Studies on the Immunogenicity of Tumour Associated Antigens in Leukaemia

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Abstract

There is an urgent need for the development of leukaemia-targeted immunotherapeutic approaches. Wilms' tumour antigen (WT1), M-phase phosphoprotein 11 (MPP11) and proteinase-3 (PR-3) proteins are overexpressed in leukemic cells and represent attractive immunotherapeutic candidates. The first part of this study explored the feasibility of using an approach to develop a novel leukaemia vaccine by modifying the sequence of low avidity HLA-A*0201-restricted peptide epitopes derived from WT1 protein (WT1-$126_{126\text{-}134}$ and WT1-$187_{187\text{-}195}$). The modified WT1-Db126 showed enhanced binding ability to the HLA-A*0201 molecule, increased the frequency of IFN-γ producing cytotoxic T lymphocyte (CTL), and boosted the lytic activity of the generated CTL against HLA-matched leukaemia cells. Interestingly, the CTL line generated with the modified epitope was able to recognize the wild-type peptide presented by target cells. The second part of this study identified a novel epitope derived from WT1 antigen (WT1-60237-251). This epitope was recognized by a $CD4^+$ T cells in an HLA-DRB1*04-restricted manner and secreted Th2 cytokines (IL-5 and IL-4). The third part of the study aimed to identify potential $CD8^+$ CTL epitopes in the sequence of MPP11 and PR-3 that may bind to HLA-A*0201 molecule and provoke specific CTL responses. A potential HLA-A*0201 binding epitope named MPP-$4_{37\text{-}45}$ derived from the MPP11 protein was identified which was used to generate a CTL line. This CTL line specifically recognized peptide-loaded target cells in both ELISPOT and cytotoxic assays. Importantly, this CTL line exerted a cytotoxic effect towards the CML leukemic cell line K562-A2.1. The study also demonstrated that PR-3-derived peptides PR-$129_{129\text{-}137}$ and PR-$9_{9\text{-}17}$ were not immunogenic since they were incapable of inducing specific $CD8^+$ T cell responses.

In conclusion, modification of the WT1-Db126 epitope resulted in enhancement of its immunogenicity without altering its antigenic specificity. The WT1-60 and MPP-4 peptides have been identified as novel CD4 and CD8 T-cell epitopes, respectively.

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List of Abbreviations

ABL	Abelson
ALL	acute lymphocytic leukaemia
ALP	alkaline phosphatase
AML	acute myelogenous leukaemia
APC	antigen presenting cell
APL	acute promyelocytic leukaemia
ATCC	American Type Culture Collection
ATRA	all-trans retinoic acid
BCG	bacillus Calmette-Guérin
BCL2	B-cell CLL/lymphoma 2
BCR	breakpoint cluster region
BIMAS	bioinformatics and molecular analysis section
BSA	bovine serum albumin
CD	cluster of differentiation
CLL	chronic lymphocytic leukaemia
CM	complete medium
CML	chronic myelogenous leukaemia
CR	complete remission
CTLA-4	cytotoxic T-lymphocyte antigen 4
CTLs	cytotoxic T Lymphocytes
DC	dendritic cells
DLI	donor lymphocyte infusions
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E.coli	Escherichia coli
E:T ratio	effector:target ratio
EBV	Epstine barr virus
EDTA	ethylenediaminetetra-acetic acid
ELISPOT	enzyme-linked immunospot assay
FAB	French-American-British
FACS	fluorescence- activated cell sorter
FCS	fetal calf serum
FI	fluorescence index
FITC	fluorescein isothiocyanate
FLT3	fms-like tyrosine kinase 3
FOXP3	forkhead box protein P3
GM-CSF	granulocyte-macrophage colony stimulating factor
GvHD	graft-versus-host disease
GvL	graft-versus-leukaemia
HLA	human leukocyte antigen

HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
IFN-γ	interferon-gamma
IFN-α	interferon-alpha
IgG	immunoglobulin G
IgVH	immunoglobulin heavy chain
IL-10	interleukein-10
IL-12	interleukein-12
IL-2	interleukein-2
IL-4	interleukein-4
IL-6	interleukein-6
Il-7	interleukein-7
KIR	killer cell immunoglobulin-like receptors
LAAs	leukaemia-associated antigens
LCL	lymphoblastoid cell lines
LPS	lipopolysaccaride
mAbs	monoclonal antibodies
MCA	methylchlanthrene
MDS	myelodysplastic syndrome
mHAs	minor histocompatibilty antigens
MHC	major histocompatibility complex
MICA	MHC-class I-related chain A
MICB	MHC-class I-related chain B
MPN	myeloproliferative neoplasm
MPP11	M-Phase Phosphoprotein 11
mRNA	messenger RNA
NE	neutrophil elastase
NK	natural killer
NKT	natural killer T
NPM1	nucleophosmin
N-terminus	amino-terminal
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pfp	pore-forming protein
Ph	Philadelphia
PHA	phytohaemagglutinin
PML-RARα	promyelocytic leukemia-retinoic acid receptor α
PR-3	Proteinase-3
PRAME	preferentially expressed antigen of melanoma
RAG	recombination-activating gene
RHAMM	receptor for hyaluronic-acid-mediated motility
RT-PCR	reverse transcriptase-polymerase chain reaction

SCID	severe-combined immunodeficiency
SEREX	serological analysis of recombinant cDNA expression libraries
STAT1	signal transducer and activator of transcription 1
TAP	transporter associated with associated with antigen processing
TCL	T cell line
TCR	T-cell receptor
TGF-β1	transforming growth factor-beta 1
Th	T helper
TILs	tumour-infiltrating lymphocytes
TMB	tetramethylbenzidine
TNF-α	tumour necrosis factor-alpha
Tregs	regulatory T cells
ULBPs	unique long16-binding proteins
VEGF	vascular endothelial growth factor
WBC	white blood cells
WHO	World Health Organization
WT1	Wilms' tumour 1
Y	tyrosine
ZAP-70	zeta-associated protein of 70 kD
γδ T cells	gamma delta T cells

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1. INTRODUCTION

1.1. Leukaemia

1.1.1. General characteristics and classification

Leukaemia is a term which refers to several types of cancers that affect the blood-forming cells in the bone marrow. Formation of blood cells takes place within the bone marrow in a process called hematopoiesis where hematopoietic stem cells (HSCs) give rise to all mature blood cell types (Figure 1.1.) In leukaemia, abnormal cells grow and multiply without control and hence accumulate in the bone marrow and peripheral blood leading to a decrease in normal blood cell production. These leukaemia cells are generally white blood cells (WBC) that are unable to carry out the functions of the normal cells. Current evidence indicates that HSCs are the target for the genetic and epigenetic events that lead to the emergence of leukemic clone (Figure 1.2; reviewed in Passegue et al., 2003). On the other hand, studies performed on mouse models of certain types of human leukemias showed that restricted progenitors or even differentiated cells may also become malignantly transformed (reviewed in Passegue et al., 2003) .

Patients with leukaemia share similar symptoms such as fatigue, shortness of breath, headaches, swollen spleen, ecchymoses, epistaxis and increased risk of infections (Resto, Caballero et al. 2000; Bozzone 2009). These symptoms are due to anaemia, neutropenia and thrombocytopenia or from infiltration of malignant cells into tissues (Devine and Larson, 1994). The exact cause of leukaemia is not known but it commonly arises as result of chromosomal rearrangements. These rearrangements consist of translocations, inversions, or deletions in genes that regulate blood cell development or homeostasis (Mitelman, 2000). However, several risk factors have been identified. These include smoking (Kane et al., 1999), exposure to ionizing radiation or to certain chemicals such as benzene (Travis et al., 1994), radiation therapy and some types of chemotherapy used to treat other cancers (Smith et al., 2003). The blood disorder myelodysplastic syndrome and myeloproliferative disorder as well as some genetic diseases such as Down's syndrome are also linked to an increased risk of leukaemia. Additionally, infection with some viruses such as human thymus derived T-cell leukaemia virus (HTLV-1) and Epstein-Barr virus (EBV) causes some forms of leukaemia (Mahieux and Gessain, 2003, Lombardi et al., 1987).

Leukaemia is classified into four major categories depending on the particular cell type affected (myeloid or lymphoid) as well as the rate of cell growth (acute or chronic). The four major types are: acute lymphoblastic leukaemia (ALL), chronic lymphoblastic leukaemia (CLL), acute myelogenous leukaemia (AML) and chronic myelogenous leukaemia (CML). Acute leukaemias are characterized by abnormal proliferation and expansion of malignantly transformed hematopoietic stem cells that accumulate in the marrow and blood and may spread to other organs of the body such as lymph nodes, spleen, liver and central nervous system (Devine and Larson, 1994). Chronic leukaemias, on the other hand, develop more slowly and involve more mature white blood cells.

Acute leukaemias are further classified into distinct subtypes using different classification schemes. The most frequently used are the French-American-British (FAB) classification and the newer World Health Organization (WHO) classification. The FAB system classified acute leukaemias according to the morphology of the predominant leukemic cell type along with some cytochemical findings (Bennett et al., 1985, Bennett et al., 1976). This system has categorized ALL into three subtypes (L1-L3) and AML into eight subtypes (M0-M7; Table 1.1). The WHO classification scheme contains more prognostic information as it integrates not only morphological but also immunological, cytogenetic, molecular genetic and clinical features in constructing its classification system (Vardiman et al., 2009). Entities of acute leukaemias recognized by WHO classification are shown in Table 1.2.

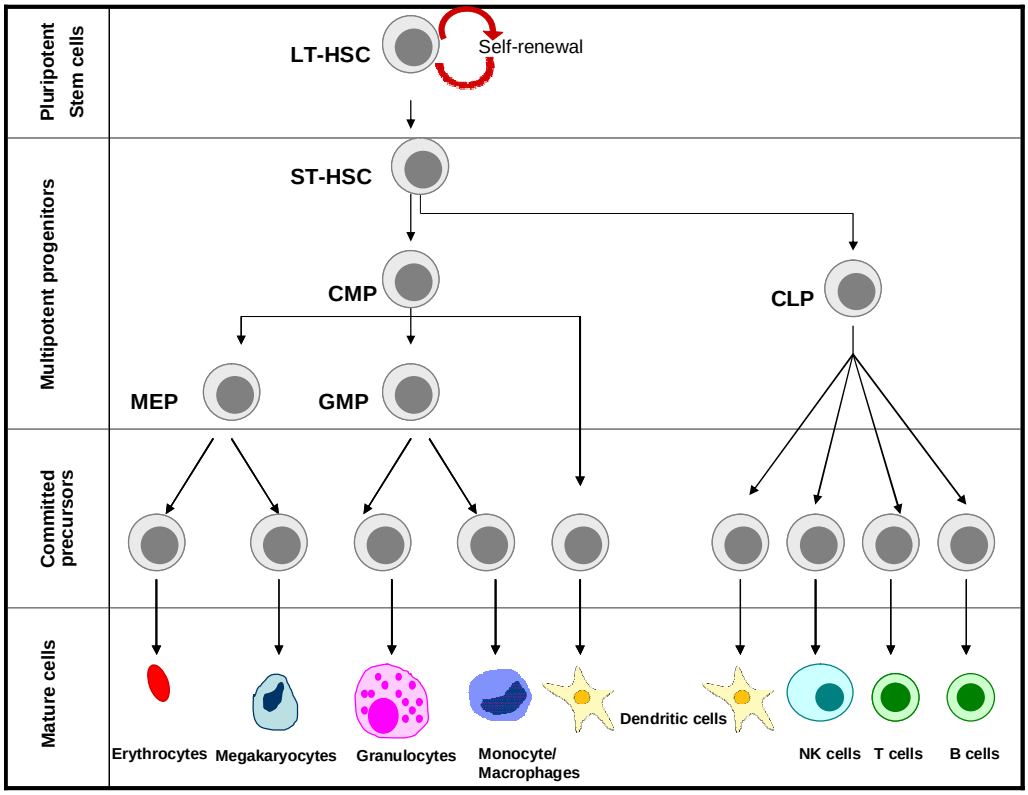

Figure 1.1. The process of hematopoiesis.

Hematopoietic stem cells (HSCs) can be divided into long-term hematopoietic stem cells (LT-HSCs) and short-term hematopoietic stem cells (ST-HSCs). The LT-HSCs are highly self-renewing cells that reconstitute the host for its entire life span whereas the ST-HSCs reconstitute the host for a limited period. The ST-HSCs give rise to two multipotent progenitors: common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). The CMPs give rise to granulocyte/macrophage progenitors (GMP), which then differentiate into monocytes/macrophages and granulocytes, and to megakaryocyte/erythroid progenitors (MEP), which produce megakaryocytes/platelets and erythrocytes. The CLPs give rise to T lymphocytes, B lymphocytes and natural killer cells. Both CMPs and CLPs give rise to dendritic cells. Figure adapted with modification from (Passegue et al., 2003).

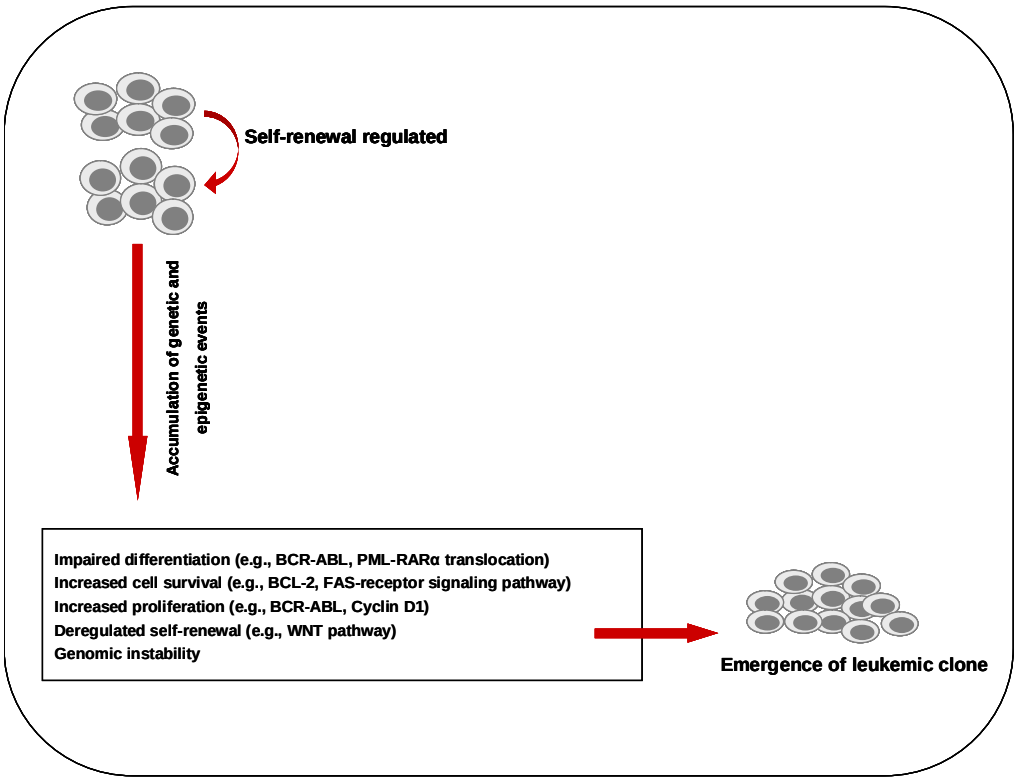

Figure 1.2. Development of leukaemia from HSCs.

Accumulation of the genetic and epigenetic events in HSC compartment eventually lead to the emergence of leukemic clone due to impaired differentiation, increased cell survival, increased proliferation capacity, increased self renewal and genomic instability. Figure adapted with modifications from (Rossi et al., 2008, Passegue et al., 2003).

Table 1.1. The French-American-British (FAB) classification of acute leukaemia, adapted with modification from (Devine and Larson, 1994).

	Morphologic Features	***Frequency***
ALL Subtypes		
L1	small homogenous cells	70% in children 30% in adults
L2	large heterogeneous cells	27% in children 65% in adults
L3	large homogenous cells	3-5% in both children and adults
AML Subtypes		
M0	large granular myeloblasts	2-3%
M1	large poorly differentiated myeloblasts	20%
M2	myeloblasts with maturation	25-30%
M3	hypergranular promyelocytes	8-15%
M4	myelomonoblasts	20-25%
M4Eo	myelomonoblasts with abnormal eosinophils	5%
M5	monoblasts, promonocytes or moncytes	10%
M6	erythroblasts	5%
M7	large and small megakaryoblasts	1-2%

Table 1.2. World health organization (WHO) classification of acute leukaemia, adapted with modification from (Vardiman et al., 2009).

Acute Myeloid Leukaemia
acute myeloid leukaemia with recurrent genetic abnormalities such as AML with t(8;21), AML with inv(16) or t(16;16), AML with t(9;11), AML with t(6;9), and acute promyelocytic leukaemia (APL) with t(15;17).
acute myeloid leukaemia with multilineage dysplasia: AML developed following myelodysplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasm (MDS/MPN).
therapy-related AML: AML developed as a complication after cytotoxic and/or radiation therapy.
acute myeloid leukaemia not otherwise specified: AML subtypes that do not fall into one of the above groups.
myeloid leukaemia associated with Down's syndrome
Acute leukaemias of ambiguous lineage
acute undifferentiated leukaemia
mixed phenotype acute leukaemia with t(9;22), or with t(v;11q23)
mixed phenotype acute leukaemia, B/myeloid
mixed phenotype acute leukaemia, T/myeloid
provisional entity natural killer (NK) cell lymphoblastic leukaemia/lymphoma
B lymphoblastic leukaemia/lymphoma
B lymphoblastic leukaemia/lymphoma, NOS
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities such as t(9;22), t(v;11q23) and t(12;21).
T lymphoblastic leukemia/lymphoma

1.1.2. Incidence and mortality

Leukaemia is among the ten most commonly occurring cancers in the whole world (Ferlay et al., 2010). In Saudi Arabia, around 5.8% of all cancer cases analyzed during the year 2005 were leukemias, making it the fifth most common cancer in this country (Al-Eid and Arteh, 2009). The overall incidence of leukaemia worldwide in year 2008 was 5.1 cases per 100.000 population and the mortality rate was 3.6 cases per 100.000 population (Ferlay et al., 2010). Furthermore, it is the most frequent type of cancer found in children accounting for more than 30% of all childhood cancers (Al-Eid and Arteh, 2009, Bozzone, 2009). The survival rate for children with leukaemia is greater than 80%, whereas only 30-40% of adult leukaemia patients survive the disease for five years after diagnosis (Faderl et al., 2003, Jabbour et al., 2005).

The incidence rate of leukaemia varies with race, age, and gender. Caucasians were found to have higher incidence rates of leukaemia (12.8 per 100.000) than that found in American Indians/Alaskan Natives (7.0 per 100.000) and Asian Americans (7.3 per 100.000;Bozzone, 2009). Adult Hispanics had lower incidence rates of leukaemia whereas Hispanic children had the greatest incidence when compared to other non-Hispanic White and Black individuals (Matasar et al., 2006). The reasons for the variation in different populations are not currently understood. Leukaemia incidence is higher in males than in females. Approximately 56% of all cases are seen in males while females account for only 44% of the cases (Bozzone, 2009). Incidence rate by age differs for each type of leukaemia. In general, children up to four years old and adults over 70 years have the highest incidence (Faderl et al., 2003, Hoelzer and Gokbuget, 2000). The most common types of leukaemia found in adults are AML and CLL while ALL is the most common form of leukaemia in children (Bozzone, 2009, Robak and Wierzbowska, 2009).

1.1.3. Prognosis

1.1.3.1. Acute myelogenous leukaemia

Prognostic factors which predict the outcome in AML patients can be divided into two categories. The first category contains factors that predict the treatment-related death. These factors are related to patient characteristics such as age and general

health condition (Dohner et al., 2010). Infants, elderly and patients with hepatic or renal dysfunction, and central nervous system involvement have poor outcome (Appelbaum et al., 2006, Juliusson et al., 2009, Merck). The other category contains factors related to characteristics of the AML clone and predicts resistance to treatment (Dohner et al., 2010). The most important factors among this category are cytogenetic analysis and molecular genetic findings performed at diagnosis (Grimwade, 2001, Grimwade et al., 1998, Byrd et al., 2002). It has been reported that 55-78% of adults with AML have cytogenetic abnormalities at diagnosis (Marosi et al., 1992, Fischer et al., 1996, Tien et al., 1995, Stasi et al., 1993). Based on these cytogenetic findings, AML patients have been categorized into three risk groups: favourable, intermediate, or adverse. The prognostic group with favourable cytogenetic is defined by the presence of chromosomal translocation between chromosome 8 and 21, translocation within chromosome 16 or chromosome 16 inversion. The intermediate prognostic group includes patients with normal karyotypes as well as patients with cytogenetic abnormalities not classified as favourable or adverse. Patients with chromosomal abnormalities such as deletion or loss of chromosome 5 or 7, chromosome 3 inversion and translocation between chromosome 6 and 9 fall into an adverse risk group (Slovak et al., 2000, Byrd et al., 2002, Grimwade et al., 1998, Dohner et al., 2010). The molecular genetic changes in AML blasts that have prognostic significance include mutations in or overexpression of specific genes. Mutations in FLT3 gene (Fms-like tyrosine kinase 3), a gene encoding for a receptor tyrosine kinase, are one of the most frequent genetic alterations occurring in AML. Patients harboring such mutations have a poorer outcome (Whitman et al., 2001, Kottaridis et al., 2001). The Wilms' tumor 1 (WT1) is a transcription factor that is overexpressed in most AML patients. Mutations in the gene encoding WT1 represent an adverse prognostic factor (King-Underwood and Pritchard-Jones, 1998, Virappane et al., 2008, Renneville et al., 2009). On the contrary, some studies have reported a favourable outcome associated with mutations in the nucleophosmin gene (NPM1) (Schnittger et al., 2005, Dohner et al., 2005). Other factors predicting resistance to treatment in AML are the presence of transmembrane transporter proteins that actively extrudes certain chemotherapeutic compounds from leukemic cells (Musto et al., 1991, Wood et al., 1994), high WBC count at time of diagnosis (Dohner et al., 2010) and having secondary AML

(therapy-related AML or AML with a previous history of MDS or MDS/MPN) compared with de novo AML (Estey et al., 1997, Estey and Dohner, 2006).

1.1.3.2. Chronic myelogenous leukaemia

The main prognostic factors for CML patients are the disease phase and presence or absence of the Philadelphia (Ph) chromosome. Philadelphia chromosome is a short chromosome 22 that results from the reciprocal translocation between chromosome 9 and 22 and leads to the generation of an abnormally active BCR-ABL1 tyrosine kinase (Nowell and Hungerford, 1960, Rowley, 1973). Patients diagnosed in the advanced phase and patients who don't have the Ph chromosome have worse outcomes (Hernandez-Boluda and Cervantes, 2009, Ezdinli et al., 1970, Theologides, 1972, Gomez et al., 1981). Further prognostic factors that are associated with poor prognosis among Ph chromosome-positive patients in the chronic phase of the disease include older age, spleen enlargement, very high or very low platelet counts, increased blood percentage of blasts and increased number of eosinophils (Jacquillat et al., 1978, Sokal et al., 1984, Tura et al., 1981).

1.1.3.3. Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia prognostic factors include age, initial WBC count, number of chromosomes and associated structural abnormalities (Mandrell, 2009). Survival decreases with increasing age (Chessells et al., 1998). It also decreases with high initial WBC count (Hoelzer and Gokbuget, 2000). The cytogenetic-molecular abnormalities occur in about 80% of children and 60-70% of adults (Faderl et al., 1998). In childhood ALL, hyperdiploid karyotype, which refers to leukemic blasts with chromosome number ranging from 51-68, is associated with a better clinical outcome; whereas having a normal number of chromosomes (diploid) or normal chromosome number with abnormal morphology (pseudodiploid) is associated with an intermediate outcome. A poor prognosis is associated with hypodiploid ALL where chromosomes number is less than 45 (Mandrell, 2009). Adult patients with either hyperdiploid (>50, 47-50, with 6q-) or diploid karyotypes have a better prognosis (Bloomfield et al., 1989). The translocation t(12;21) is the most frequent translocation occurring in childhood ALL cases and it indicates a favourable

prognosis (Mandrell, 2009), whereas the t(9;22) translocation is most frequently found in adult patients and has been linked with poor prognosis (Faderl et al., 1998).

1.1.3.4. Chronic lymphoblastic leukaemia

Prognostic factors associated with a shorter survival time in patients with CLL include rapid lymphocyte doubling time, expression of B-cell CLL/lymphoma 2 (BCL2), elevated serum thymidine kinase, CD38 positivity, absence of mutations in genes encoding immunoglobulin heavy chain (IgVH), and expression of the intracellular signaling molecule zeta-associated protein of 70 kD (ZAP-70) (Crespo et al., 2003, Faderl et al., 2002, Hallek et al., 1999, Hamblin et al., 1999, Jelinek et al., 2001, Montserrat et al., 1986). The cytogenetic abnormality associated with favourable prognosis is deletion of 13q. An intermediate risk is associated with 11q deletions, 12q trisomy and normal karyotypes whereas the worst prognosis is associated with 17p deletions (Dohner et al., 2000).

1.1.4. Treatment

Current treatment modalities for leukaemia include chemotherapy, radiation therapy and immunotherapy. The choice of treatment depends on the leukaemia subtype, patients' risk factors, age and general health. Immediate treatment is required in case of acute leukemias due to rapid progression of malignant cells which can be fatal within weeks or months (Devine and Larson, 1994). Usually treatment of acute forms consists of two phases: remission-induction and post-remission therapy. The induction therapy is usually a chemotherapy that aims to reduce the number of leukemic cells to achieve a complete remission (CR), a condition where patients have normal peripheral blood counts and less than 5% of leukemic cells in their bone marrow (Devine and Larson, 1994). The goal of post-remission therapy is to prolong the remission period as long as possible by destroying the remaining undetectable leukemic cells which could contribute to relapse. Strategies for post-induction therapy include treatment with intensive high-dose chemotherapy (termed consolidation therapy), or low-dose chemotherapy (termed maintenance therapy) or chemoradiotherapy with either allogeneic or autologous stem cell transplantation (see section 1.3.1) (Devine and Larson, 1994, Tallman et al., 2005).

The outcome of patients with particular leukemic subtypes has been greatly improved with the use of specific targeted therapies such as all-trans retinoic acid (ATRA), tyrosine kinase inhibitors and monoclonal antibodies. The efficacy of these targeted therapies was superior when used in combination with chemotherapy. All-trans-retinoic acid is used to treat APL patients with the translocation t(15;17) which occurs in more than 95% of the APL patients. The t(15;17) translocation results in a fusion transcript that gives rise to the promyelocytic leukaemia-retinoic acid receptor α (PML-RARα). Treatment with ATRA brings on complete remissions in most APL patients by forcing APL-blasts to differentiate into mature granulocytes (Huang et al., 1988, Tallman et al., 1997). Selective tyrosine kinase inhibitors that block the activity of the deregulated BCR-ABL1 tyrosine kinase are used to treat patients with Ph chromosome. Approximately 95% of CML patients and 15-30% of adults with ALL have the Ph chromosome (Bernstein, 1988, Faderl et al., 1998). Imatinib, the first tyrosine kinase inhibitor used, has become the standard therapy for CML patients as it induces high rates of complete responses and improves survival (O'Brien et al., 2003, Druker et al., 2001b). Philadelphia-positive ALL patients used to have the worst prognosis; however, promising results have been achieved with the use of imatinib (de Labarthe et al., 2007, Yanada et al., 2006, Druker et al., 2001a). The monoclonal antibodies rituximab (anti-CD20) and alemtuzumab (anti-CD52) have been shown to be effective in patients with ALL, B cell-CLL and refractory CLL (Byrd et al., 2003, Stilgenbauer and Dohner, 2002, Thomas et al., 2006). A number of monoclonal antibodies have been used to treat AML patients. Among these, gemtuzumab ozogamicin which consists of an anti-CD33 antibody linked to an immunotoxin is being evaluated in randomized trials (Robak and Wierzbowska, 2009). Preliminary results indicate that the addition of gemtuzumab ozogamicin to standard chemotherapy has improved outcomes in AML patients (Chevallier et al., 2008).

