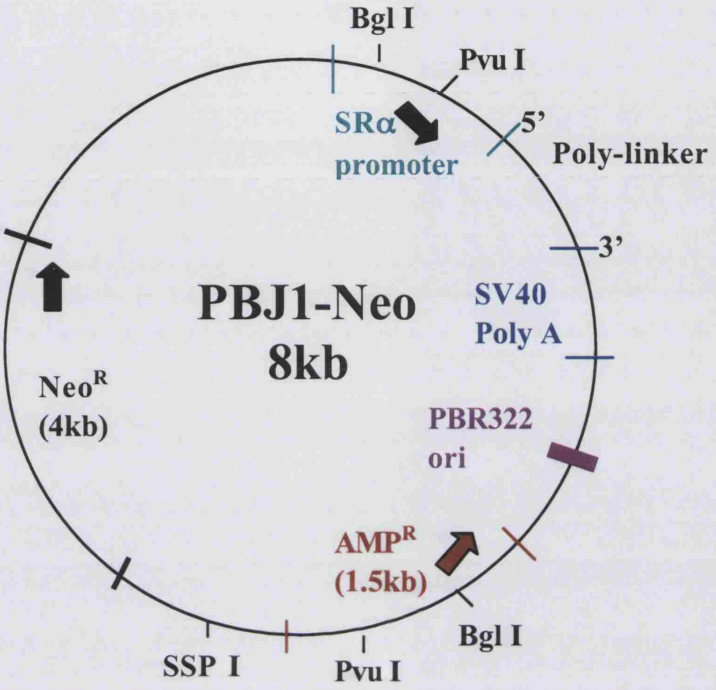
2.2 pET-3d vector



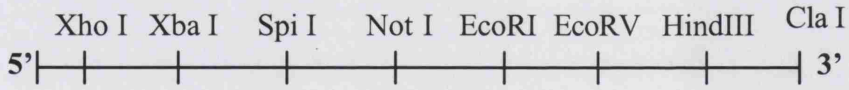


pET-3a-d cloning/expression region

2.3 pBJ1Neo vector



Poly-linker



APPENDIX 3: Publications and presentations

3.1 Publications

Madrigal, J.A, **Rusakiewicz, S.**, Dodi, I.A., Rees, R. (2004)
Immunotherapy with alloreactive T-cells? The Hematology Journal **5**:S91-S95.

I.A. Dodi[1], **S. Rusakiewicz**[1], G. Aubert[1], R.E. Clark[2], C. Creaser[3], P. Bonner[3], R. Rees[3], P.J. Travers[1], J.A. Madrigal[1]. Definition of the Breakpoint fusion peptides for HLA-B8 as well as HLA-A3 in CML cells. HLA 2002 Immunobiology of the Human MHC (In Press)

Loconto, J; **Rusakiewicz, S**; Madrigal, JA; Dodi, IA. (2003), Peptide vaccination for cancer: Chronic Myeloid Leukaemia as a model. BSHI newsletter **53**: p1-6

Rusakiewicz, S., Aubert, G., Clarke, R., Rees, R., Travers, P.J., Madrigal, J.A. and Dodi, I.A.: Definition of the presence in HLA-A3 b3a2+ CML cells of breakpoint fusion peptides. Their use to generate tetramers and screen for circulating peptide specific T cells in patients in vivo. Eur.J. Immunogen. 28, 475, BSHI Abstr.4.1, 2001.

Symons, MC; **Rusakiewicz S** ; Rees RC ; Ahmed SI (2001) , Hydrogen peroxide: a potent cytotoxic agent effective in causing cellular damage and used in the possible treatment for certain tumours. Medical Hypotheses **57** (1): p56-58.

3.2 Oral presentations

Rusakiewicz, S., Aubert, G., Clark, R. E., Christmas, S. E., Creaser, C. S., Bonner, P. L., Rees, R. C., Travers, P., Madrigal, A. J. & Dodi, I. A. Definition of the breakpoint fusion peptides for HLA-A3 and HLA-B8 in CML cells. Tetramer generation and detection of peptide specific T cells *in vivo*. British Society of Immunology; Immunity 104, suppl. 1 (2001).