1.2. Tumour Immunology

1.2.1. Tumour immune surveillance theory

The ability of the immune system to survey the body and destroy any transformed cells before they appear clinically was first suggested by Paul Ehrlich in the early 20th century (Ehrlich, 1909). In 1949, Sir Macfarlane Burnet published his theory of

acquired immunological tolerance which states that self-reactive lymphocytes are deleted in prenatal life during the development of the immune system (Burnet, 1949). Burnet's theory was experimentally verified by Medawar and colleagues (Billingham et al., 1953) and led to the abandonment of the concept proposed by Ehrlich. However, tumour transplantation experiments performed during the 1950s in genetically identical mice strongly suggested the existence of antigens associated with tumour cells enabling them to be recognized and eliminated by the immune system (Baldwin, 1955, Foley, 1953, Prehn and Main, 1957). In these experiments, mice could reject transplanted autologous tumour when challenged with a second injection of the same tumour cells. Consequently, Burnet and Lewis Thomas adopted Ehrlich's early idea and proposed that major function of the immune system is to protect from neoplastic disease (Burnet, 1970, Burnet, 1957, Thomas, 1959). The term immunological surveillance was coined by Burnet who assumed that lymphocytes play the key role in identifying and eliminating malignant cells (Burnet, 1967, Burnet, 1970, Burnet, 1964).

The validity of the theory was tested for the first time when the nude strain of mice became available to researchers. These mice lack a functional thymus making them severely immunocompromised. According to the immune surveillance theory, immunocompromised individuals will be more susceptible to develop tumours than those with a competent immune system. Nevertheless, early experiments, carried out in 1970s, showed that there was no increase in the incidence of either spontaneously arising or chemically induced tumours among the athymic nude mice compared with their normal wild-type counterparts (Rygaard and Povlsen, 1974, Stutman, 1974, Burstein and Law, 1971). In contrast, several studies confirmed that the immune surveillance mechanisms were able to eliminate the growth of tumour cells induced by oncogenic viruses in mice (Fefer et al., 1968, Fefer et al., 1967, Klein, 1976). These findings suggested that the immune surveillance in mice targeted transforming viruses but not tumours themselves. Shortly afterwards, the hypothesis was retrieved when it became clear that nude mice were not completely immunocompromised, since detectable populations of functional αβ T cells were found in this strain of mice (Ikehara et al., 1984, Maleckar and Sherman, 1987). In addition to αβ T cells, other components of the immune system, able to develop totally outside the thymus, have been discovered and found to be present in large

numbers in nude mice such as natural killer (NK) cells and gamma delta (γδ) T cells (Hayday, 2000). Repetition of the experiments carried out by Stutman (1974) using mice with different genetic backgrounds revealed that athymic nude mice did form more tumours than the immunocompetent controls after treatment with different doses of the chemical carcinogen, methylcholanthrene (MCA; (Engel et al., 1996). Moreover, it has been found that tumour formation induced by MCA was greater in immunodeficient severe-combined immunodeficiency (SCID) mice than in their normal counterparts (Engel et al., 1997).

Stronger evidence for the existence of immune surveillance was gathered using genetically altered strains of mice and monoclonal antibodies (mAbs) specific for various components of the immune system. Interferon-gamma (IFN-γ) is a pleiotropic cytokine secreted by activated NK cells and activated thymus-derived (T) cells (Boehm et al., 1997). Several studies have shown that this cytokine plays a critical role in protecting the host against the growth of transplanted tumours, as well as the formation of chemically induced and spontaneously arising tumours (Dighe et al., 1994, Kaplan et al., 1998, Shankaran et al., 2001). One of these studies showed that transplanted murine immunogenic fibrosarcoma cell line (Meth A) grew faster in mice treated with anti-IFN-γ than their control counterparts (Dighe et al., 1994). These tumour cells were made insensitive to IFN-γ by the insertion of a genetically engineered truncated IFN-γ receptor α-subunit. The insensitive tumour cells displayed enhanced tumorgenicity and were resistant to LPS induced tumour rejection, as compared with control tumours when transplanted into naïve syngeneic hosts (Dighe et al., 1994). Another study showed that IFN-γ-deficient mice developed more spontaneous lymphomas and lung tumours compared with genetically matched wild type controls (Street et al., 2002). Mice lacking either IFN-γ receptor α chain or signal transducer and activator of transcription 1 (STAT1), the transcription factor necessary for mediating interferon receptor signaling, were found to be more susceptible to MCA-induced tumours than their wild-type controls (Kaplan et al., 1998). Additionally, IFN-γ-insensitive mice lacking the genes encoding for the tumor-suppressor protein p53 developed tumours more rapidly than wild-type mice (Kaplan et al., 1998). These mice also developed a wider spectrum of tumours compared with mice lacking only p53 (Kaplan et al., 1998). In vivo depletion studies showed that adoptively transferred lymphocytes were not able to

induce regression of established tumours in mice depleted of IFN-γ (Barth et al., 1991, Tuttle et al., 1993).

Perforin is another key component that has been shown to play an important role in tumour surveillance. It is a pore-forming protein (pfp) stored in cytoplasmic granules of cytotoxic lymphocytes ($CD8^+$ T cells and NK cells). It mediates the main effector mechanism used by cytotoxic lymphocytes to induce apoptosis in target cells (Russell and Ley, 2002). Compared with perforin-competent controls, mice lacking perforin were more susceptible to tumour formation induced by MCA (Smyth et al., 2000a, van den Broek et al., 1996, Street et al., 2001). Perforin-deficient mice also had an increased incidence of spontaneously disseminated lymphomas (Smyth et al., 2000b, Street et al., 2002, Street et al., 2004). When syngeneic tumour cell lines were injected into perforin-deficient mice and their normal counterparts, tumour cells were eliminated 10-100-fold more efficiently by perforin-sufficient mice (van den Broek et al., 1996).

A study carried out using mice lacking recombination activating gene 1 and 2 proteins (RAG1 and RAG2) strongly supported the concept of lymphocytes protecting the host against tumour growth (Shankaran et al., 2001). RAG1 and RAG2 proteins are responsible for the rearrangement of VDJ DNA segments that leads to the formation of antibody molecules as well as T-cell receptors. Mice deficient in either of these genes fail to rearrange lymphocyte antigen receptors and thus completely lack natural killer T (NKT), T and B lymphocytes (Shinkai et al., 1992). Mice lacking the gene RAG-2 had a higher incidence of spontaneous and chemically induced tumours than matched wild-type controls. Interestingly, mice lacking both RAG2 and STAT1 genes developed spontaneous breast tumours that were not observed in wild type or RAG2-deficint mice (Shankaran et al., 2001). The role of NK cells in protecting against tumour formation and metastasis was highlighted in several studies. These studies showed that NK cell-deficient mice have an increased incidence of spontaneous tumours and cancer metastasis (Haliotis et al., 1985, Gorelik et al., 1982, Talmadge et al., 1980, Smyth et al., 2000a). In addition, mice that were depleted of both NK and NKT cells by the anti-NK1.1 monoclonal antibody were two to three times more susceptible to MCA-induced tumour formation than control mice (Smyth et al., 2001).

In humans, the concept of tumour immune surveillance has gained more supporting evidence through several clinical observations. One of the observations was the increased risk of developing malignancies in immunosuppressed transplant recipients. Some follow-up studies of these patients have shown a higher incidence of virally induced tumours compared with the normal population whereas the incidence of non-virally induced tumours was not increased (Penn, 1988, Sheil et al., 1997). Nevertheless, other studies have also shown that the incidence of developing a variety of non-virally induced cancers is higher in immunosuppressed patients than in the general population (Birkeland et al., 1995, Pham et al., 1995, Sheil, 1986, Penn, 1996, Vajdic et al., 2006). Another observation was the existence of a significant relationship between the presence, location, and activation state of lymphocytes in a tumour (tumour-infiltrating lymphocytes; TILs) and improved prognosis. Such a relationship was first observed in melanoma patients in whom the presence of TILs in the vertical growth phase of primary cutaneous melanomas was correlated with survival (Clemente et al., 1996, Clark et al., 1989, Mihm et al., 1996). The presence TILs was also correlated with improved outcome in patients with broad range of cancers including ovarian, breast, colorectal, gastric, oesophageal and squamous cell lung carcinoma (Naito et al., 1998, Schumacher et al., 2001, Yoshimoto et al., 1993, Zhang et al., 2003, Ishigami et al., 2000, Villegas et al., 2002). Finally, spontaneous regression of cancers has been occasionally seen in patients with melanoma, neuroblastoma and renal cancer indicating that the immune system may develop efficient anti-tumour immune responses (Bodurtha et al., 1976, Menzies and McCarthy, 1997, Everson and Cole, 1966).

1.2.2. Cancer immunoediting theory

Despite the evidence in support of immunosurveillance, cancers continue to occur in immunocompetent hosts, which suggests failure of the immune system to prevent tumour growth and metastasis even in immunocompetent environments (Dunn et al., 2002). As a result, an updated immunosurveillance hypothesis called cancer immunoediting has been proposed by Schreiber and his colleagues (Dunn et al., 2002). This hypothesis proposes that the immune system not only controls the growth of tumour cells but also indirectly shapes the neoplastic disease. According to the hypothesis, the process of immunoediting consists of three stages: elimination,

equilibrium and escape. The first stage represents the original concept of the immunosurveillance theory. It starts when tissues surrounding the growing transformed cells induce inflammatory signals that lead to recruitment of innate immune cells including NKT, NK, γδ T cells, macrophages and dendritic cells (DCs). The infiltrating lymphocytes (NKT, NK and γδ T cells) recognize the transformed cells and release IFN-γ which induces tumour cell death. Chemokines, released from tumour cells themselves as well as from surrounding tissues, also promote death of the tumour cell by preventing angiogenesis. Immature DCs ingest tumour cell debris and home to draining lymph nodes where they activate tumour-specific naïve T cells. The activated tumour-specific T cells migrate to the tumor site and destroy antigen-bearing tumour cells. In the equilibrium stage, lymphocytes and IFN-γ exert a selective pressure on tumour cell variants that have survived the elimination process leading to selection of tumour cells with reduced immunogenicity. This process is perhaps the longest of the three stages and may occur over a period of many years in humans. Tumour cell variants that have acquired insensitivity to immunological detection enter the escape phase, where they expand in an uncontrolled manner resulting in clinically detectable malignant disease (Dunn et al., 2002).

1.2.3. Immune recognition of tumour cells

The immune system is a network of organs and tissues as well as humoral factors that work together to eradicate any invading pathogens and malignant cells. It has the ability to discriminate between self and non-self. It is composed of two main arms, innate and adaptive arms. Innate immunity is the first line of defence, whereas adaptive immunity is involved in the late phase of host defence. Innate immunity includes physical barriers such as skin, soluble factors such as complement and cellular components. The cellular components of innate immunity are neutrophils, monocytes, macrophages, NK, NKT, and γδ T cells. Receptors for these cells are fixed in the genome, requiring no rearrangements and recognize conserved molecular patterns that are specific to pathogens. Adaptive immunity mediates highly specific immune responses and displays immunologic memory, therefore subsequent exposure to the same antigen leads to faster and more vigorous responses. It includes B and T lymphocytes. These cells have receptors with great

variation, able to recognize an unlimited number of highly specific pathogens through rearrangements of receptor gene segments (Melief, 1992). Both arms of the immune system play an essential role in the elimination of cancer cells. The major effector cells of innate immunity are NK cells and of adaptive immunity are T cells (Caligiuri et al., 2004).

1.2.3.1. Natural killer cells

Natural killer cells are characterized by their potent cytolytic activity against tumor and virally infected cells. Unlike T lymphocytes, NK cells do not possess clonally distributed antigen receptors. However, they share certain functions with T cells such as cytolytic effector mechanisms and cytokine production. In this, NK cells use perforin and granzymes to achieve the cytolytic activity against target cells and secrete cytokines such as IFN-γ, tumour necrosis factor–alpha (TNF-α) and granulocyte macrophage-colony stimulating factor (GM-CSF) that mediate inflammatory responses (Biron et al., 1999, Moretta, 2005).

NK cells recognize their targets through an array of surface receptors that have either inhibitory or activating function (Moretta et al., 1996, Long and Wagtmann, 1997, Lanier, 1998, Moretta et al., 2000). The engagement of NK inhibitory receptors to their ligands on target cells generates a signal that inhibits NK cell lysis-a mechanism known as missing self recognition, whereas engagement of activating NK receptors to their ligands delivers a signal that lead to target lysis-a mechanism known as induced self recognition. The ligands for the inhibitory receptors are major histocompatibilty complex (MHC) class I molecules and these are of two main types; the killer cell immunoglobulin-like receptors (KIR) which specifically recognize human leukocyte antigen (HLA)-A, -B, and –C alleles, and CD94/NKG2A which identifies the polymorphic HLA-E molecules (Braud et al., 1998, Moretta et al., 1996, Vilches and Parham, 2002). Loss or down regulation of MHC class I molecules is frequently seen in tumours (Marincola et al., 2000). Ligands that have been identified to be recognized by activating receptors are stress-inducible molecules such as MHC-class I-related chain A/B (MICA/B), and the unique long 16-binding proteins (ULBPs). These molecules are highly expressed by cells suffering from stress resulting from either viral infections or neoplastic transformation and are recognized by the activating receptor, NKG2D (Bauer et al.,

1999, Cosman et al., 2001). Therefore, tumour cells that have lost or express low levels of class I MHC molecules and tumour cells that have up regulated ligands for activating receptors, even in the presence of normal levels of MHC class I molecules, will be targeted by NK cells. Besides their potent anti-tumour cytolytic activity, activated NK cells facilitate adaptive anti-tumour immune responses by stimulating the maturation of DCs (Degli-Esposti and Smyth, 2005).

1.2.3.2. T lymphocytes or T cells

T lymphocytes are considered to be the most efficient cells in mediating anti-tumour immune responses. They are characterized by the presence of a unique receptor on their cell surface called T-cell receptor (TCR) which is a heterodimer consisting of either αβ or γδ chains. The majority of T cells express αβ TCR chains and either CD4 or CD8 molecules. $CD4^+$ T lymphocytes generally function as cytokine-secreting T helper cells (Th), while CD8+ T lymphocytes generally function as cytotoxic T cells (CTLs) (Delves and Roitt, 2000). Besides helper T cells, there are also CD4+ regulatory T cells (Tregs) which are able to control effector responses (Thornton and Shevach, 1998, Takahashi et al., 1998).

Unlike B cells which recognize free antigens, the TCR recognizes peptide antigens that are displayed on the cell surface in combination with MHC molecules. MHC molecules are highly polymorphic cell surface glycoproteins encoded by genes within the major histocompatibility complex. Class I molecules are made up of a heavy α chain and a non-covalently associated light chain, β2-microglobulin. Class II molecules are made up of α and β chains. The presentation of protein antigens to T cells in the context of MHC molecules involves two distinct intracellular processing pathways. The endogenous pathway generally presents peptides derived from cytoplasmic proteins in the context of class I MHC molecules to CD8+ T cells, whereas the exogenous pathway mainly presents peptides derived from processing of engulfed extracellular proteins in the context of class II MHC molecules to CD4+ T cells (Doyle and Strominger, 1987, Norment et al., 1988). Antigen processing and presentation by the MHC class I pathway involves three major steps: processing of endogenous proteins by the proteasome, transporting the generated peptides by transporter associated with antigen processing (TAP) and peptide binding to nascent MHC molecules (Figure 1.3). All nucleated cells express MHC class I molecules on

their surfaces. Therefore, malignantly transformed cells presenting epitopes derived from tumour antigens could be recognized and eliminated by effector $CD8^+$ T cells. In contrast class II molecules are only expressed by professional antigen presenting cells i.e. DCs, B cells and macrophages. For optimal activation of $CD8^+$ T cells, the signal generated by the interaction between the peptide-MHC complex and the T-cell receptor is not sufficient. A second signal designated as co-stimulation is required. It is provided by the interaction between CD28 on the T cell surface and members of the B7 family (CD80/B7-1, CD86/B7-2) located on the surface of antigen presenting cells (Lenschow et al., 1996). If a naïve T cell - i.e., a cell that has not yet encountered a specific antigen - recognizes an antigen in the presence of adequate costimulatory signals, cells will be primed and activated. Activated T cells proliferate by clonal expansion and differentiate into both effector and memory cells (van Stipdonk et al., 2001). On the other hand, if T cell stimulation occurs in the absence of appropriate costimulatory signals, this will lead to T cell unresponsiveness (anergy) or apoptosis and death -a mechanism which gives rise to the induction of peripheral T cell tolerance (Lenschow et al., 1996). Additionally, T cells express inhibitory receptors, such as cytotoxic T lymphocyte-4 (CTLA-4/CD152) and programmed death-1 (PD-1) that negatively regulate T cell function (Walunas et al., 1994, Carreno and Collins, 2002).

$CD8^+$ T cells induce cytolysis of their target cells through at least two different mechanisms: the granule exocytosis pathway which is mediated by perforin and granzymes or Fas-mediated pathway by upregulation of membrane-bound Fas ligand (FasL/CD95L) which interacts with the Fas receptor (CD95) on target cells. $CD8^+$ T cells also produce a number of cytokines including TNF-α and IFN-γ (Delves and Roitt, 2000).

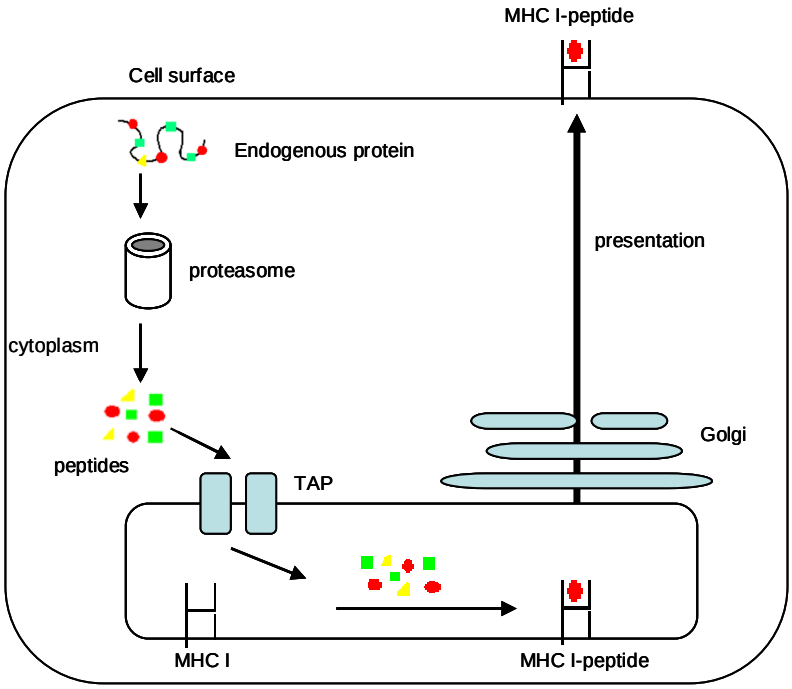

Figure 1.3. Major histocompatibility complex class I presentation pathway.
Three major steps contribute to this pathway. The first step is the degradation of endogenous proteins into smaller fragments by the proteasome which is a large multienzyme complex in the cytosol. The resulting peptides are translocated into the lumen of endoplasmic reticulum by TAP where they bind to nascent MHC class I molecules and transported through Golgi apparatus to the cell surface. Figure adapted with modifications from (Heath et al., 2004)

1.2.4. Mechanisms of tumour escape

Surviving tumours developed different mechanisms to escape recognition by the host immune system. Some of these mechanisms involve alterations in the expression of effector molecules crucial for T cell recognition. These mechanisms include: alterations in the expression of HLA class I and class II molecules, mutations of β2-microglobulin, downregulation of TAP, loss of tumour antigens, decreasing the expression of the co-stimulatory molecules such as B7 molecules, and enhancing the expression of co-inhibitory molecules such as B7-H1 molecule (Dorfman et al., 1997, Marsman et al., 2005, Jager et al., 2001, Marincola et al., 2000, Dong et al., 2002, Dermime et al., 1997, Bicknell et al., 1996, Vitale et al., 1998, Ghebeh et al., 2008). Impaired binding of perforin to the tumour cell membrane is a mechanism used by tumour cells to resist killing by cytolytic cells (Lehmann et al., 2000). Down regulation of Fas and mutations in Fas as well as in TNF-related apoptosis-inducing ligand receptors have also been reported in some tumours (Real et al., 2001, Shin et al., 2001). Tumours can kill infiltrating immune cells by expressing FasL without damaging themselves (Whiteside, 2002). In addition, tumors and a subset of DCs constitutively express the enzyme indoleamine 2,3-dioxygenase which induces tryptophan deficiency in the tumour microenvironment and sensitizes activated T cells to apoptosis (Uyttenhove et al., 2003, Munn et al., 2004).

Tumours can also evade immune recognition by secreting a number of immunosuppressive mediators such as IL-6, IL-10, macrophage-colony stimulating factor (M-CSF) vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), soluble FasL and soluble MICA. These suppressive mediators promote tumour growth by inhibiting anti-tumour immune responses and/or recruiting immune cells with regulatory functions favourable for the tumour. IL-10, TGF-β and VEGF suppress DCs differentiation and maturation (Gabrilovich et al., 1998, Kim et al., 2006). Immature DCs in turn induce either T-cell unresponsiveness or Tregs, a suppressor subset of $CD4^+$ T cells that have been shown to inhibit anti-tumour specific T cells (Almand et al., 2001, Jonuleit et al., 2000, Dhodapkar et al., 2001, Hawiger et al., 2001, Zou, 2005, Lehe et al., 2008). IL-6 and M-CSF secreted by tumour cells and tumour environmental macrophages switch DC differentiation towards macrophage differentiation (Menetrier-Caux et al., 1998). Soluble FasL and

soluble MICA inhibit anti-tumour activity of both cytotoxic T cells and NK cells (Doubrovina et al., 2003, Webb et al., 2002). Besides the induction of immature DCs and Tregs, tumour mediators promote the recruitment of myeloid suppressor cells which inhibit priming of naïve T cells (Bronte et al., 2001).

1.3. Immunotherapy for Leukaemia

1.3.1. Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cells transplantation (HSCT) is an efficient therapy for a variety of haematological malignancies, including leukemias. It was developed with the aim of restoring the hematopoietic stem cell compartment in patients following a myeloablative conditioning regimen i.e. high dose chemotherapy and/or total body irradiation. It is now known that donor immune cells transplanted with the stem cell allograft, or arising from it, recognize and eliminate residual leukaemia cells in the recipient - an immunological effect known as graft-versus-leukaemia (GvL). The donor immune cells may also attack normal host tissues such as skin, gastrointestinal tract and liver giving rise to a graft-versus-host disease (GvHD) complication. Another possible consequence of the HSCT is graft rejection, occurring when recipient immune cells attack and destroy the transplanted graft, leading to a host-versus-graft (HvG) reaction (Appelbaum, 2001).

The existence of a GvL effect has been supported by studies showing that relapse rates following allogeneic transplantation were lower in patients who developed GvHD compared with those who did not (Weiden et al., 1979, Weiden et al., 1981). The relapse rates were also lower in patients who received allogeneic transplants compared with those who received syngeneic transplants i.e. from their identical twin (Gale et al., 1994, Fefer et al., 1987). However, the most important evidence for the existence of the GvL effect came form the astonishing success of donor lymphocyte infusions (DLI). In DLI, patients relapsing following allogeneic HSCT are treated with infusions of lymphocytes obtained from the peripheral blood of the original stem cell donor. Using this immunotherapeutic approach, complete remissions could be re-induced in most relapsed CML and some AML patients (Kolb et al., 1995, Kolb et al., 1990). The reactions occurring during the GvL effect are mainly mediated by donor $CD4^+$ and $CD8^+$ T lymphocytes. Evidence for this

came from increased relapsed rates in patients who received T cell-depleted grafts in an attempt to prevent GvHD activity (Apperley et al., 1986, Hale et al., 1988). Besides T cells, NK cells have also been shown to contribute to the GvL response in cases of mismatched hematopoietic transplants (Ruggeri et al., 2002, Giebel et al., 2003). The GvL effect could be separated form GvHD in some patients because relapsed rates were reduced in a subset of patients who received allogeneic HSCT but did not develop GvHD and remissions could be achieved after DLI without developing GvHD (Giralt and Kolb, 1996, Kolb and Holler, 1997, Horowitz et al., 1990, Collins et al., 1997). These findings led to efforts to develop T-cell based therapy for leukaemia patients that aim to augment the GvL effect without causing GvHD.

1.3.2. Potential target antigens of the GvL effect

Targets recognized by alloreactive donor T cells during a GvL response include antigenic peptides that are either derived from polymorphic minor histocompatibility antigens (mHAgs) or derived from antigens that are associated with the malignant phenotype, known as leukaemia antigens (Appelbaum, 2001). The mHAgs are immunogenic peptides derived from intracellular proteins that differ between the donor and the recipient as a result of genomic polymorphisms. These antigens have been shown to be targets of alloreactive T cells in both GvL and GvHD reactions (Warren et al., 1998, Goulmy et al., 1983, Goulmy et al., 1996). Some of the identified mHAgs, including HA-1 and HA-2, are derived from proteins expressed only in hematopoietic cells, whereas other mHAgs are broadly expressed in all or most tissues (den Haan et al., 1998, den Haan et al., 1995, de Bueger et al., 1992). Several studies have demonstrated that T cells specific for the hematopoietic restricted mHAgs, but not the broadly expressed antigens represent ideal immunotherapeutic targets that would enhance the GvL activity with minimal GvHD (Akatsuka et al., 2003, Mutis et al., 1999a, Bonnet et al., 1999, Mutis et al., 1999b, Dickinson et al., 2002, Stumpf et al., 2009, Ofran et al., 2010).

Leukaemia antigens fall into two major categories: leukaemia-specific antigens and leukaemia-associated antigens (LAAs). Examples for each category are presented in Table 1.3 (Borrello and Sotomayor, 2002). The specific antigens are those resulting

from mutations or chromosomal translocations as well as antigens that are derived from viral proteins. Examples of this group include BCR-ABL found in CML and PML-RARα found in APL patients. The BCR-ABL is a chimeric protein that results from a t(9;22) translocation found in 95% of CML patients. Chromosomal translocation is a common finding in leukaemia that results in the generation of fusion genes coding for chimeric proteins. The joining region of the chimeric proteins generates unique epitopes that are tumour specific and therefore represent attractive targets for immunotherapy. Several CTL epitopes derived from the joining region of the BCR-ABL have been identified. These epitopes were found to bind to several HLA alleles, including HLA-A2, -A3, A11 and -B8 molecules and to elicit specific CTL responses in vitro (Yotnda et al., 1998, Clark et al., 2001, Bocchia et al., 1996, Osman et al., 1999a). The associated antigens are normal proteins that are aberrantly expressed or over-expressed in leukemic cells compared with normal cells. This category can be subdivided into two groups according to their expression patterns in normal and malignant tissues. These groups are overexpressed antigens and cancer-germ line antigens. Overexpressed antigens are normal proteins that are expressed in normal tissues at low levels and expressed constitutively in tumour cells. Since these proteins are self normal proteins, immunological tolerance may exist and prevent their recognition by T cells. However, the overexpression of these normal proteins can break the immunological tolerance towards these antigens (Dermime et al., 2002). The WT1 and proteinase-3 (PR-3) are examples of overexpressed leukaemia antigens that have been shown to be recognized by autologous CTLs. M-Phase Phosphoprotein 11 (MPP11) and the receptor for hyaluronic-acid-mediated motility (RHAMM/CD168) are overexpressed antigens that were identified as potential LAAs by serological screening of myeloid leukaemia patients (Greiner et al., 2003, Greiner et al., 2002). In addition, an HLA-A0201-restricted CTL epitope derived from RHAMM has been also identified (Greiner et al., 2005). Cancer-germ line antigens result from reactivation of genes which are normally silent in normal cells and become activated in different tumours. Their expression in normal tissues is restricted to placental trophoblasts and testicular germ cells. They represent attractive targets for specific immunotherapy since germ line cells lack MHC class I and class II molecules, epitopes of these antigens will not be expressed by these tissues (Borrello and Sotomayor, 2002). Preferentially expressed antigen of melanoma (PRAME) is a cancer-testis antigen

that has been shown to elicit humoral immune response in 47% of AML patients (Greiner et al., 2000).

Table 1.3. Tumour antigens expressed in leukaemias, adapted with modification from (Borrello and Sotomayor, 2002).

Category of Antigen	***Leukaemia type***
Specific-leukaemia antigens	
Fusion gene products	
-BCR-ABL	Chronic myelogenous leukaemia
-DEK-CAN	Acute myelogenous leukaemia
-AML/ETO	Acute myelogenous leukaemia
-PML-RARα	Acute Promyelocytic leukaemia
Viral-Associated Antigens	
- HTLV-1	Adult T-cell leukaemia
Associated-leukaemia antigens	
Cancer-germ line antigens	
-PRAME	Acute myelogenous leukaemia
Overexpressed Antigens	
- Proteinase-3	Acute myelogenous leukaemia and Chronic myelogenous leukaemia
- WT-1	Acute myelogenous leukaemia, Chronic myelogenous leukaemia and Acute lymphoblastic leukaemia
-MPP11	Acute myelogenous leukaemia and Chronic myelogenous leukaemia
-RHAMM	Acute myelogenous leukaemia and Chronic myelogenous leukaemia

1.4. Candidate antigens for leukaemia immunotherapy

1.4.1. Wilms' tumor 1 (WT1)

The WT1 gene encodes a zinc finger transcription factor that is normally expressed in tissues of mesodermal origin during embryogensis, including the kidney, gonads, heart, mesothelium and spleen. It has been shown to be responsible for childhood renal cancer (Call et al., 1990). It is overexpressed in acute myelogenous and lymphoblastic leukaemia, chronic myelogenous leukaemia and also in various types of solid tumours including lung and breast cancer (Oji et al., 1999, Bergmann et al., 1997, Menssen et al., 1995, Loeb et al., 2001, Oji et al., 2002). The higher expression levels of WT1 in leukaemia patients have been found to be associated with poor prognosis (Bergmann et al., 1997, Inoue et al., 1997, Boublikova et al., 2006). Therefore, WT1 is important as a prognostic marker as well as in the detection and monitoring of minimal residual disease in leukaemia and myelodysplastic syndrome (Inoue et al., 1994). WT1-derived HLA class I peptide epitopes with various HLA restrictions including HLA-A201, -A24, -A206 and -A01 have been identified (Azuma et al., 2002, Bellantuono et al., 2002, Oka et al., 2000, Li et al., 2008, Asemissen et al., 2006). Class II epitopes derived from WT1 have also been identified (Kobayashi et al., 2006, Fujiki et al., 2007, Knights et al., 2002, May et al., 2007). Furthermore, natural specific humoral immune responses to the WT1 antigen have been reported in patients with haematological malignancies (Elisseeva et al., 2002, Wu et al., 2005). For these reasons, WT1 has been considered as an attractive target for cancer immunotherapy.

1.4.2. M-Phase Phosphoprotein 11 (MPP11)

MPP11 was discovered by Matsumoto-Taniura et al (1996) using MPM2 monoclonal antibody, which recognizes several important mitosis phosphoproteins found to be present during mitosis (Davis et al., 1983). Recently, it has been reported that MPP11 function as a ribosome-associated molecular chaperon (Hundley et al., 2005, Otto et al., 2005). MPP11 maps to the critical region of chromosome 7q22-31.1, which is a critical common position in human cancer (Resto et al., 2000, Zenklusen and Conti, 1996). MPP11 has been detected by serological analysis of cDNA expression libraries (SEREX) in solid tumours and

haematological malignancies including melanoma, breast, renal cell carcinoma, small cell lung cancer and leukaemia (Gure et al., 2000, Greiner et al., 2003). The humoral immune responses to MPP11 have been detected in patients with AML and CML, but not in healthy donors or patient with autoimmune diseases (Greiner et al., 2003). Moreover, this protein has been found to be expressed extensively in AML, and CML patients as compared to normal controls (Schmitt et al., 2006, Greiner et al., 2003, Greiner et al., 2004). Immuno-histochemical staining of primary tumour sections and Western blot analysis of head and neck squamus cell cancer (HNSC cell lines revealed tumour-specific overexpression of MPP11 protein (Resto et al., 2000). Additionally, fluorescence in situ hybridization analysis carried out on HNSCC cell lines showed an increase in the copy number of MPP11 along with chromosome 7 suggesting an oncogenic role for MPP11 (Resto et al., 2000). The specific overexpression of MPP11 by tumour tissues (with the exception of very low expression in testis, kidney, and lung) makes it an attractive target for cancer immunotherapy (Greiner et al., 2004).

1.4.3. Proteinase-3 (PR-3)

PR-3 is a serine protease enzyme that is induced at the promylocytic stage of differentiation and stored in azurophilic granules of promyelocytes (van der Woude et al., 1985). PR-3 is known to be a target of anti-neutrophil cytoplasmic antibody (Hernandez-Caballero et al.) in patients who have the autoimmune disease Wegener's granulomatosis (Williams et al., 1994). Furthermore, proliferative T cell responses against purified PR-3 protein has been documented in these patients (Ballieux et al., 1995). PR-3 belongs to the group of primary granule proteins, which are aberrantly expressed in myeloid leukaemias. It is highly overexpressed by cells from CML patients and differentially expressed by some AML cells (Muller-Berat et al., 1994, Yong et al., 2008). Importantly, it may be involved in the process of leukemogenesis because antisense oligodeoxynucleotides against PR-3 were able to inhibit cell proliferation and induce differentiation in an HL-60 promyelocytic leukaemia cell line (Bories et al., 1989). Therefore, PR-3 is of great interest as a source of leukaemia antigens for immunotherapy (Barrett and Rezvani, 2006, Fujiwara et al., 2005).

An antigenic HLA-A*0201-restricted epitope, named PR1 (VLQELNVTV), derived from the PR-3 antigen has been identified (Molldrem et al., 1996) and is being used in clinical trials for leukaemia patients (Heslop et al., 2003, Clinical Trials.gov, 2006, Clinical Trials.gov, 2009, Qazilbash et al., 2004). This epitope was identified by Molldrem *et al.* (1996) who used BIMAS algorithm software program to identify PR-3-derived peptides with high binding affinities to the most frequent HLA-A allele, HLA-A*0201. The predicted peptides were used to elicit peptide-specific CTLs in vitro from healthy HLA-A*0201 donor lymphocytes which specifically lysed HLA-A*0201 positive myeloid leukemias overexpressing proteinase-3 (Molldrem et al., 1996). In a subsequent study, Molldrem and his colleagues showed that the same CTLs were able to inhibit the colony formation in vitro by leukemic but not normal $CD34^+$ cells (Molldrem et al., 1997). Furthermore, both cell lysis and inhibition of progenitor formation correlated with the amount of intra-cytoplasmic PR-3 expression by the leukaemia target cells (Molldrem et al., 1996, Molldrem et al., 1997). The PR-1 T cell epitope was then thoroughly investigated. Only T cells with high avidity exerted an anti-leukemic effect following isolation of high and low avidity anti-PR-1 T cells from normal individuals (Molldrem et al., 2003). In accordance with previous results that showed the presence of PR-1-specific T cells in healthy individuals at low frequencies (Molldrem et al., 1999), Molldrem *et al.* reported high frequencies of cytotoxic T cells specific to the PR-1 epitope in CML patients responding to interferon-α {} and also in a group of patients responding to allogeneic bone marrow transplantation (BMT) treatment. This suggests that the PR-1-specific CTLs expand in responding patients and contribute to remission (Molldrem et al., 2000). The existence of natural immune response to PR1 that can be amplified in the presence of leukemic cells was demonstrated by the Barrett group {Rezvani, 2003 #119} who found that PR-1-specific memory T-cell frequencies of around 1/100 000 exist in healthy donors, while blood taken from patients in remission showed one and two log increases over the normal baseline. PR-1 peptide sequence is also expressed by neutrophil elastase (NE) which is another granular serine protease found to be highly expressed in CML progenitors (Fujiwara et al., 2004, Yong et al., 2008). Interestingly, it has been reported that a high expression levels of both PR-3 and NE in $CD34^+$ progenitors were associated with longer survival in patients with CML suggesting T-cell mediated responses against these overexpressed antigens (Yong et al., 2007, Yong et al., 2006).

1.4.4. Approaches for the identification of tumour antigens

Different approaches have been used to identify tumour antigens that induce immune responses in humans. The first approach used is based on the identification of targets that were recognized by patients' tumour-specific CTLs clones. To identify such antigens, genomic DNA libraries or cDNA libraries prepared from tumour cells were transfected into cells expressing the appropriate MHC molecule. The tumour-specific T cells were then tested for their ability to recognize the antigen by releasing cytokines or lysing specific target cells (van der Bruggen et al., 1991, Kawakami et al., 1994). The majority of genes encoding tumour antigens in solid malignancies have been identified using this approach (Wang and Rosenberg, 1996).

Another approach based on a biochemical purification method is also used. This method involves acid-elution of peptides from MHC molecules purified from tumour cells and separation of the eluted peptides by high performance liquid chromatography. Peptide fractions are then loaded onto MHC-matched APCs to test their ability to elicit T-cell responses. The reactive peptides are further analyzed using tandem mass spectrometry which allows the isolation and sequencing of individual peptides. Once the amino acid sequence is identified, database searches can be used to determine the source of the antigen (Hunt et al., 1992, Cox et al., 1994, Skipper et al., 1999, van Bergen et al., 2007, den Haan et al., 1995).

SEREX (serological analysis of recombinant cDNA expression libraries) as well as proteomics are used for identification of tumour antigens based on detection of proteins that elicit spontaneous humoral immune responses in cancer patients. In the SEREX method, a cDNA library is constructed from a fresh tumour specimen, packaged into a lambda-phage vector and expressed in Escherichia coli (E.coli). The recombinant proteins are transferred onto nitrocellulose membranes and screened for recognition by high-titer IgG antibodies present in the patient’s serum (Sahin et al., 1995, Chen et al., 1997). Some T cell defined antigens could be also detected using SEREX analysis, indicating that this approach is useful in identifying tumour antigens that are recognized by antibodies (Sahin et al., 1995). More importantly, at least two of the newly SEREX-identified antigens were subsequently shown to elicit specific T-cell responses (Chen et al., 1997, Greiner et al., 2005). The other method, proteomics, analyzes cellular extracts as well as serum proteins from normal and

cancer patients to compare and identify differentially expressed tumour antigens. It uses two-dimensional western blotting experiments to separate proteins followed by incubation with sera from cancer patients. Mass spectrometry and database search are then used to identify reactive target proteins (Le Naour, 2007, Chen et al., 2002, Cui et al., 2005).

Analyzing gene expression profiles in cancer cells and comparing them with those in normal counterparts is used to identify any abnormal genes (mutated or virally-derived) or normal but overexpressed genes that are aberrantly expressed in tumour cells. The techniques used to analyze the expression of genes include gene microarrays, serial analysis of gene expression (SAGE) and cDNA subtraction (Velculescu et al., 1995, Wang et al., 2000).

Candidate tumour antigens that have been identified by approaches such as SEREX or gene expression analyses can be searched for their epitopes using computer based prediction algorithms available on the worldwide web. This approach is known as reverse immunology or epitope deduction. It has been used to identify CTL epitopes not only from tumour antigens but also from viral antigens, microbial antigens and autoantigens (Satoh et al., 2005, Capo et al., 2005, Hassainya et al., 2005). It is based on the identification of peptides that are predicted to bind to a given MHC class I allele. Subsequently the immunogenicity of the identified peptides is studied by challenging $CD8^+$ T cells with APCs-loaded peptides. The basic steps of this method are shown in Figure 1.4. Nowadays other steps involved in the antigen processing and presentation by MHC class I pathway i.e. proteasome processing and TAP translocation, can be also predicted. Table 1.4 list algorithms that have been established for prediction of either one step of the presentation pathway or a combination of these steps. Among these the bioinformatics and molecular analysis section (BIMAS) and the SYFPEITHI database are the most commonly used algorithms (Kessler and Melief, 2007).

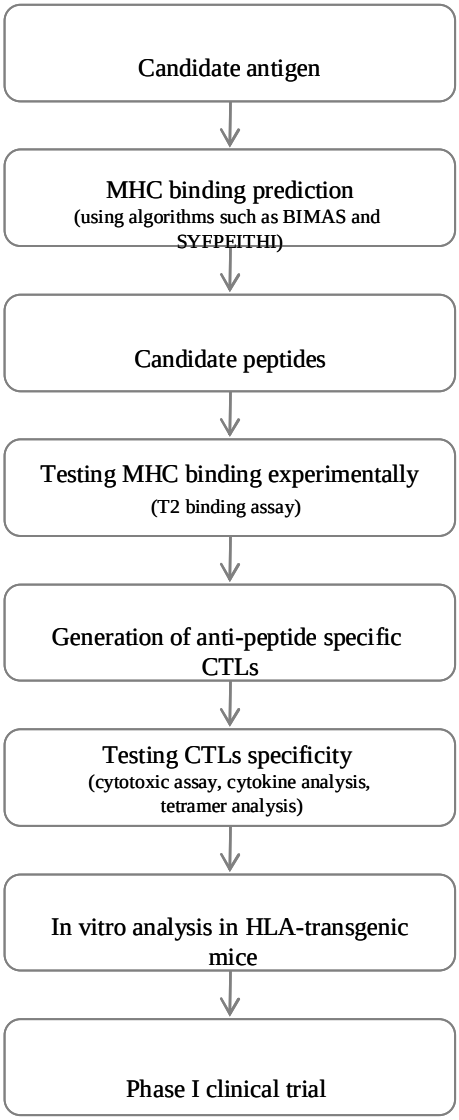

Figure 1.4. The basic steps of reverse immunology approach for the identification of immunogenic epitopes.

A candidate antigen is selected and screened for peptides that bind well to a particular class I allele using computer programs. The predicted peptide sequences are synthesized and their binding affinities are measured using assays such as the T2 binding assay (see Materials and Methods, section 2.4). Peptides with high binding affinities are pulsed onto APCs and peptide-specific CTLs are generated in vitro. The generated CTLs are tested for their specificity and subsequently for their recognition of tumour cells expressing the antigen of interest. The next step is to test the immunogenicity of the epitope in HLA-transgenic mice. If the epitope gives positive results, candidate antigens can be classified as tumour antigen and tested in clinical phase I trials. Figure adapted with modifications from (Schultze and Vonderheide, 2001).

Table 1.4. Internet-based prediction algorithms, adapted with modification from (Kessler and Melief, 2007).

Program	*Prediction of*			*URL*
	MHC class I	**Proteasome cleavage**	**TAP**	
BIMAS	+			http://bimas.dcrt.nih.gov/molbio/hla_bind
HLA-A2 (no name)	+			http://zlab.bu.edu/SMM/
IEDB (HLA class 1)	+			http://tools.immuneepitope.org/analyze/html/
MAPPP (combined)		+		http://www.mpiib-berlin.mpg.de/MAPPP/expertquery.html
MAPPP (Fragpredict)		+		http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html
MHC II (no name)	+			http://bioinfo.weizmann.ac.il/mhc2motifs/
MHC-BPS	+			http://bidd.cz3.nus.edu.sg/mhc/
MHCPred			+	http://www.jenner.ac.uk/MHCPred
Multipred	+			http://research.i2r.a-star.edu.sg/multipred/
NetChop		+		http://www.cbs.dtu.dk/services/NetChop
NetMHC	+			http://www.cbs.dtu.dk/services/NetMHC
nHLAPred	+			http://www.imtech.res.in/raghava/propred1/index.html
PAProC		+		http://paproc.de
Pcleavage		+		http://www.imtech.res.in/raghava/pcleavage/
PREDEP	+			http://margalit.huji.ac.il/
PREDTAP			+	http://antigen.i2r.a-star.edu.sg/predTAP/
ProPred-I	+	+		http://www.imtech.res.in/raghava/propred1/index.html
RANKPEP	+	+		http://bio.dfci.harvard.edu/Tools/rankpep.html
SVMHC	+			http://www-bs.informatik.uni-tuebingen.de/SVMHC/
SVRMHC	+			http://svrmhc.umn.edu/SVRMHCdb/
SYFPEITHI	+			http://www.syfpeithi.de
TAPPred			+	http://imtech.res.in/raghava/tappred/

1.4.5. Modalities of T-cell-mediated immunotherapy

The identification of several leukaemia antigens that can be specifically recognized by antigen-specific T cells, has led to the development of immunotherapeutic regimens using T cells. There are two types of T-cell-mediated immunotherapy: the passive adoptive T-cell transfer and active immunostimulatory vaccination (Kessler and Melief, 2007). The widely used form of adoptive transfer of T cells is the infusion of unmanipulated donor lymphocytes to treat leukemic patients who have relapsed after allogeneic HSCT. This therapy is curative in a significant percentage of patients (Kolb et al., 1995). The other form of adoptive therapy utilizes the identification and expansion of autologous or allogeneic antigen-specific cells that specifically target leukemic cells, followed by administration to cancer patients (Rosenberg et al., 2008). T cells specific for leukaemia antigens can be isolated from the leukaemia patient or can be generated in vitro using leukaemia antigen-loaded APCs (Amrolia et al., 2003). Another method is the transfer of a TCR gene specific for leukaemia antigen, such as WT1 via lymphocyte transfection (Stauss et al., 2008). Active vaccination based on the use of either defined or undefined tumour antigens, include the following strategies:

1.4.5.1. Peptide-based vaccines

Peptide-based vaccines deliver a peptide or multiple synthetic peptides of tumour antigens that can be recognized by the patient immune system in an MHC-restricted manner. Several phase I and II clinical vaccine trials that investigate the clinical significance of MHC class I-restricted peptides derived from BCR-ABL, WT1, PR-3 as well as RHAMM have been performed in leukaemia patients and have showed some encouraging results (Cathcart et al., 2004, Qazilbash et al., 2004, Oka et al., 2004, Mailander et al., 2004, Schmitt et al., 2008, Pinilla-Ibarz et al., 2000). A number of strategies have been established to improve this type of vaccination. These strategies include injection of a peptide vaccine together with an adjuvant such as montanide or peptide loaded onto DCs (Osman et al., 1999b, Qazilbash et al., 2004, Pinilla-Ibarz et al., 2000). It is well known that a $CD4^+$ T-helper response is required for robust and persistent $CD8^+$ T-cell responses (Janssen et al., 2003, Kumaraguru et al., 2004). Therefore, a tumour-specific Th1 epitope can be incorporated along with the CTL epitope to enhance the CTL-mediated tumour

destruction (Smits et al., 2009). Another strategy known as epitope enhancement has also been used. Epitope enhancement strategies rely on the use of heteroclitic (modified) peptides. These peptides are synthetic variants of the natural peptide sequence with the same HLA-binding specificity and avidity, but with enhanced affinity to class I MHC molecules or to TCR (Smits et al., 2009). This strategy has been used to enhance the immunogenicity of peptides derived from leukaemia antigens such as BCR-ABL, CD33 and WT1 as well as peptides derived from melanoma associated antigens such as MART-1/Melan-A and gp100 (Tsuboi et al., 2002, Pinilla-Ibarz et al., 2005, Valmori et al., 1998, Parkhurst et al., 1996, Bae et al., 2004a).

1.4.5.2. Full-length tumour antigen vaccines

Recombinant viral vectors or naked DNA plasmids that encode defined full-length tumour antigens are used to deliver full-length tumour antigen vaccines (Kessler and Melief, 2007). Viral vectors have the ability to initiate immune responses with inflammatory reactions occurring as a result of the viral infection. According to the danger theory (Matzinger, 1994), viral infection should attract professional APCs necessary for antigen presentation (Dermime et al., 2002). In a mouse model, vaccination with adenovirus encoding the WT1 antigen was shown to elicit potent anti-WT1 responses, protect mice from leukemic cell challenge and retarded tumour growth (Osada et al., 2009). A DNA-based vaccine targeting the leukaemia antigen PML-RARα in a mouse model induced protective immune responses against leukaemia progression when combined with the conventional ATRA therapy (Padua et al., 2003).

1.4.5.3. Whole-tumour cell vaccines

Whole-tumour cell vaccines are among the earliest forms of cellular therapy that has been used to vaccinate cancer patients. It usually consisted of autologous irradiated tumour cells or tumour-derived lysates. In order to induce the required immune response in tumour-bearing patients, tumour-cell vaccines were mixed with bacterial adjuvants such as Bacillus Calmette-Guerin (BCG) or cytokines such as IL-2 and GM-CSF (Powles et al., 1975, Powles et al., 1977, Zhang et al., 2005). Nowadays, tumour cells can be genetically modified to express high levels of co-stimulatory molecules, such as CD80 and /or cytokines, such as IL-2 and GM-CSF (Chan et al.,

2005, Stripecke et al., 2000). When using this kind of vaccination, all antigens that could serve as targets for the immune system will be included in the vaccine without the need for their prior identification. However, the main limitation for this approach is the need for a personalized vaccine. This problem has been solved by the use of allogeneic tumour-cell vaccination in which cell lines derived from the tumour type which is the therapeutic target can be established in vitro, genetically modified, and used as a single reagent (Dermime et al., 2002).

1.4.5.4. Dendritic cell-based vaccines

Dendritic cells represent the most powerful APCs capable of priming effective T cell responses (Banchereau and Steinman, 1998). Immature DCs constitutively express high levels of MHC molecules, costimulatory and adhesion molecules and possess an extraordinary capacity to capture and process antigens. Upon maturation, DCs are able to stimulate $CD4^+$ T cells via HLA class II and $CD8^+$ T cells via interaction of HLA class I-a process known as cross-priming. DCs also stimulate NK cells and B cells (Banchereau and Palucka, 2005). Besides these properties, the possibility to generate large numbers of functional human DCs ex vivo has opened the way to use these cells in vaccination trials. Two general strategies have been used to obtain human DCs for vaccine therapy: immature DC precursors can be purified from peripheral blood or DCs can be derived in vitro from peripheral blood $CD14^+$ monocytes or $CD34^+$ hematopoietic stem cells using GM-CSF and IL-4 (Borrello and Sotomayor, 2002). Different approaches for the development of DC-based vaccines have been used in leukaemia patients and many of which were effective in inducing anti-leukaemia immune responses. DCs were loaded with peptides derived from the leukaemia antigens PML-RARα, or transfected with mRNA encoding WT1 antigen (Osman et al., 1999b, Smits et al., 2009). DCs were also loaded with undefined leukaemia antigens derived from apoptotic or necrotic leukaemia cells, leukaemia cell lysates or acid eluted peptides (Delluc et al., 2007, Weigel et al., 2006, Jarnjak-Jankovic et al., 2005, Lee et al., 2004, Spisek et al., 2002). Leukaemia cells induced to express DCs properties by differentiation, fusion or genetic transfer have been also used (Klammer et al., 2005, Roddie et al., 2000, Roddie et al., 2006). Importantly, these leukemic cells maintain expression of leukemic antigens but also have some immuno-stimulatory features of DCs.

1.5. Aims of this Study

In order to improve the T-cell-based immunotherapeutic approaches for leukaemia patients, this study aimed to enhance the immunogenicity of previously identified epitopes derived from WT1 antigen as well as to identify novel potential epitopes derived from other leukaemia-associated antigens such as MPP11 and PR-3.

The $Db126_{126\text{-}134}$ (126N=RMFPNAPYL) and $WH187_{187\text{-}195}$ (187N=SLGEQQYSV) are HLA-A*0201-resticted epitopes derived from the WT1 antigen. These epitopes have been shown to elicit specific CTL responses. The aim of the first part of the study was to improve the immunogenicity of these epitopes using an epitope enhancement approach consisting of replacing the first amino acid of the epitope sequence with a tyrosine. The modified peptides may then display stronger binding affinities to the HLA-A*0201 molecule and enable a more efficient trigger of peptide-specific CTLs, recognizing the naturally occurring epitope. The CTL lines will be generated by in vitro stimulation of CD8+ T cells, purified from normal human HLA-A*0201donor, using autologous-mature DCs loaded with the WT1-derived peptides. The immunogenicity of the CTLs generated against each analogue peptide will be compared with that of the native sequence using cytotoxicity and ELISPOT assays against WT1-loaded autologous LCLs and T2 cells and HLA-matched leukaemia cells. Importantly, the CTLs specific for the analogue peptides will be tested for their ability to recognize the native sequences.

Our group has successfully generated Treg clones specific for a peptide derived from WT1 antigen, WT1-84-333-347 (RYFKLSHLQMHSRKH), in an HLA-DR04 restricted manner and showed the existence of this population in leukaemia patients. The aim of the second part of the study was to investigate whether Tregs specific for WT1-84 peptide could also be generated from other HLA-DR04-matched donors. The T cell lines will be generated by in vitro stimulation of PBMCs obtained from three normal HLA-$DR04^+$ donors using autologous-mature DCs loaded with the WT1-Pepmix. The proliferative activity of the generated T cell lines will be tested for their ability to recognize WT1-84 peptide using proliferation assay against WT1-Pepmix/ WT1-84 peptide loaded autologous APCs.

The third part of the study aimed to identify novel $CD8^+$ CTL epitopes in the gene sequence of MPP11 and PR-3 which may bind to the HLA-A*0201 molecule and provoke specific CTL responses. Identification of such novel CTL epitopes derived from these leukaemia-associated antigens is a crucial step for the development of leukaemia-specific therapies. These epitopes will be used as targets for the generation of antigen specific T-cells which subsequently could be utilized in adoptive therapy or the development of vaccines efficient in eradicating minimal residual leukemic cells in leukaemia patients expressing elevated levels of MPP11 and/or PR3. The identification of the novel CTL epitopes will be carried out by screening the amino acid sequence of MPP11 and PR-3 antigens for potential HLA-A*0201 binding peptides using epitope prediction programs. CTL lines will be generated against candidate peptides by in vitro stimulations of $CD8^+$ T cells, purified from normal human HLA-A*0201donors, using APCs-loaded peptides. The generated CTL lines will be tested for the ability to generate anti-peptide specific responses using cytotoxicity and ELISPOT assays against peptide-loaded target cells and HLA-matched leukaemia cells.

2. MATERIALS AND METHODS

2.1. Patients and Donors

All samples were obtained with informed consent at King Faisal Specialist Hospital and Research Centre (KFSH&RC), Saudi Arabia. Blood samples were obtained from patients with AML and ALL patients. Information such as the percentage of the blast cells in the patients' blood samples as well as the patients' status were obtained from the clinic. Leukocyte-rich Buffy coats not older than eight hours were obtained from healthy volunteers from the Blood Bank.