Rusakiewicz, S., Aubert, G., Clark, R. E., Rees, R. C., Travers, P., Madrigal, A. & Dodi, I. A. Definition of the presence in HLA-A3 b3a2+ CML cells of breakpoint fusion peptides. Their use to generate tetramers and screen for circulating peptide specific T cells in patients in vivo. British society of Histocompatibility and Immunogenetics. (BSHI); European Journal of Immunogenetics, 28:4 (2001).

Rusakiewicz, S., Aubert, G., Clark, R. E., Creaser, C., Bonner, P., Rees, R. C., Travers, P.J., Dodi, I.A., Madrigal, J.A. HLA-associated tumour specific antigen for chronic myelogenous leukaemia: their antigenicity and immunogenicity in vitro. Royal society of Medicine, President's prize evening:Clinical Immunology and Allergy (2002).

Rusakiewicz, S., Aubert, G., Clark, R. E., Creaser, C., Bonner, P., Rees, R. C., Travers, P.J., Dodi, I.A., Madrigal, J.A. Definition of the breakpoint fusion peptides for HLA-A3 and HLA-B8 in CML cells. Their use to generate tetramers and to expand and detect peptide specific T cells in vivo. European Journal of Immunogenetics, 29:2 (2002).

Rusakiewicz, S., Dodi, I.A., Aubert, G., Macintyre A.R., Clark, R.E., Rees, R. C., Travers, P.J., Christmas S.E., Madrigal, J.A. CD8+ T cells reactive to bcr/abl junctional peptides circulate in CML patients and may be expanded in vitro. Progress in Vaccine Against Cancer (PIVAC). Cambridge (2001)

Dodi, I.A., **Rusakiewicz, S.**, Aubert, G., Clark, R.E., Creaser C., Bonner P., Rees, R. C., Travers, P.J., Madrigal, J.A. CD8+ T cells reactive to BCR/ABL junctional peptides circulate in CML patients and may be expanded in vitro. GVL/GvHD meeting, Munich Germany. (2001)

Rusakiewicz, S. Can the Bcr/Abl translocation be exploited for immunotherapy of CML? Teaching session. 4th UK. Cord Blood Immunology Group Meeting 2003 (Teaching Session 1). Newcastle University UK.

3.2 Posters

Institut Pasteur Euroconferences : Advances in Immuno-Intervention. (Paris, France, June 2002)
Keystone symposia: Recent Advances in Tumor Immunology. (Keystone, USA, February 2003)
Progress in Vaccination against Cancer (Oxford, UK, August 2003)
ASH 43rd Annual Meeting: abstract # 1768, session 873-II

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Ackerman, A. L. and Cresswell, P.: Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat Immunol 5:* 678-84, 2004

Aderem, A. and Ulevitch, R. J.: Toll-like receptors in the induction of the innate immune response. *Nature 406:* 782-7, 2000

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Albert, M. L., Sauter, B., and Bhardwaj, N.: Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature 392:* 86-9, 1998

Alfonso, C. and Karlsson, L.: Nonclassical MHC class II molecules. *Annu Rev Immunol 18:* 113-42, 2000

Altman, J. D., Moss, P. A., Goulder, P. J., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J., and Davis, M. M.: Phenotypic analysis of antigen-specific T lymphocytes. *Science 274:* 94-6, 1996

Amrolia, P. J., Muccioli-Casadei, G., Yvon, E., Huls, H., Sili, U., Wieder, E. D., Bollard, C., Michalek, J., Ghetie, V., Heslop, H. E., Molldrem, J. J., Rooney, C. M., Schlinder, J., Vitetta, E., and Brenner, M. K.: Selective depletion of donor alloreactive T cells without loss of antiviral or antileukemic responses. *Blood 102:* 2292-9, 2003

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