2.2. Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs from leukaemia patients and healthy donors were isolated from heparinized blood by density gradient centrifugation over Ficoll–Paque PLUS (Amersham Biosciences) according to the manufacturers' instructions. Briefly, blood samples were diluted with 2-4 volumes of phosphate-buffered saline containing 0.5% fetal calf serum (PBS/FCS).Each sample was then carefully layered over a Ficoll-Paque Plus layer in sterile centrifuge tubes. The samples were centrifuged at 2000 rpm. The interface cells (PBMCS i.e. lymphocytes and monocytes) were aspirated and placed in a sterile centrifuge tube and washed in cold PBS/FCS. The cells were further washed twice and cryopreserved by resuspending in 90% FCS $^{+}$ 10% DMSO, and aliquoted into freezing containers (Nalgene cryogenic vials) for 2h at –80˚C and then in liquid nitrogen at -192°C until further processing. The HLA typing of these samples was carried out at the routine Immunopathology Laboratory.

2.3. T lymphocyte Epitope Prediction and Peptide Synthesis

2.3.1. WT1-pepmix

A pool of 110 peptides spanning the entire length of the WT1 protein designated as WT1-Pepmix and a micro-scale WT1 peptide set containing each peptide in a single well were obtained from JPT Peptide Technologies (Jerini AG, GmbH) with a purity of more than 70% by HPLC. The peptides were 15 amino acids long with an overlap of 11 amino acids.

2.3.2. WT1 peptides

A tyrosine (amino acid substitution in the sequence of the following 9-mer WT1 peptide) was carried out at the N-terminus in: Peptide Db126 for arginine (wild type 126N126-134 = RMFPNAPYL, analogue 126Y = **Y**MFPNAPYL), and in peptide WH187187-195 for Serine (wild type 187N = SLGEQQYSV, analogue 187Y = **Y**LGEQQYSV). We compared the predictive binding ability of the wild-type (126N and 187N peptides with their corresponding analogues (126Y and 187Y) to the HLA-A*0201 molecule using a computer-based epitope prediction program, BIMAS. The score predicted by BIMAS algorithm is based on the calculation of the theoretical half-time of the peptide dissociation to HLA class I molecules (Parker et al., 1994).

Individual 15-mer WT1 peptides used were WT1-60 $_{237\text{-}251}$ (TMNQMNLGATLKGVA), WT1-61$_{241\text{-}255}$ (MNLGATLKGVAAGSS), WT1-79$_{313\text{-}327}$ (SASETSEKRPFMCAY), WT1-80$_{317\text{-}331}$ (TSEKRPFMCAYPGCN), WT1-84$_{333\text{-}347}$ (RYFKLSHLQMHSRKH), and WT1-96$_{381\text{-}395}$ (KPFQCKTCQRKFSRS). In addition to peptides derived from the WT1 sequence, hTert$_{672\text{-}686}$ (RPGLLGASVLGLDDI) HLA-DR*07–restricted peptide derived from the human telomerase reverse transcriptase (hTRT) (Schroers et al., 2002), was obtained and used as a positive control.

2.3.3. MPP11 and PR-3 peptides

MPP11 peptide sequences predicted to bind to HLA-A*0201 molecule were obtained using the SYFPEITHI prediction program. For PR-3, peptide sequences predicted to bind to HLA- A*0201 molecule were obtained using three computer-based epitope prediction programs (BIMAS, SYFPEITHI and RANKPEP).The SYFPEITHI prediction algorithm is a database comprising more than 4500 peptide sequences known to bind class I and class II MHC molecules. The scoring system of the SYFPEITHI program is based on the presence of certain amino acids in certain positions along the MHC-binding groove (Rammensee et al., 1999). RANKPEP server prediction is based on the similarity of an entered protein sequence to a set of peptides known to bind to a given MHC molecule. The degree of similarity is scored using a Position Specific Scoring Matrixes (PSSM) derived

from the aligned peptides that are known to bind to a particular MHC molecule (Reche et al., 2002).

The amino acid sequences of MPP11 and PR-3 were entered into the specified programs and candidate epitopes were selected based on their predicted ability to bind to the HLA- A*0201 molecule. Four 9-mer peptides derived from MPP11 and two 9-mer peptides derived from PR-3 were selected as potential epitopes for the generation of CTL responses. The four MPP11-derived peptides were: MPP-$1N_{421-429}$ (QLLIKAVNL), MPP-$2_{419-427}$ (DLQLLIKAV), MPP-3_{13-21} (AIMLLLPSA) and MPP-4_{37-45} (STLCQVEPV). The PR-3-derived peptides were: PR-$129_{129-137}$ (NLSASVATV) and PR-9_{9-17} (ALASVLLAL). In addition to the predicted peptides, we modified the sequence of the first predicted peptide derived from MPP11 by introducing a Y at its N-terminus (wild type MPP-1N= QLLIKAVNL, analog MPP-1Y= **Y**MFPNAPYL). Furthermore, PR-$169_{169-177}$ (VLQELNVTV), a previously described HLA-A*0201 epitope derived from PR-3 antigen, was used as a positive control (Molldrem et al., 1996). The amino acid sequences of the nonameric peptides with a purity of ≥90% were synthesized and purified by Alta Bioscience (Birmingham University, UK).

2.4. T2 Binding Assay

Peptide binding capacity to the HLA-A*0201 molecule was measured using the TAP-deficient, HLA-A*0201-positive cells as described previously (Molldrem et al., 1996). T2 cells are a hybrid human cell line produced by fusion of a B lymphoblast line 174 with a T lymphoblast line CEM. The cell line has a homozygous deletion of the MHC class II region on chromosome 6 including the known transporter proteins for antigenic peptide TAP1 and TAP2 genes and proteasome genes. They contain the gene HLA-A*0201, but express very low levels of cell-surface HLA-A*0201 and are unable to present endogenous antigens (Zweerink et al., 1993). Stable HLA-A*0201 expression in T2 cells is observed only when peptides that are capable of binding to and stabilizing the cell surface expression of the HLA-A*0201 molecule are added exogenously. The principle of the assay is as illustrated in Figure 2.1. Briefly, T2 cells were washed three times, suspended at 10^6 cells/mL and incubated for 18 hours in serum-free medium

containing different concentrations of peptides and 1 μg/mL β2-microglobulin (Sigma). Cells were then washed twice with cold FACS buffer (PBS containing 2% FCS). Purified rabbit IgG (Sigma) was added to the cell suspension and incubated for 15 minutes on ice in order to block the FC receptors. FITC-conjugated mouse anti-HLA-A2 antibody (clone: BB7.2; AbD Serotec) was added to the cell suspension (1μg/10^6 cells) and incubated for 30 minutes at 4°C in the dark. The cells were washed twice with cold FACS buffer and fixed with PBS/4% paraformaldehyde. The level of HLA-A2 expression was analyzed using fluorescence-activated cell sorter (FACS) Scan (Becton & Dickinson, Immunocytometry Systems, CA, USA). HLA-A2 expression was quantified as fluorescence index (FI) according to the following formula: fluorescence index = (median fluorescence intensity with peptide - median fluorescence intensity without peptide)/ median fluorescence intensity without peptide. All T2 binding assays were carried out in duplicate.

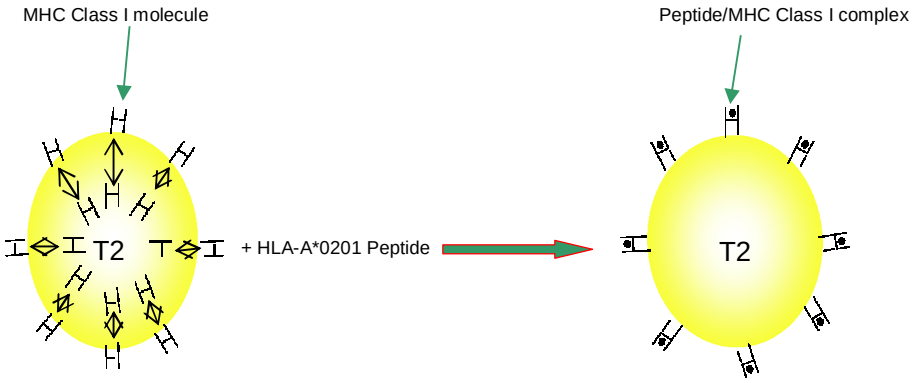

Figure 2.1. Principles of T2 binding assay.

T2 cells are incapable of presenting endogenously synthesized antigens due to a deletion in genes that encode the transporter associated with antigen processing (TAP1 and TAP2). Therefore, the HLA-A2 molecules expressed by these cells are unstable and disassociate in the absence of peptides. The stability of HLA-A2 molecules at the cell surfaces can be achieved by adding exogenous peptides with high affinity for HLA-A2. The amount of stable HLA-A2 can be followed using an anti-HLA-A2 monoclonal antibody and quantified by flow cytometry.

2.5. Culture and Generation of Target Antigen Presenting Cells (APCs)

2.5.1. Cell lines

The mutant TAP-deficient cell line T2 (ATCC number CRL-1992) and the HLA-class I and II negative human CML cell line K-562 (ATCC number CCL-243) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). K-562 cells transfected with HLA-A*0201 gene were kindly provided by Dr. Wolfgang Herr (University of Mainz, Germany). The T2 and K562 cells were maintained in complete medium (CM) consisting of RPMI 1640 medium (Sigma, MO, USA) supplemented with 10% Fetal Calf Serum (FCS) (Cambrex Bio Science, MD, USA), 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/mL streptomycin (Sigma). K562/A*0201 were cultured in CM containing 0.5 mg/mL geneticin (G418, Sigma).

2.5.2. Generation of B-lymphoblastoid cell lines

B-lymphoblastoid cell lines (LCLs) were established from normal donors by transformation of B cells using Epstein-Barr virus (EBV) according to a standard technique (Neitzel, 1986). The EBV virus stock was produced by the B95.8 cell line and was kindly provided by the Derartment of Genetics (Research Centre, KFSH&RC). Briefly, PBMCs (10^7 cells) were incubated with the virus (500 µL) for an hour at 37ºC. Then cells were cultured in CM containing 1µg/mL PHA (PHA-P, Sigma) and 0.1 ug/mL cyclosporin A and maintained by twice-weekly refreshing of CM. Established LCLs were used after 3-4 weeks of culture.

2.5.3. Generation of PHA blast

PHA blasts were prepared as described previously (Atanackovic et al., 2003). PBMCs were seeded onto 24-well plates (BD Biosciences, USA) at a density of 1-2 x 10^6 cells /well into a 24-well culture plate in T cell culture medium. The T cell culture medium consisted of X-vivo 15 medium (BioWhittaker, MD), supplemented with 10% heat-inactivated human AB serum (Sigma). A concentration of 10 µg/mL phytohemagglutinin (PHA) was added to the culture on day 0. Half of the medium was replaced with a complete medium containing 20 IU/mL recombinant human interleukin-2 (rhIL-2; eBioscience) and 40 ng/mL rhIL-7 on day 3, and this step was

subsequently repeated every 3 days. The activated T cell-APCs (T-APCs) were checked for class II, CD80, and CD86 expression and then used as target cells around day 20 of the culture.

2.5.4. Generation of mature dendritic cells from autologous monocytes

2.5.4.1. Isolation of monocytes for subsequent dendritic cell generation

Monocytes ($CD14^+$ cells) were isolated from PBMCs of healthy HLA-A*0201 positive donors either by negative selection using a MACS human Monocyte Isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) or by plastic adherence. Monocyte magnetic isolation was done according to the manufacturer's instructions. Briefly, 10^7 PBMCS were suspended in 30 µL of ice-cold MACS buffer (PBS supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA) and 10 µL of FcR blocking reagent (human Ig). Non-$CD14^+$ T cells were indirectly magnetically labelled by incubating the cells with 10 µL of biotin-conjugated antibodies cocktail against CD3, CD7, CD16, CD19, CD56, CD123, and Glycophorin A. After 10 minutes of incubation at 4–8°C, 20 µL of anti-biotin micro beads (clone: Bio3-18E7.2; mouse IgG1) and buffer were added. Following an additional 15 minutes of incubation at the same temperature as above, cells were washed and resuspended in 500 µL of buffer. The MS column (Miltenyi Biotec) were prepared by placing it in the magnetic field of a MACS separator and rinsing with 500 µL of MACS buffer. The cell suspension was applied onto columns and allowed to pass through and effluent was collected (unlabeled cells), representing the enriched monocyte fraction.

For monocyte isolation by plastic adherence, PBMCs were cultured at 5 x 10^6 cells/mL/well of a 6-well plate (Sigma) in X-vivo 15 medium supplemented with 2 mM L-glutamine (DC-medium), and allowed to adhere in a 5% CO_2 incubator at 37°C for 90-minutes. Non-adherent cells and media were removed and fresh DC-medium was added to the cells. After a second incubation period, non-adherent cells and media were removed and the adherent cells were washed carefully with pre-warmed medium. The isolated monocytes were resuspended in fresh DC-medium.

2.5.4.2. Generation of mature conventional dendritic cells

The isolated monocytes were cultured in six-well plates in DC-medium in the presence of 50 ng/mL rhIL-4 and 100 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF; R&D Systems, MN). Immature dendritic cells (DC) were stimulated into maturity by adding 20 ng/mL recombinant human tumour necrosis factor- α (rhTNF-α; R&D Systems), 10 ng/mL rhIL-1β (eBioscience, CA) and 25 mg/mL polyinosinic acid:poly-CMP (Poly I:C; Sigma) on day 6 and mature dendritic cells (DCs) were harvested on day 7.

2.5.4.3. Generation of mature fast dendritic cells

Fast dendritic cells (DC2d) were generated according to previously published protocols (Dauer et al., 2003, Ho et al., 2006) with minor modifications. Briefly, DC-medium supplemented with 100 ng/mL rhGM-CSF and 50 ng/mL rhIL-4 was added to monocytes cultured in 6-well plates. Maturation cytokines, 10 ng/mL rhTNFα, 10 ng/mL rhIL-1β, 10 ng/mL rhIL-6 (R&D Systems) and 1 μM PGE2 (Sigma), were added to the cultures on the next day. Cultures were incubated for an additional day before mature DC2d were harvested.

2.6. Generation of T lymphocyte lines

Different approaches have been used to generate $CD8^+$ T lymphocyte lines. These approaches include the use of mature conventional DCs or mature fast DCs to prime naïve $CD8^+$ T cells. Two rounds of T cell-stimulation were sufficient to prime the T cells. Following priming, DCs or monocytes were used to expand the generated T cell lines. Another approach employed the use of T2 cells to prime and to expand naïve $CD8^+$ T cells. A single approach was used to generate $CD4^+$ T lymphocyte lines. In this approach, mature conventional DCs were used to prime naïve $CD4^+$ T cells and monocytes or T-APCS were used to expand the generated T cell lines.

2.6.1. Isolation of $CD8^+$ T lymphocytes

$CD8^+$ T lymphocytes were negatively separated from PBMCS obtained from healthy HLA-A0201 positive donors using MACS $CD8^+$ T Cell Isolation Kit II (Miltenyi Biotec). Following the manufacturer's protocols, 10^7 PBMCS were suspended in 40

µL of ice-cold MACS buffer. Non-$CD8^+$ T lymphocytes were indirectly magnetically labelled by incubating the cells with 10 µL of a cocktail of biotin-conjugated antibodies against CD4, CD14, CD16 CD19, CD36, CD56, CD123, TCR γ/δ and Glycophorin A. After 10 minutes of incubation at 4–8°C, 20 µL of anti-biotin microbeads (clone: Bio3-18E7.2; mouse IgG1) and buffer were added. After an additional 15 minutes of incubation at the same temperature, cells were washed, resuspended in 500 µL of buffer, and applied to the prepared MS columns (Miltenyi Biotec). The effluent (unlabeled cells) was collected, representing the enriched $CD8^+$ T cell fraction.

2.6.2. Generation of anti-peptide specific $CD8^+$ T lymphocytes

Some of the $CD8^+$ T cell lines were generated using protocol adapted from previous studies (Molldrem et al., 1996, Houbiers et al., 1993). Briefly, T2 cells were washed three times in serum-free medium and incubated with each peptide at concentrations of 50 µM/mL and 1 µg/mL β2-microglobulin at 37 °C, 5% CO_2 for 2 hours. The peptide-pulsed T2 cells were then irradiated with 7500 rad, washed once and added to freshly purified $CD8^+$ in T lymphocyte culture medium (X-vivo 15 medium supplemented with 2 mM L-glutamine and 10% human serum) at a T lymphocyte: T2 ratio of 5:1. After 7-10 days of T cell stimulation, the second stimulation was performed and on the following day, 20 IU/mL of rhIL-2 was added. The cultured T lymphocytes were maintained by restimulations with peptide-pulsed T2 cells. After the third in vitro stimulation, peptide-stimulated cells were tested for their cytotoxicity using chromium release assays. DC-loaded peptides were used to in vitro prime some of the purified $CD8^+$ T lymphocytes. Briefly, the mature DCs growing in DC medium were harvested, washed once with the same medium and incubated with the peptides at concentration of 50 µM/mL and 5 µg/mL β2-microglobulin for 2 hours at 37 °C, 5% CO_2. The peptide loaded DCs were then X irradiated (2500 rads), washed once and added to the $CD8^+$ T lymphocytes at 20:1 to 5:1 T lymphocytes: DCs ratio. rhIL-7 (20 ng/mL) and rhIL-12 (100 pg/mL) were added to each culture. The cells were incubated in 2 mL T cell culture medium in 24-well plates. After 7-10 days, T lymphocyte were harvested, washed and re-stimulated with peptide loaded DC2d as previously described and rhIL-2 (20 IU/mL) was added the following day. The cultured T lymphocytes were maintained

by re-stimulation with autologous peptide-loaded antigen presenting cells (DCs or freshly prepared adherent monocytes) and fed with rhIL-2 the next day. T lymphocyte lines against the different peptides were generated. Responder T lymphocytes were tested for their specificity and cytotoxic activity using Enzyme-Linked Immunospot Assay (ELISPOT) Assay and Chromium Release Assay against different targets.

2.6.3. Generation of anti-peptide specific $CD4^+$ T lymphocytes

Irradiated DCs (2500 rads) were pulsed with 10 μg/mL WT1-Pepmix in DC medium and incubated for 4 h at 37°C, 5% CO_2. DCs were co cultured with autologous PBMCs at a 10:1 T-cell to DC ratio. T cell culture medium containing 10 ng/mL rhIL-7, and 20 pg/mL rhIL-12 (R&D Systems) was used. After 7-10 days of co-culture, a similar second stimulation was performed and 260 IU/mL rhIL-2 was added 2 days after re-stimulation. T cells were re-stimulated with autologous peptide-loaded cells (freshly prepared adherent monocytes or T-APC) and 260 IU/mL rhIL-2 was added 2 days after each re-stimulation.

After several rounds of antigen-specific re-stimulation, dynabeads CD3/CD28 T cell expander (Invitrogen Dynal, USA) was used to activate T cells according to the manufacturers' protocol. The beads are superparamagnetic polystyrene beads coated with monoclonal antibodies against the CD3 and CD28 cell surface molecules of T cells. For T cell stimulation, 1 bead/5-10 T cells was added to T cells cultured in T cell culture medium containing 260 IU/mL rhIL-2. Cells were expanded for up to 2 weeks before antigen-specific re-stimulation was repeated.

2.7. Generation of T Lymphocyte Clones

2.7.1. Cloning of WT1 T cell line

2.7.1.1. T cell cloning by limiting dilution assay

WT1-TCL57 was cloned using a limiting dilution method as mentioned above except that HLA-matched allogeneic LCLs were used to stimulate the T cell line. The T cells were seeded at 1, 2 and 5 cells/well/200 μL in T cell culture medium in 96-well U-bottomed plates. Allogeneic PBMCs were irradiated with 2500 rad and

used as a feeder layer (25 x 10^3 cells/well). LCLs were incubated separately in X-Vivo 15 with 50 μM/mL of WT1-peptides for 2 hr at 37°C. LCLs were then irradiated with 7500 rad, washed once and used for T cell stimulation (50 x 10^3 cells/well) in the presence of 130 IU/mL of rhIL-2.

2.7.1.2. T cell cloning using dynabeads

The T cells were seeded at 5 cells/well/200 μL T cell culture medium containing dynabeads CD3/CD28 T cell expander (1 bead/5-10 T cells) and rhIL-2 (130 IU/mL) in 96-well V-bottomed plates. The cultures were fed with fresh medium containing rhIL-2 (130 IU/mL) every 3-4 days and kept for 2-4 weeks. Growing clones were transferred to 96-U-bottomed plates and re-stimulated with irradiated peptide-pulsed T-APC. Clones were tested for their specificity against target APC-pulsed with or without WT1-peptides (WT1-60, WT1-61, and WT1-80) using the proliferation assay.

2.7.2. Cloning of MPP11 T cell line

T lymphocytes lines were cloned by limiting dilution as described before (Dermime et al., 1996, Ho et al., 2006) with minor modifications. T lymphocytes were seeded at 1, 2 and 5 cells/well/200 μL T cell culture medium in 96-well U-bottomed plates. Allogeneic PBMCs were X-irradiated (2500 rads) and used as a feeder layer (25 x 10^3 cells/well). T2 cells were incubated separately in X-Vivo 15 (5 X 106 cells/mL) with 5 μg/mL β2-microglobulin and 50 μM/mL of MPP-4 peptide for 2 hours at 37°C. T2 cells were then irradiated (7500 rads), washed once and used for T cell stimulation (50 x 10^3 cells/well) in the presence of 130 IU/mL of rhIL-2. 5ng/mL rhIL15 (R&D Systems) and 30ng/mL OKT3 were also added to the wells. The plates were incubated for 2-4 weeks. Cultures were re-fed with fresh medium containing rhIL-2 (130 IU/mL) every 3-4 days. Wells with positive signs of growth were selected for expansion. Growing clones were transferred to 96-well flat-bottomed plates, and each clone was stimulated with irradiated peptide- pulsed T2 cells. The activation cycle was repeated after 7-10 days of culture at lymphocyte: T2 ratios of 20:1 to 5:1. Clones were screened for their lytic activity against T2-pulsed with or without peptide using a Chromium Release Assay.

2.8. Flow Cytometry Analysis

Cells were collected into FACS tubes (Falcon) and washed with cold FACS buffer. Purified rabbit IgG (Sigma) was added to the cell suspension and incubated for 15 minutes on ice in order to block the FC receptors. Fluorochrome-conjugated mouse anti-human antibody was added to the cell suspension (1 μg/10^6 cells), with a control sample labelled with isotype-matched monoclonal antibody. The cells were incubated on ice for 30-60 minutes in the dark, washed twice with cold FACS buffer, fixed with 300 μl of PBS/4% paraformaldehyde and analyzed using FACS Scan. The following antibodies were purchased from AbD Serotec, NC, anti-CD14-FITC (clone: TuK4). Antibodies purchased from Dakocytomation, CA, were anti-CD8-FITC (clone: DK25), anti-CD3-APC (clone: UCHT1) and anti-CD4-FITC (clone: MT310). Anti-CD1a-FITC (clone: HI149) and anti-CD83-PE (clone: HB15e) were obtained from BD Biosciences, USA. In addition, Tregs were phenotyped using a kit obtained from eBioscience and used according to the manufacturer's instructions. Briefly, the T cells were stained with a cocktail of anti-CD4-FITC and anti-CD25-PE, fixed, permeabilized using the Foxp3 staining buffers and subsequently stained with anti-Foxp3-APC. Analyses were performed using FACS Scan (Becton & Dickinson, Immunocytometry Systems, CA, USA).

2.9. Cytotoxicity Assay (Chromium Release Assay)

Cytotoxicity assays were performed 5 days after the in vitro stimulation. Target cells were removed from culture, washed in RPMI1640 serum-free medium, re-suspended in a minimal volume (± 50 μL) of RPMI1640 and incubated with Cr^{51} (100 μCi) per target for 90 minutes. Target cells labeled with peptide were concurrently incubated with the appropriate peptide at 50 μM/mL. The cells were then washed and placed in 96 V bottomed wells at 10^3 cells/100 μL/well. The effector T lymphocytes were washed, and added in triplicate at varying quantities to the target cells, to give varying effector to target (E:T) ratios in a final volume of 200 μL/well. The plates were spun for 5 minutes (300g) and incubated for 4 hours at 37 ˚C and 5% CO_2 before 100 μL supernatants were removed and transferred to 1450 Microbeta Plus Wallac plates (Wallac, Turku, Finland). 150 μL scintillation

fluid, Optiphase HiSafe 2 (Wallac), was added to each well and the plates were heat-sealed using a Microsealer system (Wallac).

Chromium release was assessed by a liquid scintillation counter (Wallac). Target cells were also incubated with 0.2% Tween 20 or medium alone to assess the maximum and minimum (spontaneous) release of the chromium respectively. Spontaneous release was maintained and never exceeded 20% of the maximum release. The percentage of specific lysis was calculated as: % Specific lysis = (experimental release - spontaneous release)/(maximal release - spontaneous release) x100. In some of the experiments, 10 µg/mL anti-class I ABC blocking antibody (clone: W6/32, AbD Serotec) was added to target cells before T lymphocytes addition.

2.10. T Cell Proliferation Assay

T-cell proliferation was assessed by tritiated thymidine ([^{3}H]-thymidine) incorporation. T cells were seeded in duplicate or triplicate wells of a 96-well U-bottomed plate. T cells were cultured with different stimulators at T cells: APCs ratio of 1:2 in a final volume of 200 µl of T cell culture medium for 3 d. [^{3}H]thymidine (1 µCi per well; Amersham) was added 18 hours before harvest and cells were subsequently harvested onto glass fiber filters (Wallac); [^{3}H]thymidine incorporation was measured using a 1450 Micro Beta PLUS liquid scintillation counter (Wallac). In some of the experiments, 60 µg/mL of the following purified and azide-free blocking antibodies were used: anti–HLA-DR (clone: G46-6, BD Biosciences), anti–HLA-DP, and anti–HLA-DQ (clones: 1a3 and B7/21 respectively, Leinco Technologies). The anti-ABC antibody (AbD Serotec) was used at 10 µg/mL and mouse isotypes were used as controls.

2.11. Enzyme-Linked Immunospot Assay (ELISPOT)

ELISPOT assay was performed using IFN-γ, granzyme B and perforin kits (Mabtech, Mariemont, OH) according to the manufacturer's protocol. $5x10^3$ to $5x10^4$ T lymphocytes/well and 2 $x10^4$ to 10^5 cells/well of different stimulators were seeded in Multiscreen 96-well plates (Millipore, MA) pre-coated overnight (4˚C) with catching-antibody. The plates were then blocked with T lymphocyte culture

medium. After 40 hours incubation (37˚C, 5% CO_2), cells were removed and after washing, biotinylated monoclonal antibodies specific for IFN-γ, granzyme B, and perforin were added and incubated for 3 hours at room temperature (RT). After washing, Streptavidin-alkaline phosphatase (ALP) or Streptavidin-horseradish peroxidase (HRP) was added to each well and incubated at RT for 2 hours. After washing, the appropriate substrate (BCIP/NBT in the case of ALP and AEC in the case of HRP) was added to each well and incubated at RT until colour developed according to the manufacturer's instructions. Spots were counted using an automated ELISPOT reader (AID, Strasberg, Germany). Antigen-specific T cell frequencies were considered to be significantly higher if they were at least two-fold higher than in the control wells.

2.12. Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants from 48-h T cell cultures were harvested and stored in aliquots at -80˚C until used. ELISA for human IFN-γ, IL-4, IL-5, IL-10, GM-CSF ELISA kits (Mabtech) and transforming growth factor (TGF)-β1 matched antibody pairs (R&D Systems) was performed according to the manufacturer's instructions. Briefly, Nunc Maxisorp 96-well plates (eBioscience) were coated with the appropriate capture antibodies overnight. To start the assay, plates were washed and blocked with blocking buffers for 1 hr at room temperature. Diluted samples and standards were added and incubated for 2 hr. The plates were washed, incubated with biotin–conjugated detection antibodies, washed again and then incubated with Streptavidin-HRP and washed. Tetramethylbenzidine (TMB) substrate was added for colour development. After suitable time, colour development was stopped by adding 2M sulphuric acid. Optical density was measured in an ELISA reader (Anthosn Lucy 3) at 450 nm.

2.13. RNA Preparation

Total RNA was isolated from PBMCSs using Trizol method. According to the manufacturer's instructions, cells were lysed with 1 ml Tri reagent (Sigma). After mixing the lysed cells with chloroform, total RNA content was collected, precipitated with isopropanol, washed once with 75% ethanol, dissolved in

diethylpyrocarbonate (DEPC)-treated water and stored at -80˚C. The concentration and purity of the extracted RNA was determined by measuring the absorbance at 260 and 280 nm with a spectrophotometer.

2.14. Reverse Transcription (RT)

RNA from each sample was treated with DNase1 (Invitrogen, CA) and then reverse-transcribed in a 25-μL reaction volume. 1 μg RNA in DEPC-treated water was incubated at 65˚C for 5 minutes and then mixed with Reaction mix. Reaction mix included RT buffer (50 mM Tris-HCl, PH 8.5; 30 mM KCl; 8 mM MgCl2), 1 mM dithiothreitol (DTT), 100 μM of dNTP mix, 200 ng of Oligo-d(T) primers, 80 U of RNAse inhibitor, and 600 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reaction mixture was then incubated at 37˚C for 2 h, boiled for 5 minutes, placed quickly on ice and stored at -80˚C until use.

2.15. Conventional Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

The WT1 and MPP11 mRNA expression was quantified in PBMCs obtained from normal donors and leukaemia patients using conventional RT-PCR. 0.1 μg mRNA equivalent of cDNA was used. Sequences of the WT-1 and MPP11 primers used were based on previous studies (Greiner et al., 2003, Greiner et al., 2004). The amplification product of the house keeping gene β-actin served as control of cDNA quality. β-actin primers were designed using Oligo 6 software. Table 2.1and Table 2.2 display the sequences of the primers and PCR conditions, respectively. mRNA isolated from the K562 cell line served as a positive control for WT1 and MPP11 genes. Distilled water served as a negative control. The PCR products were electrophoresed on a 1% agarose gel. Expected band size was 1243 bp, 790 bp for MPP11 and WT1, respectively.

Table 2.1. Primer sequences

Antigen	Forward primer	Reverse primer
Mpp11	5'AAGATCATTATGCAGTTCTTGGAC 3'	5'CCAATAACATCTTTGGCAGTTCT3'
WT1	5'ATGAGGATCCCATGGGCCAGC 3'	5'CCTGGGACACTGAACGGTCCCCGA3'
β-actin	5'CCCAGCACAATGAAGATCAAGATCAT 3'	5'ATCTGCTGGAAGGTGGACAGCGA 3'

Table 2.2. PCR conditions

Antigen	Denaturation	Annealing	Elongation	Cycles	$MgCl_2$
MPP11	94˚C (1 min)	60˚C (1min)	72 ˚C (1 min)	28	1.5mM
WT1	94˚C (1 min)	64˚C (1 min)	72 ˚C (1 min)	30	1.5mM
β-actin	94˚C (1 min)	60˚C (1 min)	72 ˚C (1 min)	28	1.5mM

2.16. Quantitative Real Time RT-PCR for WT1 Expression in Patients' Leukemic Cells

The WT1 mRNA expression was quantified using Light Cycler FastStart DNA master SYBR Green 1kit (Roche, Mannheim, Germany) in a Light Cycler (Roche). PBGD was used as house-keeping gene. 2 μL cDNA from the K562 cells was used to generate standard curves (Greiner et al., 2004) in 5-log steps for WT1 and PBGD. Amplification was conducted in a total volume of 20 μL for 40 cycles/10 sec at 95˚C, 4 sec/64˚C and 35 sec/72˚C. Samples were run in triplicates and their relative expression was determined by normalizing the expression of each target to PBGD and then comparing this to the normalized expression in a reference sample (K562) to calculate a fold change-value. Primers were designed using Oligo 6 software. The WT1 forward primer: TTCATCAAACAGGAGCCGAGC and reverse primer: GGTGCGAGGGCGTGTGA were used. The PBGD forward primer: CATGTCTGGTAACGGCAATG and reverse primer: TCTTCTCCAGGGCATGTTCAA were used. All primers were obtained from Aragene Laboratory (KFSH&RC).

2.17. Statistical Analysis

The statistical methods used were the mean, the median, the standard deviation and the Student t test. The mean is the average of a set of data. The standard deviation is used to measure the spread or dispersion of data around the mean. The mean and the standard deviation were calculated using Excel. The median is defined as the numeric value separating the higher half of a sample, a population, or a probability distribution, from the lower half. It was calculated using CellQuest software. The Student t test was used for comparative analysis as it is the most commonly used method to evaluate the differences in means between two groups. A P value less than or equal to 0.05 was deemed to be significant in all experiments.

3. WT1 PEPTIDE ANALOGOUE WT1-126Y ENHANCES LEUKEMIA LYSIS

3.1. Introduction

The Wilms tumour antigen 1 (WT1) has been shown to be expressed at low levels in some normal cells such as $CD34^+$ stem cells (Ellisen et al., 2001, Menssen et al., 1997, Inoue et al., 1997). Therefore, many of the potential CTL epitopes against this antigen may be absent or suboptimal. This is mainly due to clonal deletion of high-avidity CTLs during maturation of the immune system (Wood et al., 1987). To this end, different groups introduced modifications in the sequence of the anchor positions of these 'sub-optimal' peptides to improve their binding to HLA class I molecules and to increase their immunogenicity (Parkhurst et al., 1996, Chen et al., 2000, Valmori et al., 1998, Bakker et al., 1997). Besides modification of anchor positions, substitution of amino acid residues pointing to the T cell receptor has been shown to enhance epitope immunogenicity (Hoffmann et al., 2002, Tangri et al., 2001, Zaremba et al., 1997). It has been shown that modified peptides used to immunized transgenic mice were able to induce potent peptide-specific CTLs that induced tumour regression (Dyall et al., 1998, El-Shami et al., 2000, Mullins et al., 2001, Overwijk et al., 2003). Some of these modified peptides have been effectively used to vaccinate cancer patients (Rosenberg et al., 1998), to improve the detection of anti-tumour immunity (Chen et al., 2000), or to reverse the unresponsiveness of T lymphocytes to wild-type tumour antigens (Hoffmann et al., 2002). Indeed, it has been reported that vaccination of cancer patients with WT1-modified peptides induced WT1-specific CTLs and resulted in tumour regression (Oka et al., 2004).

3.2. Aim

The aim of the study was to explore the feasibility of using an approach that enhances the immunogenicity of low-avidity restricted peptides without altering their antigenic specificity (Tourdot et al., 2000). This approach consists of replacing the first amino acid of the epitope by a tyrosine (P1Y). The advantage of this strategy is that the P1Y variants are able to efficiently trigger in vivo wild-type peptide-specific CTLs which also recognized the naturally occurring epitope (Tourdot et al., 2000).

Given that WT1 specific CTLs isolated from leukaemia patients exert a low-avidity response (Rezvani et al., 2003, Scheibenbogen et al., 2002), experiments were

carried out where a Y amino acid substitution in the sequence of the Db126 and WH187 WT1-derived peptides was introduced with the aim of improving their binding affinities to MHC I and enhancing the immunogenicity of the peptides. Two peptides were selected as they have been reported to be presented by the most common Caucasians HLA allele (HLA-A*0201 present in approximately 50% of the population) and exert specific lysis against WT1-expressing leukaemia cells (Oka et al., 2000). In order to identify leukaemia AML and ALL samples which were positive for WT1 for subsequent use in cytotoxicity assays, forty samples were analysed for WT-1 expression

3.3. Results

3.3.1. WT1 antigen screening in leukaemia patients and normal donors

The mRNA expression pattern of the WT1 antigen was evaluated in PBMCs of AML (n=20), ALL (n=20) and in PBMCs of healthy volunteers (n=28) using conventional RT-PCR. This screening was performed for two purposes. The first purpose was to compare the WT1 gene expression level in acute leukaemia patients and in normal donors. The second purpose was to be able to select the HLA-A*0201^{+} leukaemia donors expressing the WT1 gene to be used (if needed) as targets for T cells generated against the WT1 peptides.

Total RNA was extracted from the PBMCs of patients and healthy donors and reverse transcriptized into cDNA using Moloney murine leukaemia virus. The extraction and reverse transcription methods are described in Chapter 2 section 2.13 and 2.14, respectively. The conventional RT-PCR was performed as described in Chapter 2 section 2.15. The amplification product of the house keeping gene β-actin served as control of cDNA quality. RNA isolated from K562 cell line, which expresses WT1, served as a positive control. Distilled water served as a negative control. The expression of the WT1 gene was classified into one of the following categories: - , no detectable mRNA expression; +, low expression; ++, moderate expression; +++, high expression. The intensity of the positive bands determined the level of antigen expression.

The results show that 16/20 (80%) of AML patients and 9/20 (45%) of ALL patients express the WT1 gene. In total, WT1 expression was detected in 25/40 (62.5%) of acute leukaemia patients. However, WT1 expression was not detected in any of the 27 normal donors tested. Results are shown in Figure 3.1 and Table 3.1. As shown in Table 3.1, there was no clear relation between WT1 expression levels and the blast number (the percentage of blasts varies between 1% and 99%).

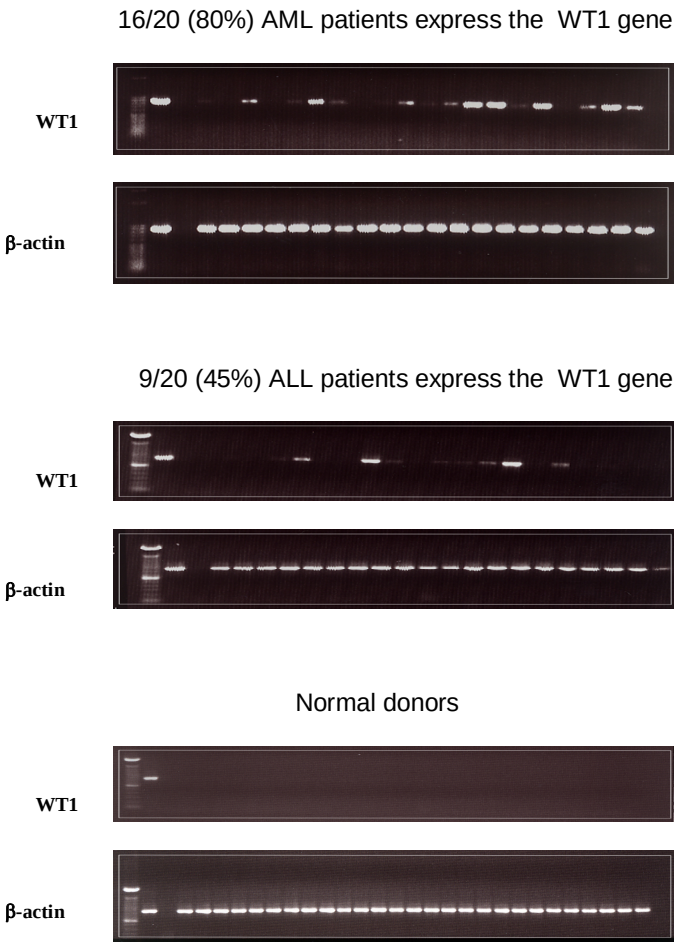

Figure 3.1. RT-PCR analysis of WT1 gene expression in PBMCs from AML, ALL patients and normal donors.

80% of AML patients and 45% of ALL patients expressed the WT1 antigen, whereas none of the normal donors expressed the WT1 antigen.

Table 3.1. Clinical features of leukaemia patients with WT1 gene expression.

Patients #	*Subtypes*	*Status*	*% Blast*	*WT1 expression*
M0	AML 3	New	84	++
M1	AML 1	New	61	+
M2	AML 5	New	1	-
	AML 7	New	61	+
	AML 8	New	20	+++
	AML 19	New	23	+
	AML 29	Rel Post chemo	81	-
M4E0	AML 13	2nd Rel post allo BMT	10	+
	AML 36	New	12	++
M4	AML 25	New	52	+++
	AML 27	New	81	+++
	AML 28	New	61	+
M5a	AML 2	New	NR	-
	AML 12	New	79	-
M5	AML 11	New	80	++
	AML 16	New	4	++
	AML 24	New	50	+++
	AML 31	New	13	++
	AML 34	Rel Post allo BMT	80	+++
M6	AML 20	New	65	++
Pre T-cell	ALL 26	New	99	++
T-cell	ALL 1	Rel	6	-
	ALL 2	Rel Post-BMT	87	-
	ALL 3	Rel	87	-
	ALL 13	New	75	+++
	ALL 16	New	NR	++
	ALL 18	New	94	-
	ALL 22	New	47	++
	ALL 23	New	84	+++
Pre B-cell	ALL 4	New	55	+
	ALL 5	New	56	+++
	ALL 20	New	5	+
	ALL 21	New	83	+
	ALL 27	New	59	-
	ALL 28	New	88	-
	ALL 29	New	2	-
	ALL 36	Rel on chemo	NR	-
B-cell	ALL 10	New	59	-
BP / Biclonal	ALL 7	New	89	-
ALL 24	NR	Rel	61	-

New, newly diagnosed; Rel, relapsed; - , no detectable mRNA expression; +, low expression; ++, moderate expression; +++, high expression

3.3.2. Identification of peptides predicted to highly bind to the HLA-A0201 molecule

Two WT1 peptides known to be HLA-A*0201 restricted peptides (Oka et al., 2000), were modified by substituting the amino acid at position 1 with tyrosine (Y). The binding of the wild-type peptides (126N and 187N) with their corresponding analogues (126Y and 187Y) to the HLA-A*0201 molecule was compared using a computer-based epitope prediction program (http://bimas.dcrt.nih.gov/molbio/hla_bind/). The amino acid sequences of each peptide (wild-types and analogues) were entered into the BIMAS program and the HLA-A*0201 molecule was selected. After entering the amino acid sequence and selecting the MHC type, the predicted binding score for each peptide was obtained. The data indicated that this tyrosine modification resulted in a 4.6-fold increase in the estimated half-time of disassociation of the modified peptides to HLA-A*0201 compared with their native counterparts as shown in Figure 3.2 .

The computer-based epitope prediction program has been suggested to have only 60–80% predictive accuracy (Pinilla-Ibarz et al., 2006). Therefore, the strength of the interaction between these peptides and the HLA-A*0201 molecules was directly measured using the T2 binding assay. The assay was performed twice; however, different concentrations of the WT1-derived peptides were used. T2 cells were pulsed with either 50, 30, 15, 7 and 1μM or with 70, 7 and 0.7 μM of each peptide overnight at 37˚C in a serum-free medium. Cells were washed, stained with FITC-conjugated anti-HLA-A2 monoclonal antibody and analyzed by flow cytometry. The HLA-A*0201 expression by the T2 cells was quantified as fluorescence index according to the following formula: fluorescence index = (median fluorescence intensity with peptide - median fluorescence intensity without peptide)/ median fluorescence intensity without peptide. Figures 3.3, 3.4, 3.5 and 3.6 show the flow cytometric analyses of T2 cells incubated overnight with different concentrations (50, 30, 15, 7 and 1μM) of either native or modified 126 and 187 peptides. Figures 3.7, 3.8, 3.9 and 3.10 show the flow cytometric analyses of T2 cells incubated overnight with different concentrations (70, 7 and 0.7μM) of either native or modified 126 and 187 peptides.

Using different concentrations of the 9-mer peptides, an enhanced binding of the 126Y analogue peptide was observed compared to the wild-type 126N peptide at all doses tested (Figure 3.11A). A concentration of 50 μM of the 126N peptide was needed to achieve a 2.5 fluorescence index, while only 15 μM of the 126Y peptide was sufficient to reach the 2.5 fluorescence index. However, modification of the 187 peptide (187Y) did not enhance its binding ability and stabilization of the HLA-A*0201 molecules (Figure 3.11 B). The same pattern of peptide binding affinity was observed when the binding assay was repeated with different peptide concentrations (70, 7 and 0.7 μM) (Figure 3.11A and B).

These data show a selective enhancement of peptide binding to HLA-A*0201 after modification, and thus demonstrated a good model to test the effect of this process on the function of the generated T cells.

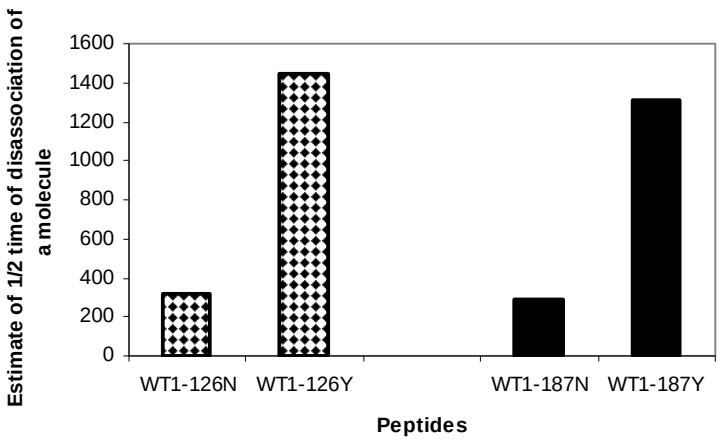

Figure 3.2. Wild-type (WT1-126N, WT1-187N) and the modified (WT1-126Y, WT1-187Y) peptides binding affinities to HLA-A*0201 molecule as predicted by the computer-based epitope prediction program BIMAS.

Using this program, we have shown that substitution of the tyrosine (Y) at position 1 resulted in 4.6-fold increase in the estimated half time of disassociation of the modified peptides compared to their native counterparts.

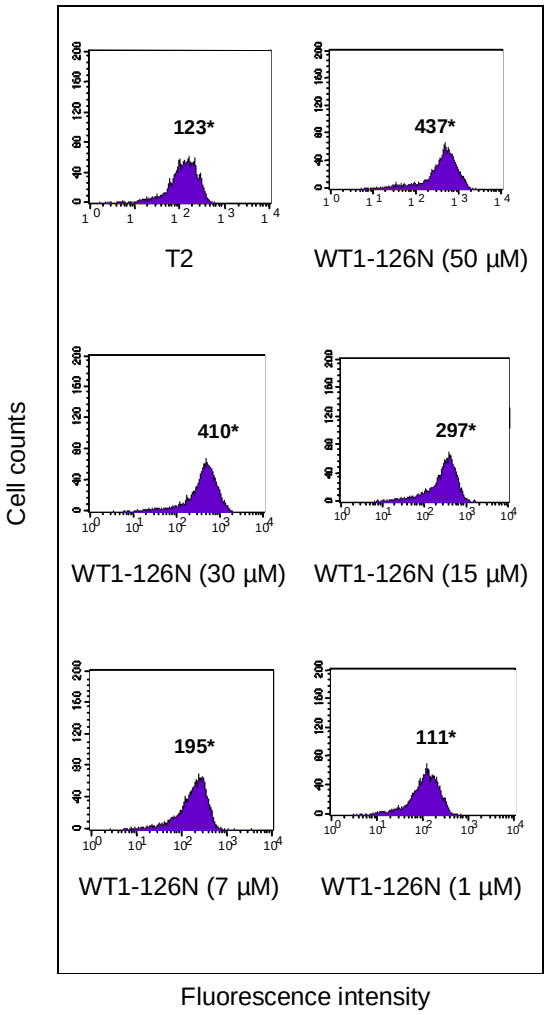

Figure 3.3. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 50, 30, 15, 7 and 1µM of the WT1-126N peptide. Following incubation with different concentrations of the native 126N peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

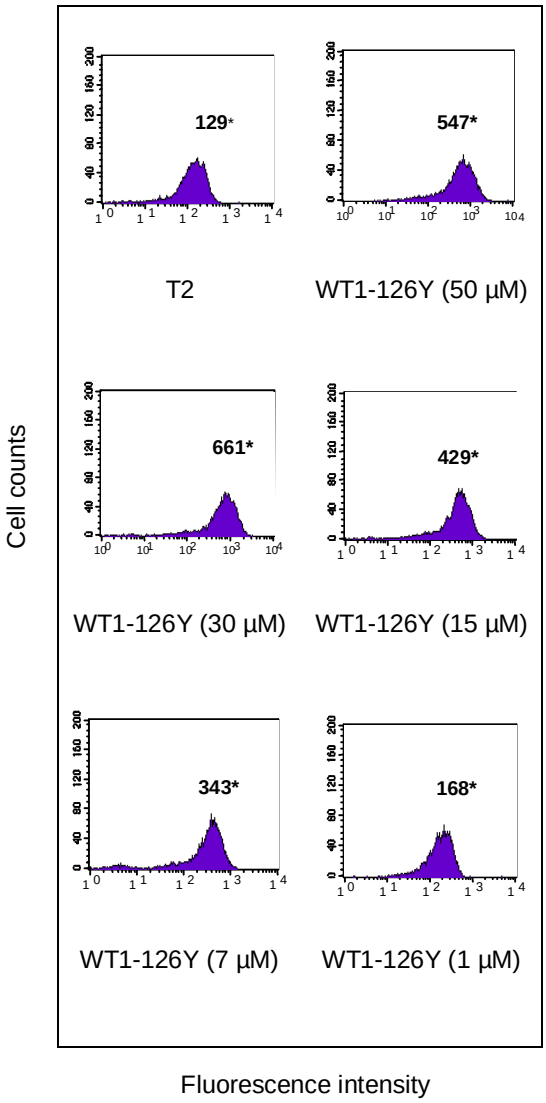

Figure 3.4. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 50, 30, 15, 7 and 1µM of the WT1-126Y peptide. Following incubation with different concentrations of the analogue 126Y peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

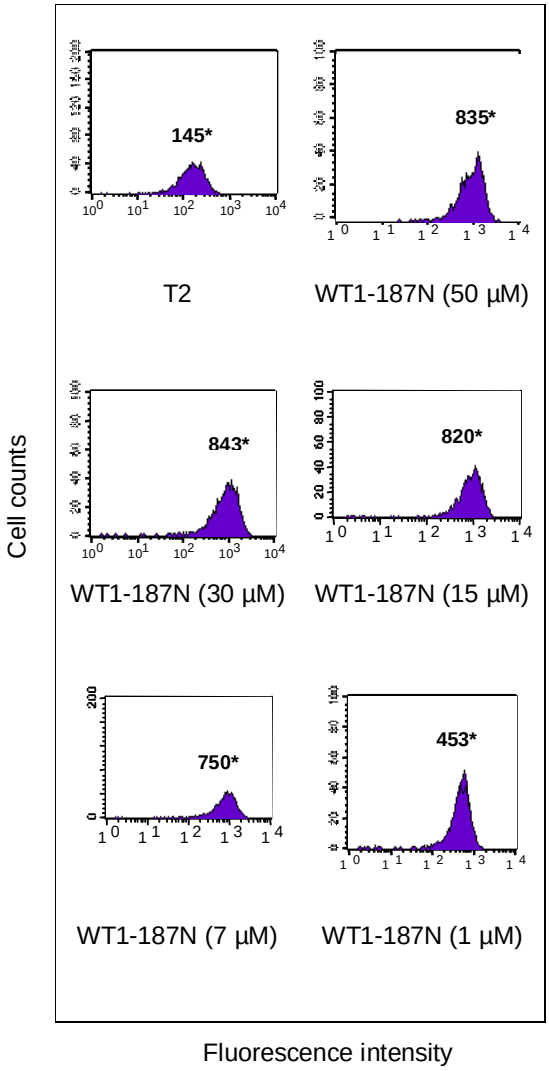

Figure 3.5. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 50, 30, 15, 7 and 1µM of the WT1-187N peptide. Following incubation with different concentrations of the analogue 187N peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

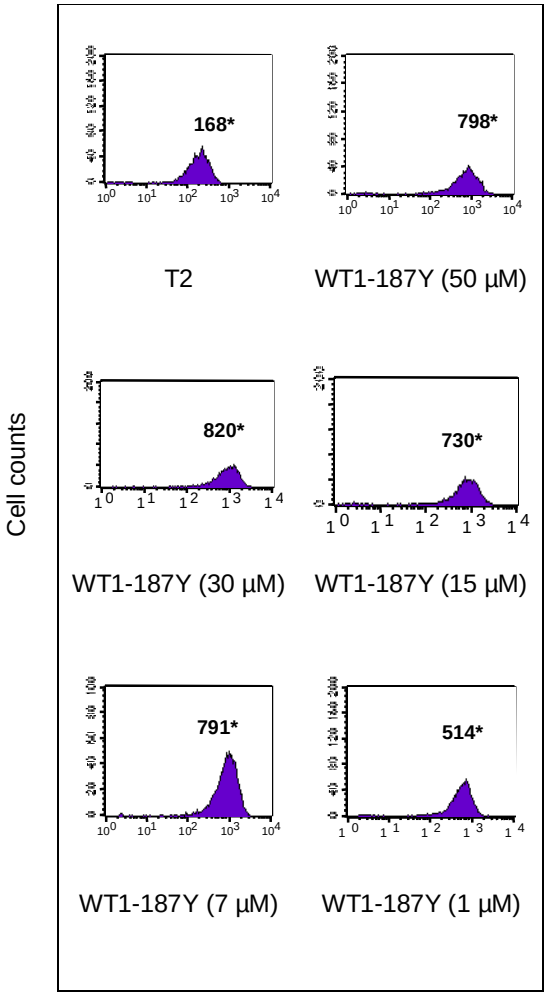

Figure 3.6. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 50, 30, 15, 7 and 1µM of the WT1-187Y peptide. Following incubation with different concentrations of the analogue 187Y peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

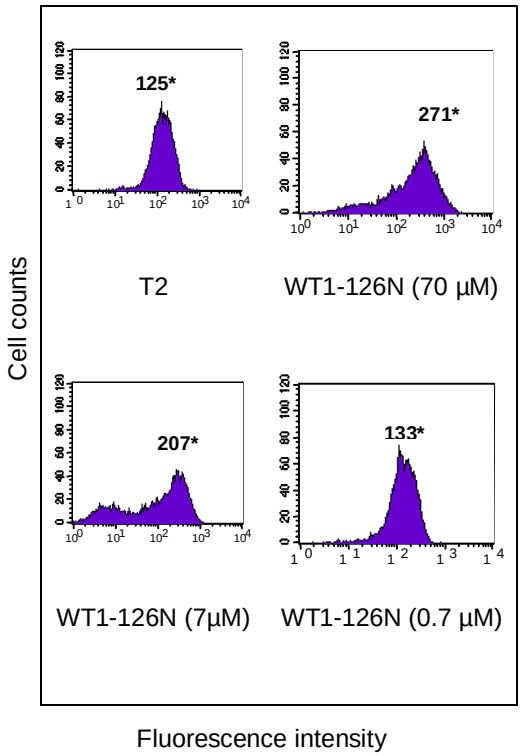

Figure 3.7. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 70, 7 and 0.7µM of the WT1-126N peptide.

Following incubation with different concentrations of the native 126N peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

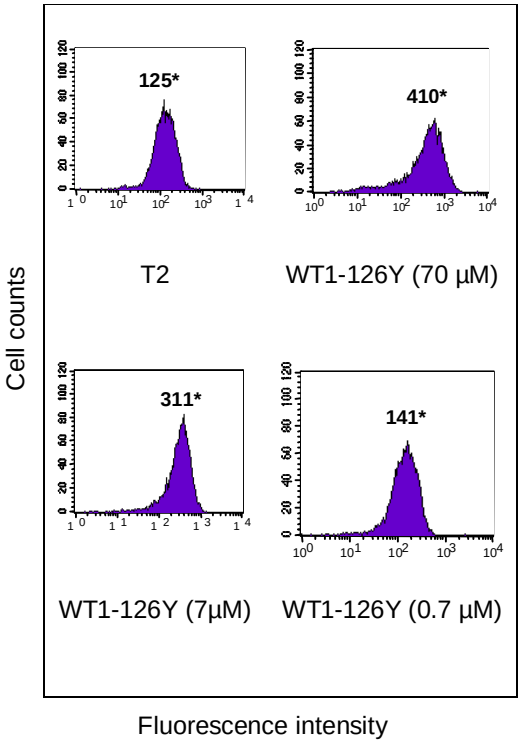

Figure 3.8. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 70, 7 and 0.7µM of the WT1-126Y peptide.

Following incubation with different concentrations of the analogue 126Y peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

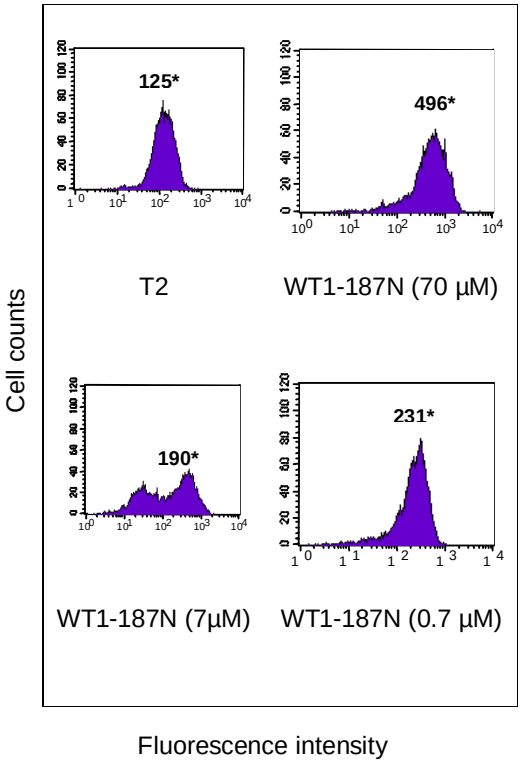

Figure 3.9. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 70, 7 and 0.7µM of the WT1-187N peptide.
Following incubation with different concentrations of the native 187N peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

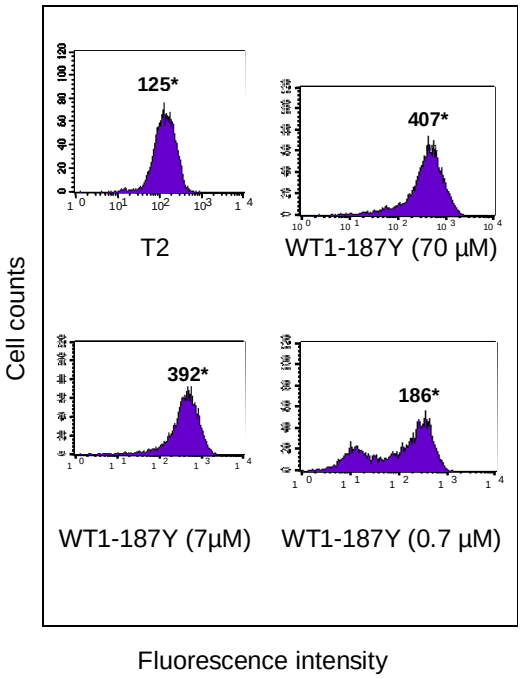

Figure 3.10. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 70, 7 and 0.7µM of the WT1-187Y peptide.

Following incubation with different concentrations of the analogue 187Y peptide, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Median fluorescence intensities of T2 cells stained with anti-HLA-A2 antibody.

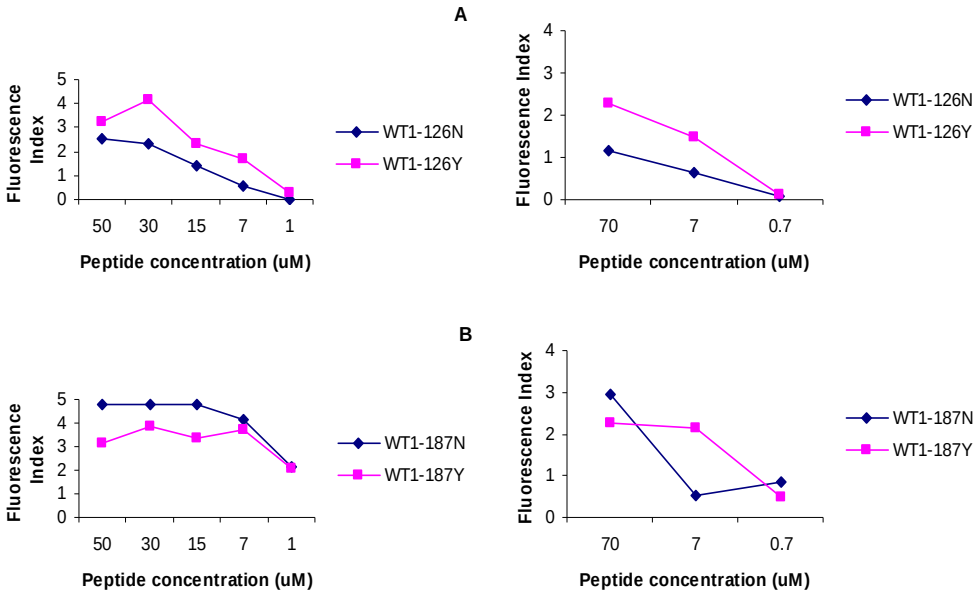

Figure 3.11. The binding ability of WT1-derived peptides to HLA-A*0201 molecule as measured by a standard T2 stabilization assay.

The experiment was performed at either 50, 30, 15, 7 and 1μM or at 70, 7 and 07 μM of each peptide. X axis represents different concentrations per well of the peptide tested. Fluorescence index is shown on the Y axis. (A): HLA-A*0201 epitope enhancement of the WT1-126N wild-type peptide by an amino acid substitution (Y) at position 1 (126Y) analogue peptide. (B) No enhancement in the case of peptide 187Y.

3.3.3. Generation of WT1 peptide-specific cytotoxic T cell lines

The effect of a tyrosine modification of the WT1-126 peptide on its ability to induce specific CTL compared with its wild-type peptide, was tested because only the 126Y analogue peptide but not the 187Y peptide showed a high binding affinity to the HLA-A*0201 molecule compared to their other peptide counterparts. An optimized T-cell activation protocol, with $CD14^+$ monocytes-derived DC as antigen presenting cells, and purified $CD8^+$ T cells as responders was used in order to generate and expand T cell lines specific to the native (126N) and the modified (126Y) peptides. The method for the generation of the T cell lines is as illustrated in Figure 3.12. The PBMCs were obtained from three normal healthy HLA-A*0201 donors designated as BC-21, BC-32 and BC-37, and used as a source of APCs and responder cells. Monocytes and $CD8^+$ T cells were isolated from BC-32 by negative selection using magnetic beads. Cells were stained with FITC-conjugated anti-CD14 antibody and analyzed by flow cytometry in order to determine the degree of purity achieved during monocyte separation. Ninety-five percent of the purified cells were $CD14^+$ as shown in Figure 3.13A, and were used to generate conventional DCs as described in Materials and Methods section 2.5.4.2. For $CD8^+$ T cells, aliquots of cells before and after the separation were collected and stained with FITC-conjugated anti-CD8 antibody. The percentage of $CD8^+$ T cells among PBMC-32 was 22% (Figure 3.13B) and the purity of the $CD8^+$ T cells fraction was 82% (Figure 3.13C) as verified by flow cytometry. DCs were used to prime $CD8^+$ T cells since DCs are the most potent stimulators of naïve T lymphocytes. The expression level of CD1a, and CD83 on the cell surface of the mature DCs were analysed by flow cytometry. Preliminary experiments that used PBMCs obtained from other normal donors were carried out to compare the expression levels of the DC-related markers by immature as well as mature DCs. These experiments showed that the mature DCs down regulated the expression level of CD1a whereas the expression level of the activation marker CD83 was up regulated. As shown in Figure 3.14A and B, only 0.5% of the mature DCs expressed the CD1a marker while 85% of them expressed CD83 marker.

To compare the immunogenicity of the native and the modified WT1-126 peptides as well as to investigate whether the generated line against the analogue peptide

could enhance stronger CTLs response and more importantly whether it cross-reacted with its naturally occurring counter-part, the CTLs were tested after 3 to 8 rounds of stimulations against different HLA-A*0201^{+} targets in the presence or absence of autologous peptide using a Chromium Release Assay and an ELISPOT assay.

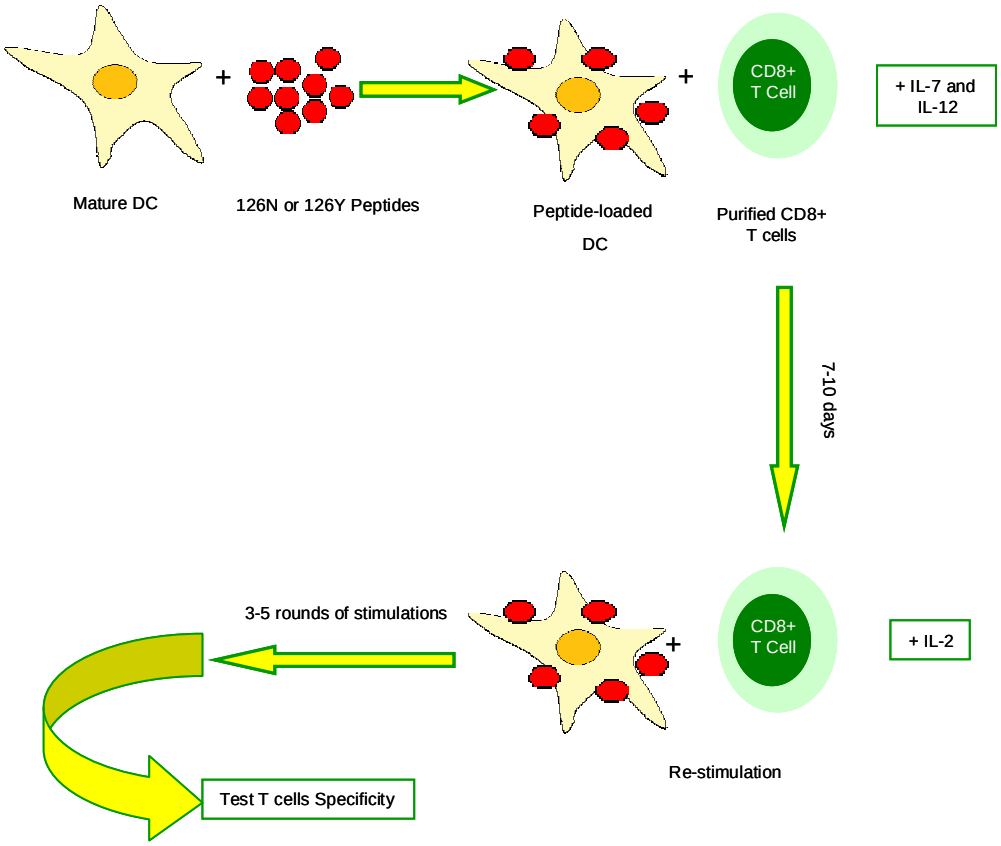

Figure 3.12. Method used to generate CTLs specific for 126N and its analogue (126Y) peptides. Peptide-pulsed autologous DCs were used to prime and re-stimulate $CD8^{+}$T cells. IL-7 and IL-12 were added at the initiation of the cultures. IL-2 was added one day after each subsequent stimulation. The immunogenicity of the generated CTL lines were tested and compared after 3-8 rounds of stimulation.

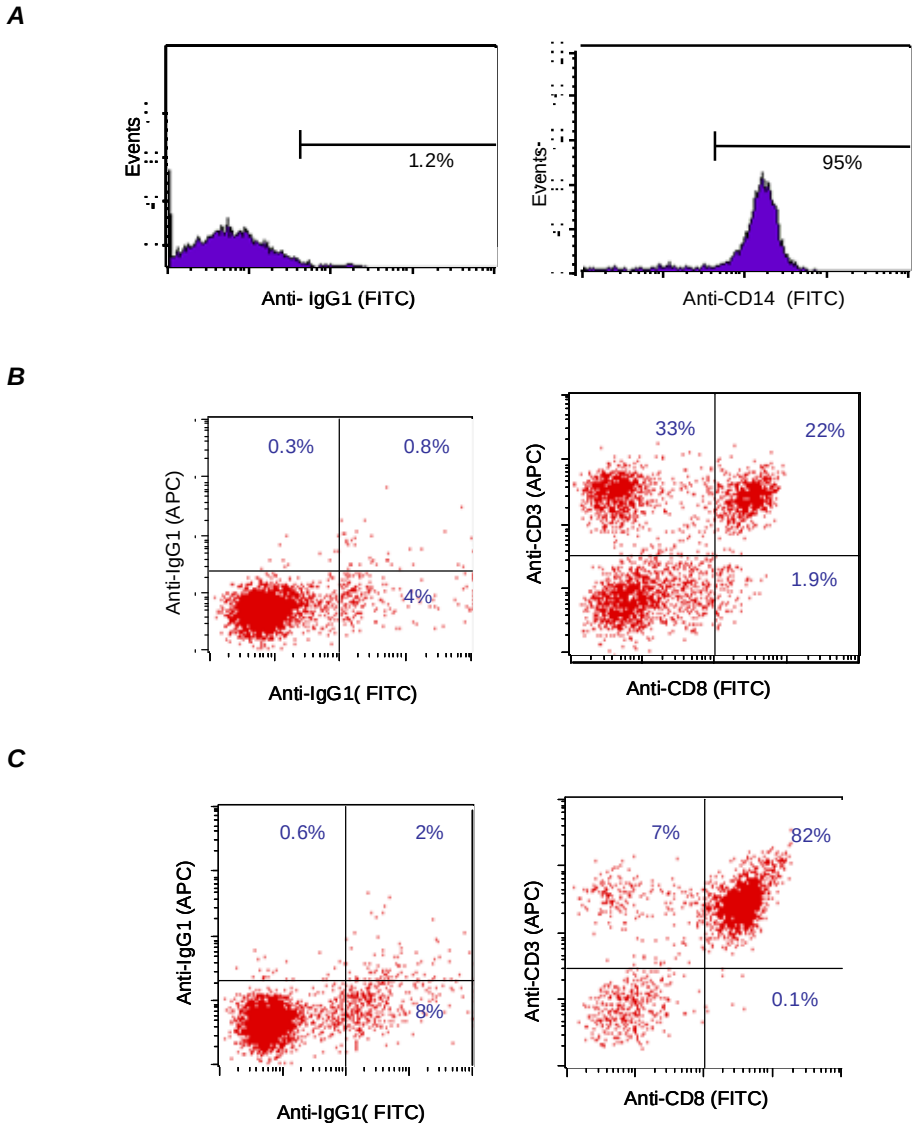

Figure 3.13. Efficiency of magnetic bead cell separation.

(A) Flow cytometry analysis of $CD14^+$ cells isolated from PBMC-32 by negative selection (B) Flow cytometry analysis of $CD8^+$ in PBMC-32 before separation (C) and after separation.

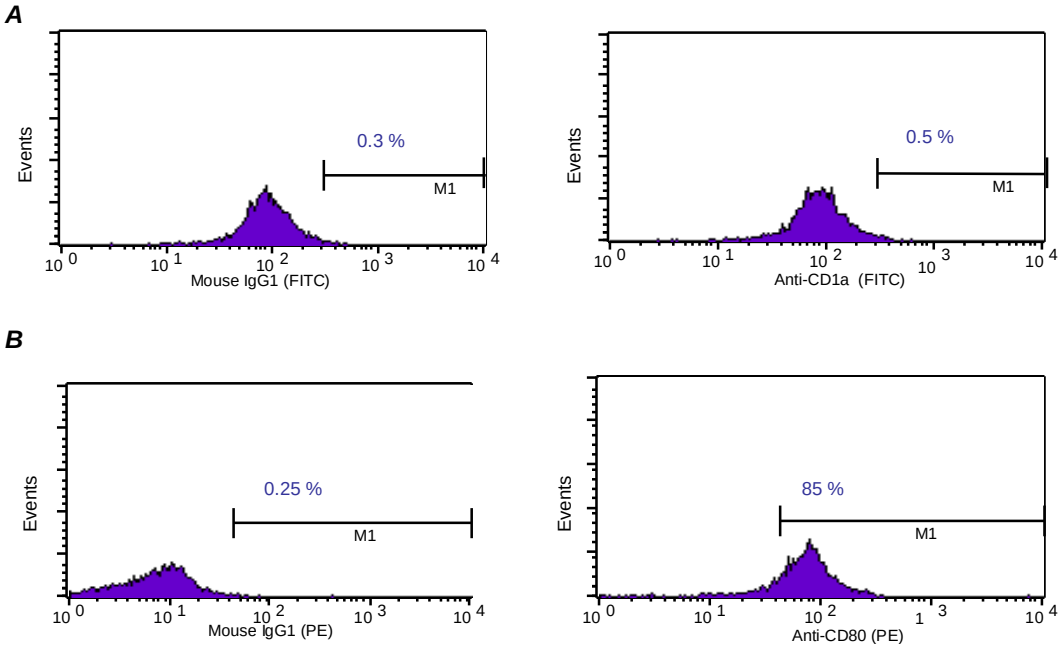

Figure 3.14. Flow cytometry analysis of CD1a and CD83 expression in the mature-monocytes derived DC-32.

(A) Histograms show that 0.5% of DC-32 expresses CD1a marker. (B) Histograms show that 85% of DC-32 expresses the activation marker CD83.

3.3.4. Enhanced cytotoxicity of CTL generated against WT1-126Y

We tested the effect of tyrosine modification of the WT-126 peptide on its ability to induce specific CTL compared with its wild-type peptide. After three to eight rounds of stimulations, the generated T cell lines were tested against the autologous LCLs or T2 cells in the presence or absence of autologous peptide using a Chromium Release Assay. At the early stages of T cell stimulation, most of the T cells' responses were not specific. Therefore, the T cell lines were subjected to weekly stimulation and the cytotoxic assay was performed five days after each antigen-specific stimulation. Cross-reactivity between the two peptides was also tested to determine if the analogue peptide 126Y is able to induce an immune response to the native peptide (126N). The lytic activity of the T cell lines generated from BC-32 against 126N and 126Y after four and seven rounds of stimulations are presented in Figure 3.15A and B, respectively. Figure 3.15A shows that the CTL raised against the native (WT1-126N) and the modified (WT1-126Y) peptides were not able to specifically lyse the peptide-loaded LCLs. The assay was done after only four rounds of T cell stimulation. Figure 3.15B shows that following the seventh antigen-specific re-stimulation, specific lysis was recorded against the T2 cells for both BC-32 T cell lines raised against the 126N peptide and its 126Y analogue, because there was no T cell response against the T2 cells in the absence of the peptides. However, a significantly (*P=0.0006*) higher lytic activity was recorded when the T cell line raised against the 126Y modified peptide was used (Figure 3.15B). Interestingly, we found cross reactivity of each T cell line with the opposite peptide. In this, the T cell line generated against the wild-type 126N peptide was able to equally lyse the T2 cells loaded with the 126N or the 126Y. Similar results were obtained for the T cell line generated against the analogue 126N peptide. The generated T cell lines were tested against different APCs loaded with the corresponding peptide such as autologous LCLs and T2 cells. Usually when the T cells showed a specific killing response against one of the peptide-loaded target cells, they will show killing responses against other peptide-loaded targets. The 126N and 126Y CTLs failed to recognize peptide-loaded LCLs because the test was done after four rounds of T cell stimulation. $CD8^+$ T cells needed more rounds of antigen-stimulation before a specific response is seen.

To confirm that the T cells response generated against both the wild-type 126N and the 126Y peptides is restricted to the HLA-A*0201 molecule, an HLA blocking assay was performed. Blocking of the HLA-ABC resulted in a strong inhibition of the cytotoxic activity of both T cell lines against peptide-loaded T2 cells (Figure 3.16). However, the isotype control did not inhibit the cytotoxic activity of this T cell lines. These data show that the tyrosine modification of the WT1-126 peptide, which enhanced their binding to HLA-A*0201, can increase its lytic activity not only to the 126Y epitope, but also to the wild-type 126N counterpart.

The lytic activity of the T cell lines generated against the native peptide 126N and its analogue 126Y from BC-21 and BC-37 was also tested and compared. However, none of these T cell lines elicit any peptide-specific cytotoxic responses. These T cell lines were challenged with T2 cells pulsed with no peptide or with each peptide at different E:T ratio starting from 100:1. Figure 3.17A and B shows the failure of the 126N and 126Y T cell lines generated from BC-21 to recognize T2 cells loaded with either the native or with the analogue peptide. Similar findings have been reported for the T cell lines generated from BC-37. As shown in Figure 3.18A and B, BC-37 T cell lines did not exert any specific cytotoxicity towards T2 cells loaded with either the native or with the analogue peptide

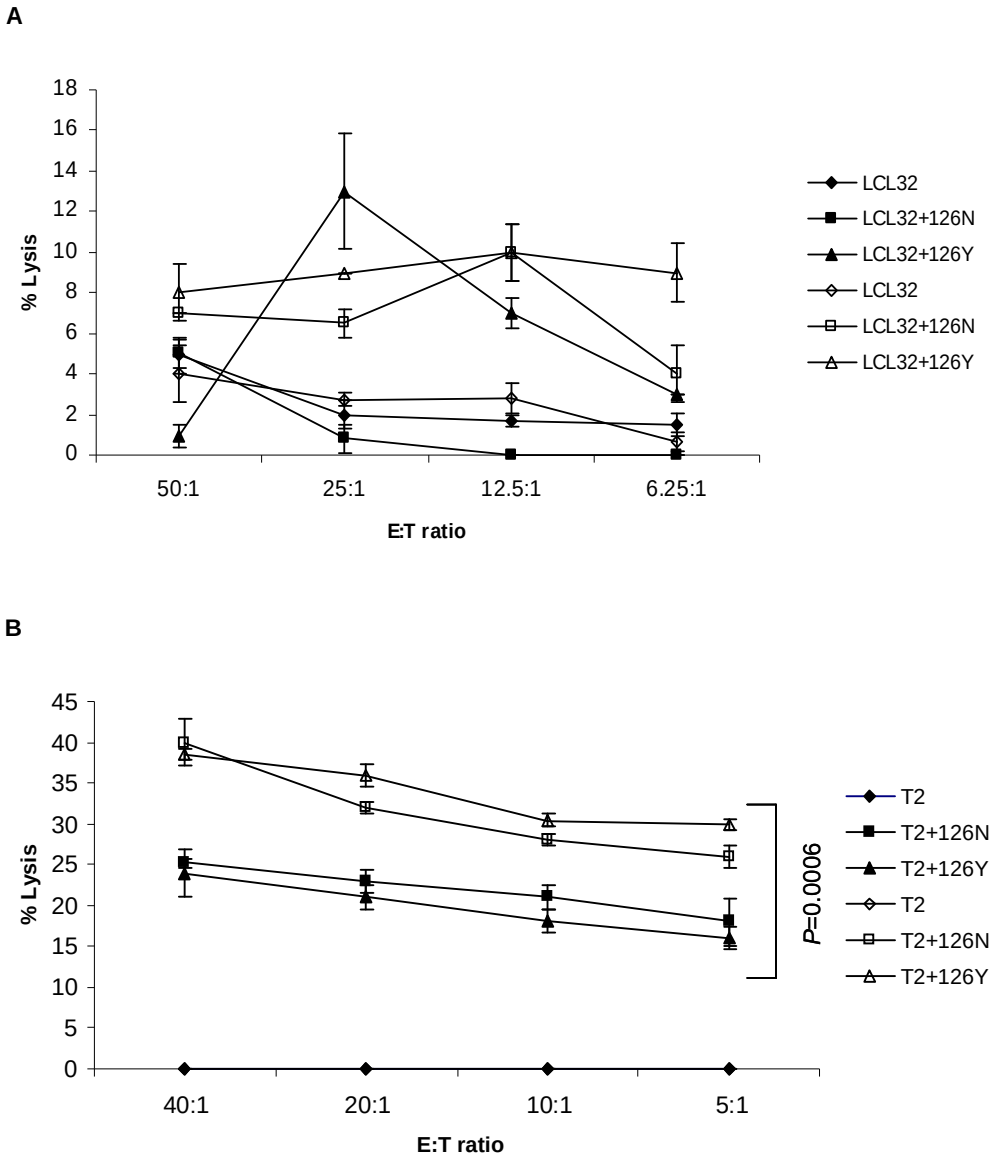

Figure 3.15. Cytotoxicity assay with T cells generated against WT1-126N and WT1-126Y peptides from a healthy HLA-A*0201 donor.

Cytotoxicity of the T cell line generated against the native 126N peptide is shown with the closed symbols. Cytotoxicity of the T cell line generated against the analogue 126Y peptide is shown with the opened symbols. (A): Non-specific lysis by CTLs generated against 126N and 126Y peptides after the fourth round of in vitro antigen-stimulation. Target cells were autologous LCLs pulsed with either no peptide (♦,◊), 126N peptide (■,□) or with 126Y peptide (▲,Δ). (B): Specific lysis by CTLs generated against 126N and 126Y peptides after the seventh round of in vitro antigen-stimulation. Target cells were T2 cells pulsed with either no peptide (♦,◊), 126N peptide (■,□) or with 126Y peptide (▲,Δ). The data are presented as the percentage specific lysis at the indicated E:T ratios. Error bars (±SD) represent triplicate wells from one experiment. *P* value between A and B=0.0006.

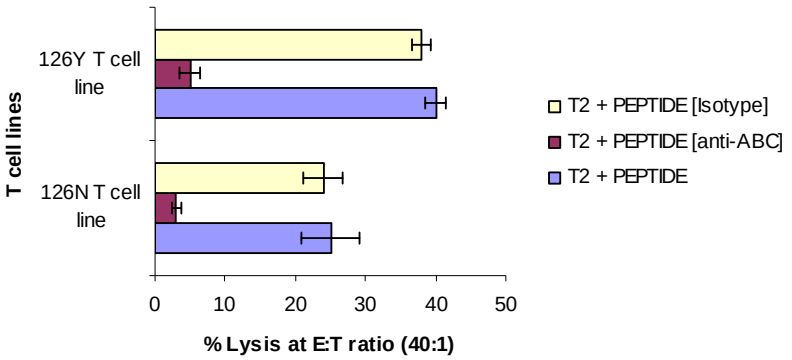

Figure 3.16. Inhibition of lytic activity of BC-32 T cell lines generated against WT1-126N and WT1-126Y peptides after blocking with an anti-ABC antibody showing the restriction response to the HLA-A*0201 molecule.

The assay was performed after the seventh round of in vitro antigen-stimulation. Ab was added to the wells containing T2 cells 20 min before the addition of T cells. The data are presented as the percentage specific lysis at E:T ratio of 40:1. Error bars (±SD) represent triplicate wells from one experiment.

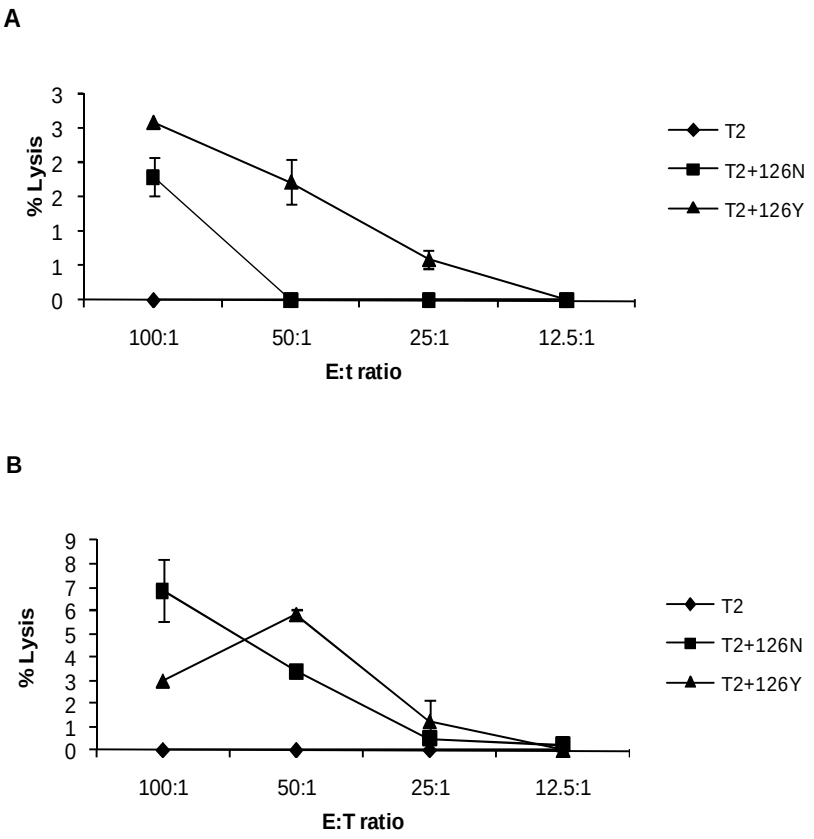

Figure 3.17. Non-specific lysis by BC-21 T cell lines generated against WT1-126N and WT1-126Y peptides as tested using a standard chromium release assay.

The T cell line generated from a normal HLA-A0201^{+} donor, BC-21, were used as effector cells. Target cells were T2 cells pulsed with either no peptide (♦), with 126N peptide (■) or with 126Y peptide (▲). (A) Shows the cytotoxicity of the T cell line generated against the native 126N peptide. (B) Shows the cytotoxicity of the T cell line generated against the analogue 126Y peptide. The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of two experiments and error bars indicate the SD.

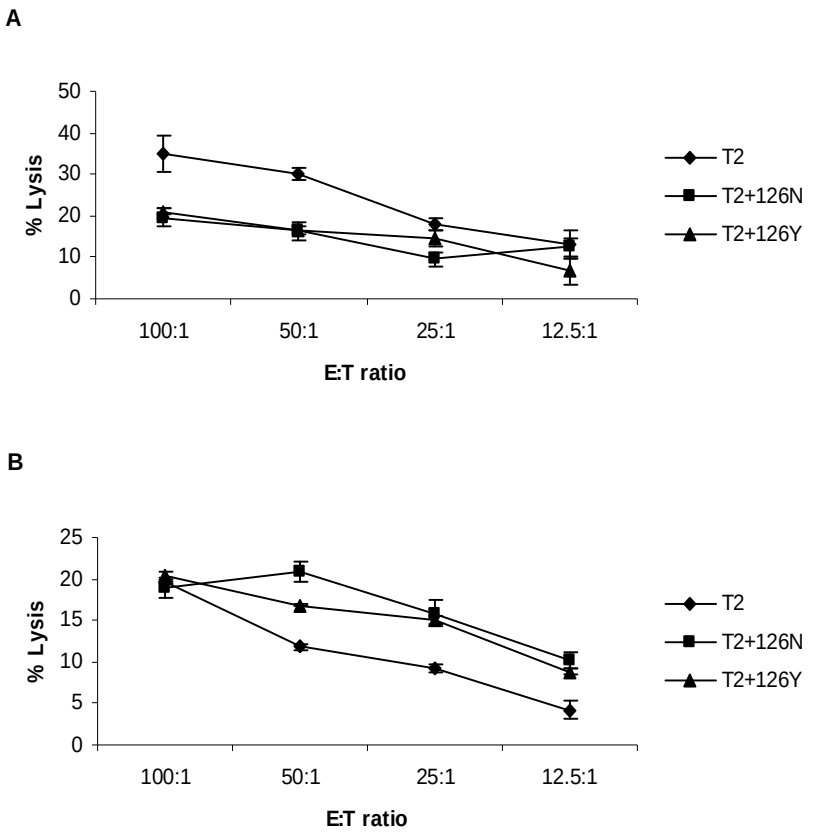

Figure 3.18. Non-specific lysis by BC-37 T cell lines generated against WT1-126N and WT1-126Y peptides as tested using a standard chromium release assay.

The T cell line generated from a normal HLA-A0201^{+} donor, BC-37, were used as effector cells. Target cells were T2 cells pulsed with either no peptide (♦), with 126N peptide (■) or with 126Y peptide (▲). (A) Shows the cytotoxicity of the T cell line generated against the native 126N peptide. (B) Shows the cytotoxicity of the T cell line generated against the analogue 126Y peptide. The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of two experiments and error bars indicate the SD.

3.3.5. WT1-126Y peptide increased the frequency of IFN-γ producing T cells

We have tested the 126N and the 126Y T cell lines for the frequency of IFN-γ producing T cells after three rounds of in vitro stimulation using an ELISPOT assay. Results obtained from two experiments are shown in Figure 3.19A. Both T cell lines generated from BC-32 produced specific IFN-γ in response to stimulation with autologous LCL loaded with their corresponding peptide (Figure 3.19A). However, the T cell line generated with the analogue 126Y peptide exerted a significantly higher number (P=0.0003) of IFN-γ producing T cells compared with that generated against the 126N wild-type peptide (Figure 3.19A). The 126N and 126Y T cell lines were challenged with the PHA to explore whether the two T cell lines did not differ in the proportion of T cells capable of IFN-γ production. As shown in Figure 3.19B, the two lines did not differ in the proportion of T cells capable of IFN-γ production. These data demonstrate that modification of the WT1-126 peptide can increase the frequency of IFN-γ producing T cells and thus making them better effector cells.

The 126N and the 126Y T cell lines generated from BC-21 and BC-37 were also tested for the frequency of IFN-γ producing T cells. Consistent with the cytotoxic findings, these T cell lines did not produce specific IFN-γ when stimulated with autologous LCL loaded with their corresponding peptide (Figure 3.20 and Figure 3.21).

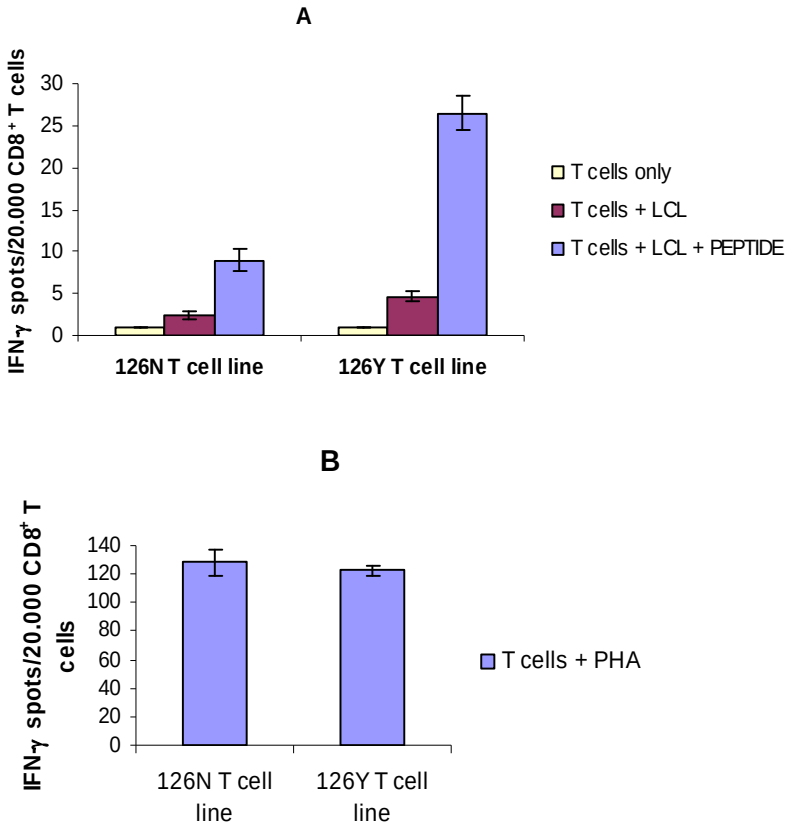

Figure 3.19. Specific production of IFN-γ by the two T cell lines generated against the wild type 126N and its analogue 126Y peptides as measured by an ELISPOT assay.

The T cell lines were established from BC-32, an HLA-A0201$^+$ donor, and tested following three to eight rounds of *in vitro* T cell stimulations. The background observed when T cells alone were used is shown. (A) T cells were stimulated with autologous LCL± peptide for 40 h in anti-IFN-γ pre-coated wells before development of the spots. T cells incubated without stimulators (T cell only) served as a baseline. (B) T cells stimulated with PHA did not differ in the proportion of T cells capable of IFN-γ production. Results are expressed as IFN- γ positive cells per 20.000 CD8$^+$ T cells and are the mean of duplicate assays (±SD).

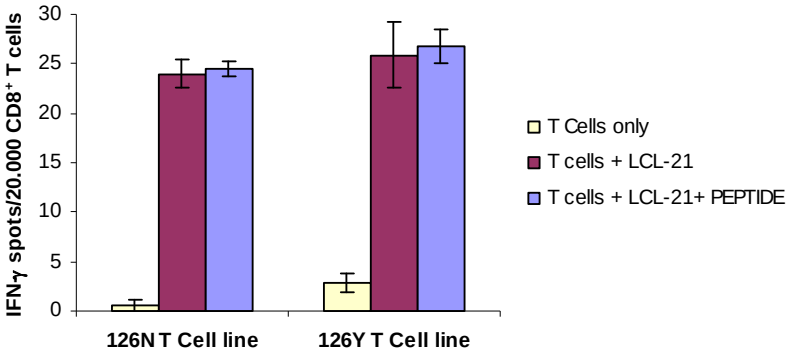

Figure 3.20. Non-specific IFN-γ release by BC-21 CTLs generated against the WT1-126N and WT1-126Y peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-21, an HLA-A0201$^+$ donor, and tested following three to eight rounds of *in vitro* T cell stimulations. Targets were autologous LCLs pulsed with either no peptide or with peptide. The background observed when T cells alone were used is shown. Results are expressed as IFN- γ positive cells per 20.000 CD8$^+$ T cells and are the mean of triplicate assays (±SD).

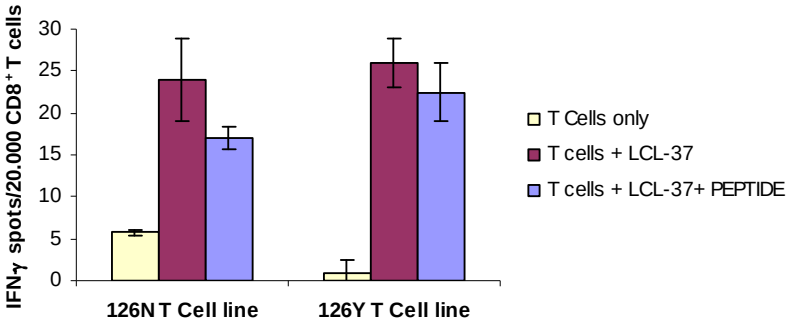

Figure 3.21. Non-specific IFN-γ release by BC-37 CTLs generated against the WT1-126N and WT1-126Y peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-37, an HLA-A0201$^+$ donor, and tested following three to eight rounds of *in vitro* T cell stimulations. Targets were autologous LCLs pulsed with either no peptide or with peptide. The background observed when T cells alone were used is shown. Results are expressed as IFN- γ positive cells per 20.000 CD8$^+$ T cells and are the mean of triplicate assays (±SD).

3.3.6. Evaluation of WT1 expression and lytic activity of anti-WT1 T cell lines against HLA-A0201-matched leukemic cells

Given the above, the feasibility of using this modification approach of the WT1-126 peptide to generate CTLs that effectively kill HLA-A*0201-matched leukemic cells was tested. The expression of the WT1 mRNA in fresh AML cells (AML-27 and AML-28) was examined using Q-RT-PCR. K562 cells, known to express high levels of WT1 (Greiner et al., 2004) were used as a positive control. PBMC-32 derived from a normal donor was used as a negative control. Higher levels of WT1 expression were observed in AML samples (Table 3.2). The lytic activity of the two T cell lines generated against the 126N and 126Y peptides were then measured against PBMCs from HLA-A*0201-matched (AML-28) and non-HLA-A0201 matched (AML-27) AML patients. Specific lytic activity was recorded for both T cell lines against the AML-28 cells expressing the HLA-A*0201 molecule (Figure 3.22). However, both T cell lines failed to recognise non-HLA-A*0201 AML-27 even though they expressed high levels of WT1 (Figure 3.22). Interestingly, the T cell line generated with the analogue 126Y peptide exerted a significantly higher lytic activity (P=0.0014) compared with that generated against the 126N wild-type peptide (Figure 3.22). Altogether, our data indicated that the modified WT1-126Y enhanced its binding to HLA-A*0201, increased the frequency of IFN-γ producing T cells, and boosted the lytic activity against HLA-matched leukaemia cells. Therefore, modification of the WT1-126 peptide provides a potential approach for the development of leukaemia vaccine.

Table 3.2. Clinical characteristics of patients and donors used in the study

Donor/Patient Samples	***HLA-A Type***	***% Blasts[1]***	***QRT-PCR[2]***	***RT-PCR[3]***	***Status[4]***	***FAB-Type[5]***
PBMC-32	A0201, 29	NA	60	-	NA	NA
AML-27	25,29	81	7950	+++	new	M4
AML-28	A0201, 26	61	1800	+	new	M4

1 % blasts were determined routinely by the hematopathology laboratory

2 Quantitative real-time PCR data for the expression level of WT1 which normalized from K562 set on 1000

3 Conventional RT-PCR data for the expression of WT1

4 New indicates that samples were taken at first diagnosis

5 French-American-British classification

NA=not applicable

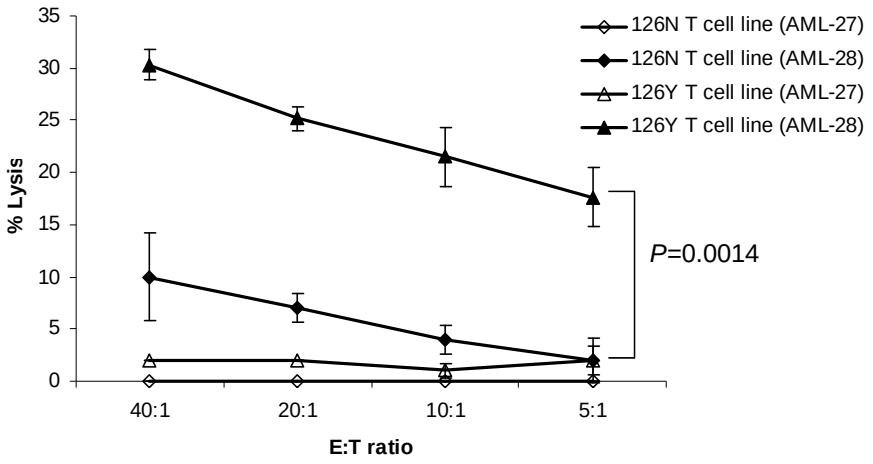

Figure 3.22. Cytotoxicity assay showing the lytic activity of the 126N and 126Y T cell lines against HLA-A*0201-matched (AML-28) and non-matched AML-27 cells.

The T cell lines generated from a normal HLA-A0201^{+} donor, BC-32, were used as effector cells. The T cell lines were tested after eight rounds of *in vitro* T cell stimulations. The data are presented as the percentage specific lysis at E:T ratio of 40:1. Error bars (±SD) represent triplicate wells from one experiment.

3.4. Discussion

Majority of tumour-associated antigens, which have been identified so far, are derived from self-antigens. Immunological tolerance can prevent the development of immune responses against such self-antigens. Therefore, most self/tumour antigens used in clinical trials so far showed poor immunogenicity. To circumvent the problem of tolerance, Stauss et al. (Bellantuono et al., 2002, Gao et al., 2000) developed an allo-restricted CTL against leukaemia. In this approach, T cells from HLA-A*0201 donors were stimulated with peptide loaded HLA-A*0201-matched APCs. This allowed the generation of high-avidity allo-restricted CTLs against an HLAA0201 WT1-derived epitope. Interestingly, the generated CTLs specifically lysed WT1, HLA-A*0201-matched leukaemia cells. More importantly, only the high-avidity CTLs were able to eliminate the progenitors of CFU-GM and BFU-GM from CD34 CML samples, whereas normal CD34 progenitors/stem cells were spared (Bellantuono et al., 2002, Gao et al., 2000). However, as allogeneic APCs usually provoke dominant CTL responses against allogeneic epitopes, the isolation of HLA-A0201-WT1 peptide-specific allogeneic CTLs was unsuccessful in many cases. Another drawback of this strategy is the difficultly in applying such an approach in clinical settings. A more promising approach has been recently developed by the same group, which rely on introducing the WT1-specific TCR into autologous T cells for possible future clinical applications (Xue et al., 2005). Because the WT1 is a self-protein expressed at low levels in some normal cells (Ellisen et al., 2001, Menssen et al., 1997), many of the potential CTL epitopes from this antigen may be absent or 'sub-optimal'. This is due to clonal deletion of high-avidity CTLs during maturation of the immune system. Therefore, the induction of antitumor immune responses frequently needs to be addressed against those 'sub-optimal' CTL epitopes to enhance their immunogenicity, frequency and hence their therapeutic potential. In addition, naturally arising $CD25^{+}CD4^{+}$ regulatory T cells (Tregs), play an important role in the maintenance of immunologic self-tolerance (Sakaguchi et al., 2006). Although this T cell population plays a key role in regulating the immune response to self antigen, it has been shown recently that Tregs can directly suppress the anti-tumour immune responses in cancer patients (Beyer and Schultze, 2006, Clarke et al., 2006, Khazaie and von Boehmer, 2006, Orentas et al., 2006). Indeed, another group demonstrated that the depletion of this T

cell population resulted in an enhancement of vaccine-mediated anti-tumour immunity in cancer patients (Dannull et al., 2005). Furthermore, a recent study showed that such response can be largely affected by the presence of $CD4^+CD25^+$ Tregs and depletion of this T cell population was necessary for the generation of an effective WT1- specific cytotoxic response (Asemissen et al., 2006).

It is known that the immunogenicity of antigenic peptides is dependent on their binding affinity to MHC class I molecules; stability of the peptide-MHC complexes; and the avidity of the TCR binding to the peptide complex (Yu et al., 2004). It has been demonstrated that the poor immunogenicity of some self/tumour antigens is due to the instability of the peptide-MHC complex rather than low affinity of T cell receptors to peptide-MHC complexes (Yu et al., 2004). Therefore, many studies have focused on introduction of a peptide modification to improve the binding of self antigenic peptides to their corresponding MHC class I molecules and consequently enhance the immunogenicity of these peptides (Bakker et al., 1997, Chen et al., 2000, Hoffmann et al., 2002, Parkhurst et al., 1996, Rosenberg et al., 1998, Tangri et al., 2001, Valmori et al., 1998). In the present study, we have applied a previously described strategy to enhance the immunogenicity of low-avidity HLA-A0201-restricted peptides without altering their antigenic specificity (Tourdot et al., 2000). Briefly, this consists of substituting the first amino acid of the epitope with a tyrosine (Y). The importance of this approach is that the resulting peptide analogues are able to efficiently trigger in vivo wild-type peptide-specific CTLs which also recognised the naturally occurring epitope (Tourdot et al., 2000). We have carried out the Y amino acid substitution in the sequence of the two known HLA-A*0201- restricted Db126 and WH187 WT1-derived peptides (Oka et al., 2000). Using a computer-based epitope prediction program, the 126Y and 187Y analogue peptides were predicted to bind 4.6-fold more to the HLA-A0201 molecule compared to their native 126N and 187N counterparts. However, a significant enhancement of the actual binding ability was recorded for the 126Y peptide analogue when we used the T2 binding assay. Therefore, we generated CTLs lines against the wild-type 126N and its 126Y peptide analogue. Although both peptides were able to induce specific cytotoxic immune responses against autologous peptide-loaded T2 cells, the CTL line generated with the 126Y peptide showed a higher and significant lytic activity compared with the CTL line generated with the

126N wild-type peptide. Interestingly, CTL generated against the 126Y analogue peptide was able to lyse the T2 target cells when loaded with the wild-type peptide. Therefore, modification of the 126 peptide resulted in enhancement of its immunogenicity without altering its antigenic specificity. Another important finding was the significant high lytic activity observed for the 126Y CTL line against HLA-A*0201-matched leukemic cells expressing the WT1 antigen. These data imply that the generated 126N and 126Y T cell lines were different. In addition, these data confirm that T cells generated against the 126Y analogue peptide cross-react also with the naturally processed 126N native peptide. These results suggest that stimulation with the 126Y peptide analogue induced CTLs with a high TCR avidity. Finally, the high lytic activity provoked by the 126Y CTL may also be attributed to the significantly high number of anti-126 T cells in this T cell line as demonstrated by γ-IFN production.

During the course of this study, Pinilla-Ibarz et al. (Pinilla-Ibarz et al., 2006) reported on a similar study based on the same strategy to modify several HLA-A0201 WT1-derived peptides including the 126N, 187N and their analogues as described in the present work. Although, this group did not find any difference in the binding affinity between the 2 126N and 126Y peptides, they reported that stimulation with the 126Y peptide analogue was able to generate an earlier and stronger specific CTL than that generated with the 126N wild-type peptide. Consistent with our findings the 126Y CTL line was able to recognise the 126N wild-type peptide on the target cells and specifically lyse HLA-A0201-matched leukaemia cells expressing the WT1 antigen. Another group has introduced a tyrosine modification in position two of a 9-mer WT1-derived peptide which is known to bind to the HLA-A*2402 molecule and showed a better binding affinity and elicitation of higher lytic activity (Tsuboi et al., 2002). Moreover, the modified peptide has been used to vaccinate cancer patients where a clear correlation was observed between an increase in the frequencies of WT1-specific CTL after vaccination and clinical responses (Oka et al., 2004).

In conclusion, this study is consistent with previously reported findings (Oka et al., 2004, Pinilla-Ibarz et al., 2006, Tsuboi et al., 2002) and has shed more light on the importance of peptide modification as a new approach in elucidating a better

immune response, needed to combat cancer. On the basis of these findings, leukaemia patients could be clinically tried for vaccination with the current WT1-126Y peptide analogue or adoptively treated with ex vivo anti-WT1-126Y T cells to specifically enhance the graft-versus-leukaemia effect and anti-WT1 CTL frequency, which are known to be very low in these patients.

4. IDENTIFICATION OF A NOVEL WT1-DERIVED EPITOPE RECOGNIZED BY HUMAN $CD4^+$ T CELLS

4.1. Introduction

Compelling evidence indicates a key role for regulatory T cells (Tregs) in the host response to cancer. In both mice and humans, Treg populations were shown to directly suppress anti-tumour immunity and to contribute to tumour growth and progression (Beyer and Schultze, 2006, Ghebeh et al., 2008, Shen et al., 2009, Ke et al., 2008, Sakaguchi, 2004, Lizee et al., 2006). Depletion or reduction of this T-cell population restored the anti-tumour immunity and resulted in an enhancement of vaccine efficacy in cancer patients (Dannull et al., 2005, Ruter et al., 2009, Nishikawa et al., 2005, Mahnke et al., 2007). Furthermore, patients with solid tumours as well as haematological malignancies have been shown to have increased prevalence of Tregs (Sasada et al., 2003, Ichihara et al., 2003, Strauss et al., 2007, Miller et al., 2006, Wang et al., 2005b, Beyer et al., 2006) . The presence of increased number of Tregs in cancer patients has been shown to be inversely related to the outcome of several malignancies (Salama et al., 2009, Heimberger et al., 2008). Tregs may directly modulate the $CD8^+$ T-cell response or alternatively promote tolerization of $CD8^+$ T cells by preventing the licensing of antigen-presenting cells (APC) by $CD4^+$ T helper cells (Alpan et al., 2004, Bourgeois et al., 2002). It has became clear that $CD8^+$ T cells generated in the absence of $CD4^+$ T cells help may have a normal primary response; however, their cytotoxic memory response is severely weakened (Janssen et al., 2003).

In our lab, we generated a T cell line and clones that specifically recognized a $WT1_{333-347}$ (RYFKLSHLQMHSRKH) peptide designated as WT1-84 in an HLA-DRB1*0402–restricted manner. These T-cell clones produced GM-CSF, IL-4 and IL-5 in response to WT1-84 peptide exerting a T helper 2 cytokine profile. Importantly, they recognized HLA-DRB1*04 matched fresh leukemic cells expressing the WT1 antigen. These clones had a $CD4^+CD25^+Foxp3^+GITR^+CD127^-$ Tregs phenotype and significantly inhibited the proliferative activity of allogeneic T cells Importantly, anti–WT1-84 interleukin-5^+/granzyme B^+/$Foxp3^+$ $CD4^+$ Tregs have been detected in five of eight HLA-$DR4^+$ acute myeloid leukaemia patients (Lehe et al., 2008).

4.2. Aim

Specific anti-WT1 immune responses have been described in which $CD8^+$ cytotoxic T cells have been generated in vitro (Asemissen et al., 2006, Oka et al., 2000, Azuma et al., 2002, Bellantuono et al., 2002, Li et al., 2008). However, a recent study showed that such response can be largely affected by the presence of $CD4^+CD25^+$ Tregs in which depletion of this T-cell population was necessary for the generation of an effective WT1-specific cytotoxic response (Asemissen et al., 2006). In our lab, we generated a Treg clones specific for the WT1-84 antigen in an HLA-DRB1*0402–restricted manner. We also showed the existence of this T cell population in leukaemia patients. The aim of this part of the study was to investigate whether this T cell type could also be generated from other HLA-$DR04^+$ donors. In this chapter, specific T cell lines and clones were generated against a mixture of peptides derived from the WT1 antigen (WT1-Pepmix) and tested for their peptide specificity by proliferation assay against different APCs loaded with WT1 peptides and also against WT1-expressing leukemic cells.

4.3. Results

4.3.1. Generation of T-cell lines against the WT1-Pepmix and analysis of their HLA restriction

In order to confirm our previous findings in more HLA-DRB1*04 positive donors, T-cell lines from three healthy individuals designated as BC-52, BC-57 and BC-62 were generated. Two of the donors, BC-52 and BC-62, share the same HLA-DR alleles i.e. 0402,1601 and matched with the donor used to generate the WT1-84 reactive T cell lines in one of the DR alleles i.e., *0402, whereas the BC-57 donor has HLA-DR-*0406, 0701 alleles (Table 4.1). PBMCs from the three donors (BC-52, BC-57 and BC-62) were stimulated for two rounds with autologous-conventional DCs generated from blood monocytes as described in Materials and Methods section 2.5.4.2. The phenotype of the generated DCs from the three donors was verified by FACS analysis (Figure 4.1A, B and C). After two rounds of stimulation, the cells were re-stimulated with different autologous APCs i.e. monocytes and activated T cells (T-APCs). The T-APCs were prepared from a single donor (BC-57) as described in Materials and Methods section 2.5.3. Mature

DCs were pulsed with WT1-Pepmix, a mixture of 110 synthetic 15-mer peptides that overlaps by 11 amino acids and spans the entire WT1 sequence. Figure 4.2 shows the sequence of the 110 peptides of the WT1-Pepmix. Corresponding T-cell lines designated TCL-52, TCL-57, and TCL-62 were generated and their proliferative activity was assessed in a thymidine uptake [^{3}H]-proliferation assay. All T cell lines were tested after the fourth and the fifth round of stimulation and showed specific proliferative activity against autologous PBMCs pulsed with WT1-Pepmix (Figure 4.3 A and B). Both TCL-52 and TCL-62 lines showed high proliferative activity against PBMCs pulsed with WT1-84 peptide. Additionally, the HLA restriction of the lines was determined in which anti–HLA-DR mAb was found to significantly inhibit their proliferation (Figure 4.3A & B). These data indicate that the two cell lines (TCL-52 and TCL-62) recognized WT1-84 peptide in an HLA-DR restricted manner and thereby confirming the previous findings. The TCL-52 and TCL-62 were extensively studied by my colleague Cynthia Lehe who published her findings in the 2008 August 1 issue of the Cancer Research Journal (Lehe et al., 2008).

The proliferation of TCL-57 against PBMCs and LCLs pulsed with the WT1-84 peptide was not very strong indicating that the T cells were not recognizing the WT1-84 peptide (Figure 4.4). However, the TCL-57 showed strong proliferative activity against autologous PBMCs and LCLs pulsed with the WT1-Pepmix as shown in Figure 4.4. The mean proliferation of TCL-57 to the Pepmix was 3.5 and 1.8 times higher than the proliferation to autologous PBMCs and LCLs pulsed with the WT1-84 peptide, respectively (Figure 4.4). To analyze the HLA restriction of the TCL-57, an antibody-blocking assay was performed using monoclonal antibodies specific to class I and class II molecules. As shown in Figure 4.5, the proliferation of TCL-57 was inhibited to some extent by the use of anti-HLA-ABC, anti-HLA-DP and anti-HLA-DQ mAb but it was significantly inhibited by using anti-HLA-DR mAb. These data indicate that the TCL-57 recognized a peptide within the 110 WT1-Pepmix peptides in an HLA-DR-restricted manner. The phenotype analysis of the generated TCL-57 using the flow cytometry revealed that that 95% of the generated T cells were $CD4^+$ T cells (Figure 4.6A). These cells were also analyzed for the expression of two important Tregs markers CD25 and Foxp3 in $CD4^+$ T cells. As shown in Figure 4.6B, 16.8% of the $CD4^+$ T cells co-express CD25 and Foxp3.

Table 4.1. HLA typing of normal donors used to generate anti-WT1 T cell lines.

Subjects	***HLA-A***	***HLA-B***	***HLA-DRB1****	***HLA-DQ***	***HLA-DP***
BC-52	ND	ND	0402,1601	0302,0502	ND
BC-57	2,3	5,5	0406,0701	0202,0402	0401,0402
BC-62	ND	ND	0402,1601	0302,0502	ND

ND= not done

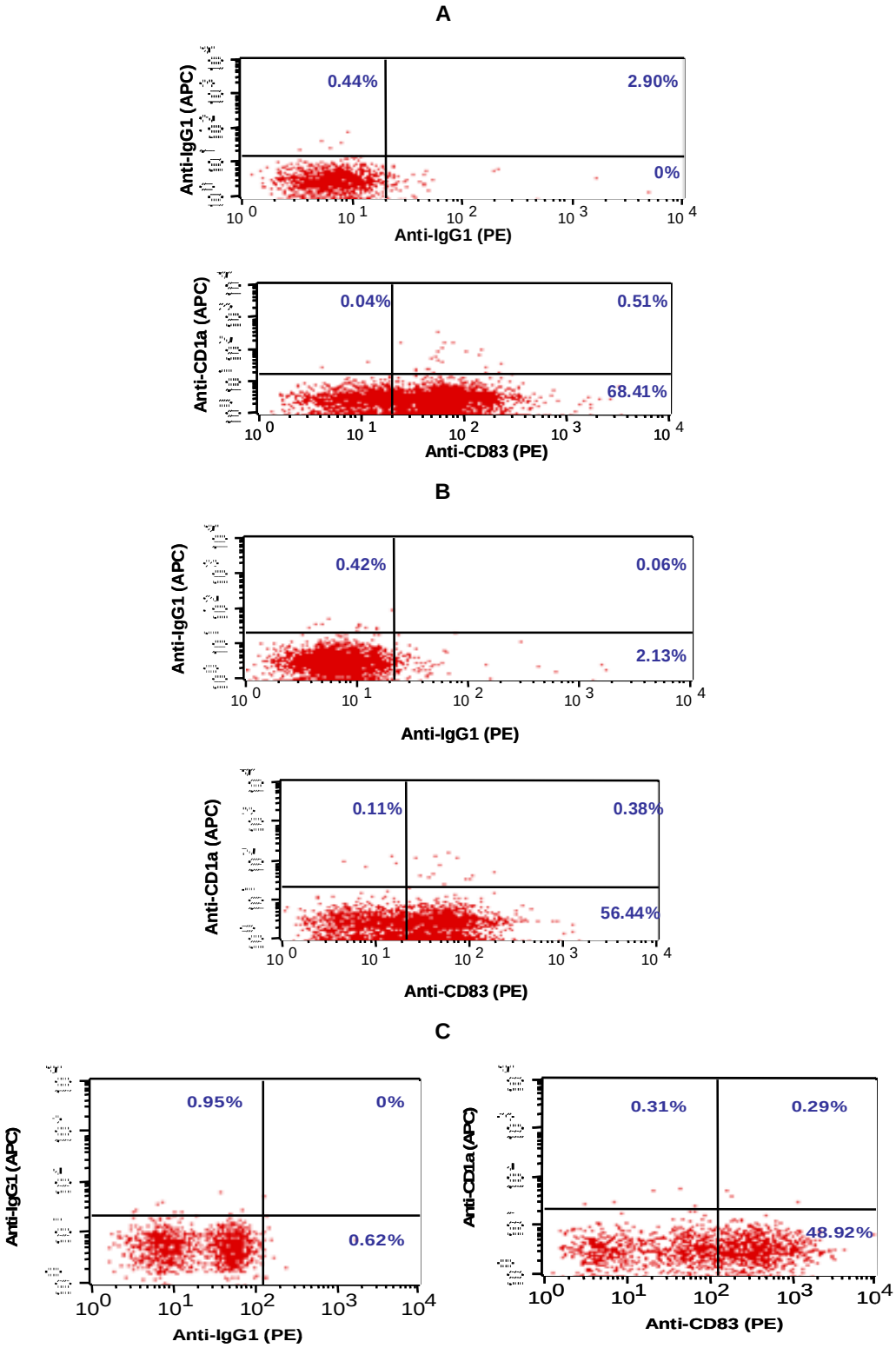

Figure 4.1. Flow cytometry analysis of CD1a and CD83 expression in the mature-monocytes derived DCs.

(A) (B) and (C) show the expression level of CD1a marker and the activation marker CD83 in DC-52, DC-57 and DC-62, respectively.

1. MGSDVRDLNALLPAV
2. VRDLNALLPAVPSLG
3. NALLPAVPSLGGGGG
4. PAVPSLGGGGGCALP
5. SLGGGGGCALPVSGA
6. GGGCALPVSGAAQWA
7. ALPVSGAAQWAPVLD
8. SGAAQWAPVLDFAPP
9. QWAPVLDFAPPGASA
10. VLDFAPPGASAYGSL
11. APPGASAYGSLGGPA
12. ASAYGSLGGPAPPPA
13. GSLGGPAPPPAPPPP
14. GPAPPPAPPPPPPPP
15. PPAPPPPPPPPPHSF
16. PPPPPPPPHSFIKQE
17. PPPPHSFIKQEPSWG
18. HSFIKQEPSWGGAEP
19. KQEPSWGGAEPHEEQ
20. SWGGAEPHEEQCLSA
21. AEPHEEQCLSAFTVH
22. EEQCLSAFTVHFSGQ
23. LSAFTVHFSGQFTGT
24. TVHFSGQFTGTAGAC
25. SGQFTGTAGACRYGP
26. TGTAGACRYGPFGPP
27. GACRYGPFGPPPPSQ
28. YGPFGPPPPSQASSG
29. GPPPPSQASSGQARM
30. PSQASSGQARMFPNA
31. SSGQARMFPNAPYLP
32. ARMFPNAPYLPSCLE
33. PNAPYLPSCLESQPA
34. YLPSCLESQPAIRNQ
35. CLESQPAIRNQGYST
36. QPAIRNQGYSTVTFD
37. RNQGYSTVTFDGTPS
38. YSTVTFDGTPSYGHT
39. TFDGTPSYGHTPSHH
40. TPSYGHTPSHHAAQF
41. GHTPSHHAAQFPNHS
42. SHHAAQFPNHSFKHE
43. AQFPNHSFKHEDPMG
44. NHSFKHEDPMGQQGS
45. KHEDPMGQQGSLGEQ
46. PMGQQGSLGEQQYSV
47. QGSLGEQQYSVPPPV
48. GEQQYSVPPPVYGCH
49. YSVPPPVYGCHTPTD
50. PPVYGCHTPTDSCTG
51. GCHTPTDSCTGSQAL
52. PTDSCTGSQALLLRT
53. CTGSQALLLRTPYSS
54. QALLLRTPYSSDNLY
55. LRTPYSSDNLYQMTS
56. YSSDNLYQMTSQLEC
57. NLYQMTSQLECMTWN
58. MTSQLECMTWNQMNL
59. IECMTWNQMNLGATL
60. TWNQMNLGATLKGVA
61. MNLGATLKGVAAGSS
62. ATLKGVAAGSSSSVK
63. GVAAGSSSSVKWTEG
64. GSSSSVKWTEGQSNH
65. SVKWTEGQSNHSTGY
66. TEGQSNHSTGYESDN
67. SNHSTGYESDNHTTP
68. TGYESDNHTTPILCG
69. SDNHTTPILCGAQYR
70. TTPILCGAQYRIHTH
71. LCGAQYRIHTHGVFR
72. QYRIHTHGVFRGIQD
73. HTHGVFRGIQDVRRV
74. VFRGIQDVRRVPGVA
75. IQDVRRVPGVAPTLV
76. RRVPGVAPTLVRSAS
77. GVAPTLVRSASETSE
78. TLVRSASETSEKRPF
79. SASETSEKRPFMCAY
80. TSEKRPFMCAYPGCN
81. RPFMCAYPGCNKRYF
82. CAYPGCNKRYFKLSH
83. GCNKRYFKLSHLQMH
84. RYFKLSHLQMHSRKH
85. LSHLQMHSRKHTGEK
86. QMHSRKHTGEKPYQC
87. RKHTGEKPYQCDFKD
88. GEKPYQCDFKDCERR
89. YQCDFKDCERRFSRS
90. FKDCERRFSRSDQLK
91. ERRFSRSDQLKRHQR
92. SRSDQLKRHQRRHTG
93. QLKRHQRRHTGVKPF
94. HQRRHTGVKPFQCKT
95. HTGVKPFQCKTCQRK
96. KPFQCKTCQRKFSRS
97. CKTCQRKFSRSDHLK
98. QRKFSRSDHLKTHTR
99. SRSDHLKTHTRTHTG
100. HLKTHTRTHTGKTSE
101. HTRTHTGKTSEKPFS
102. HTGKTSEKPFSCRWP
103. TSEKPFSCRWPSCQK
104. PFSCRWPSCQKKFAR
105. RWPSCQKKFARSDEL
106. CQKKFARSDELVRHH
107. FARSDELVRHHNMHQ
108. DELVRHHNMHQRNMT
109. RHHNMHQRNMTKLQL
110. HNMHQRNMTKLQLAL

Figure 4.2. Sequence list of the 110 single WT1-derived micro-scale peptides

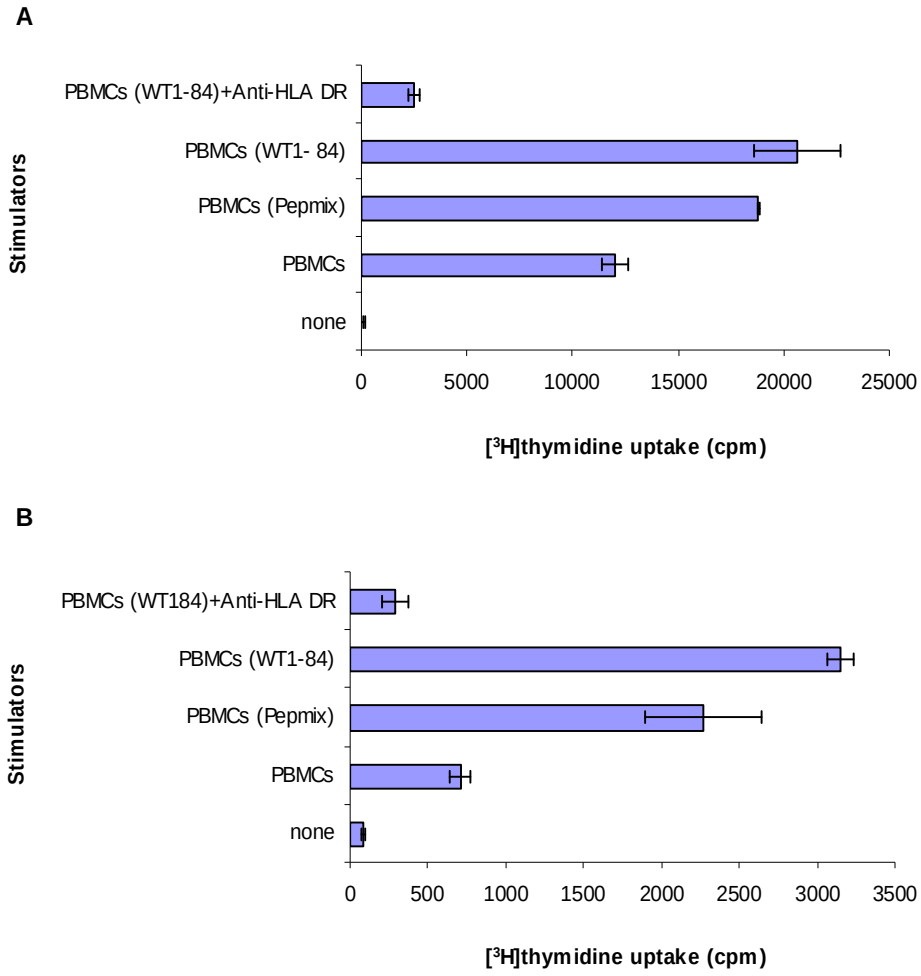

Figure 4.3. Proliferative activity of T-cell lines generated against the WT1-Pepmix.
T cells ($25x10^4$ per well) from two T-cell lines (TCL-52 and TCL-62) were challenged for 72 h with irradiated (2500 rads, XR) autologous PBMCs (10^5 per well) in the absence or presence of either WT1-Pepmix or WT1-84. Cells were pulsed with [^{3}H] thymidine for an additional 18 h and then harvested. T cells only (none) were used as a negative control. HLA-DR mAbs were added to the wells containing PBMCs 20 min before the addition of T cells to confirm that the response was restricted to the HLA-DR molecule. (A): the proliferative activity of TCL-52 mixed with irradiated autologous PBMCs pulsed with the WT1-Pepmix or WT1-84 peptide. (B): the proliferative activity of TCL-62 mixed with irradiated autologous PBMCs pulsed with the WT1-Pepmix or WT1-84. Data represent the mean of two different experiments performed in duplicate. Error bars indicate the SD.

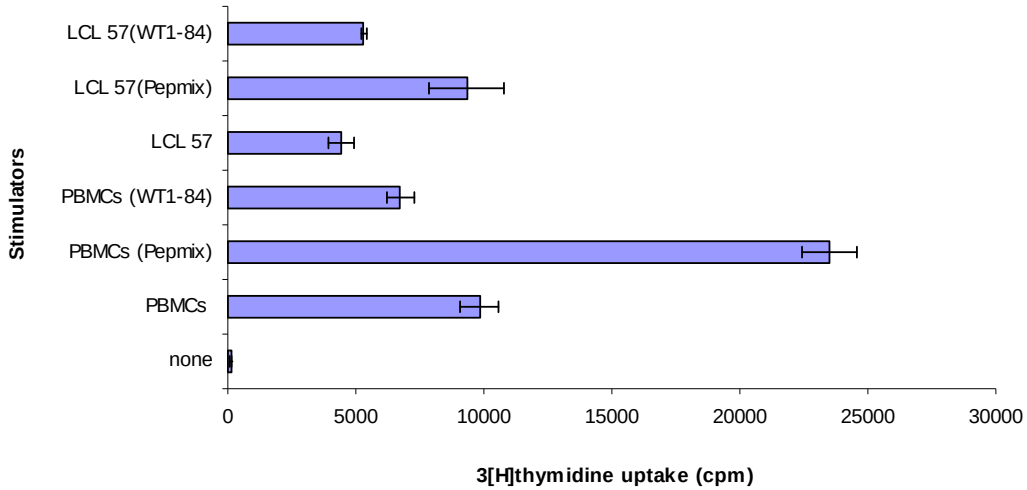

Figure 4.4. Proliferative activity of the T-cell line 57 generated against the WT1-Pepmix. T cells ($25x10^4$ per well) from a healthy donor were challenged for 72 h with irradiated (2500 rads, XR) autologous PBMCs (10^5 per well) in the absence or presence of either WT1-Pepmix or WT1-84. Cells were pulsed with [^{3}H] thymidine for an additional 18 h and then harvested. T cells only (none) were used as a negative control. Data represent the average of two different experiments performed in duplicate. Error bars indicate the SD.

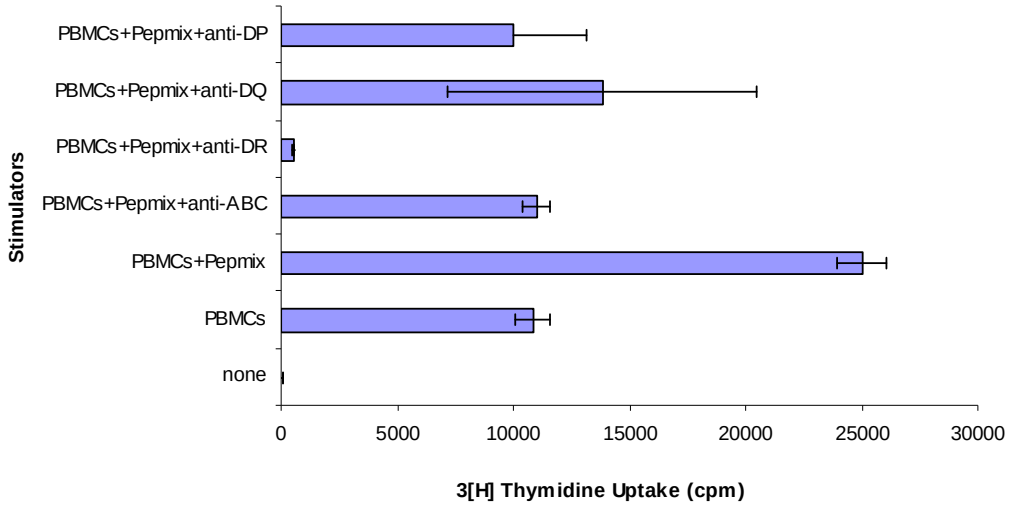

Figure 4.5. Effect of blocking antibodies on the proliferative responses of TCL-57.
T cells ($25x10^4$ per well) from a healthy donor were challenged for 72 h with irradiated (2500 rads, XR) autologous PBMCs (10^5 per well) in the absence or presence of WT1-Pepmix. In order to assess T cell restriction, HLA-ABC, HLA-DR, HLA-DQ and HLA-DP azide-free mAbs were added to the corresponding wells containing PBMCs 20 min before the addition of T cells. T cells only (none) were used as a negative control. Cells were pulsed with [^{3}H] thymidine for an additional 18 h and then harvested. Data represent the average of two experiments performed in duplicate. Error bars indicate the SD.

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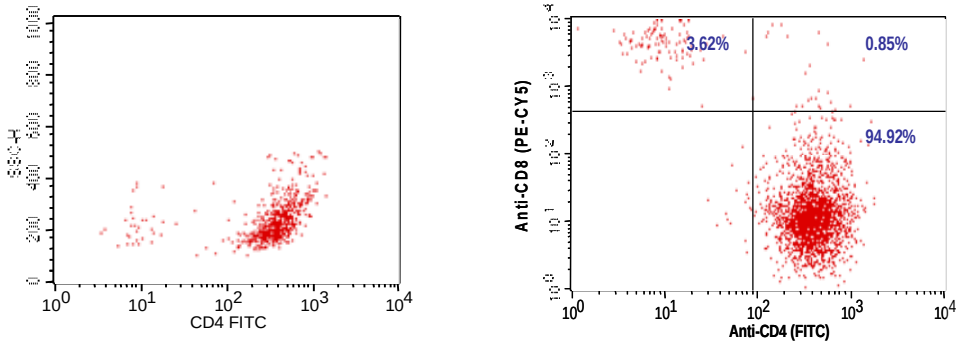

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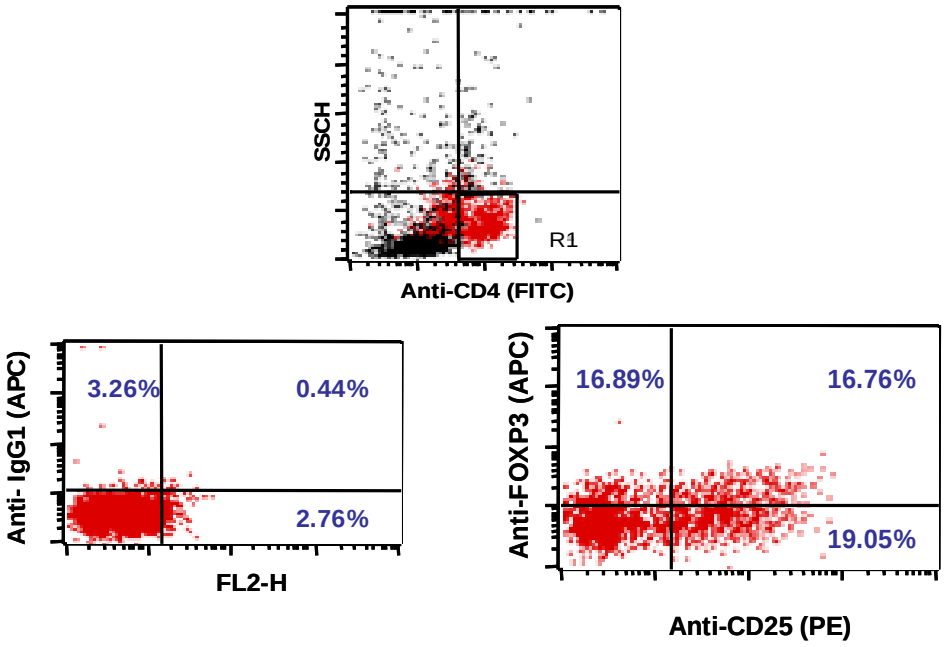


Figure 4.6. Flow cytometry analysis of CD8, CD4, CD25 and FOXP3 expression in the TCL-57.

(A): 95% T cells are $CD4^+$ T cells. (B): 16.8% of $CD4^+$ T cells co-express CD25 and FOXP3 molecules.

4.3.2. Generation of specific responses against WT1-Pepmix using dynabeads

The dynabeads T cell expander was used to activate the TCL-57 after several rounds of antigen-specific re-stimulations with autologous-APCs (DCs or monocytes) pulsed with the Pepmix. The dynabeads are polystyrene beads coated with monoclonal antibodies against the CD3 and CD28 cell surface molecules. The beads were used because the T cells were poorly proliferating which may have been due to the age of the T-cell cultures.

After the 9^{th} stimulation, the T cells were divided into two fractions. One of these fractions was re-stimulated with autologous monocytes loaded with the WT1-Pepmix and the other fraction was re-stimulated with the dynabeads. After two more rounds of stimulation, the cells were tested in a proliferation assay to evaluate the efficiency of bead-stimulation. Although the T cells stimulated with monocytes showed a specific proliferative response against WT1 pepmix, a very high proliferative capacity (7.5 fold) was recorded for T cells stimulated with the dynabeads when challenged with PBMCs pulsed with the Pepmix (Figure 4.7). The result demonstrated that the dynabeads T cell expander was very efficient in activating the exhausted T cells after several rounds of antigen-specific re-stimulation.

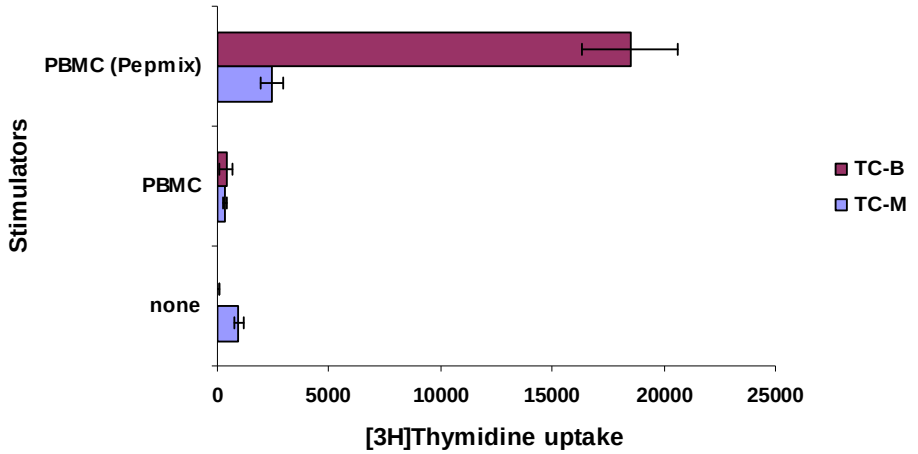
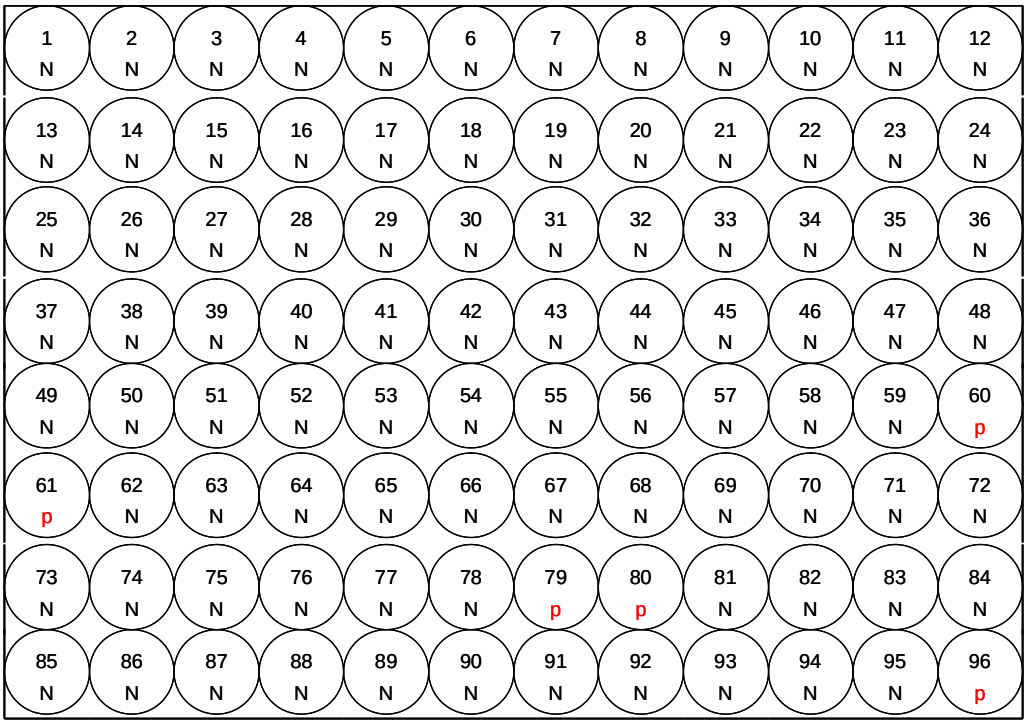


Figure 4.7. The efficiency of the dynabead T cell expander in activating exhausted T cells. T cells ($25x10^4$ per well), either re-stimulated with monocytes pulsed with WT1-Pepmix (TC-M) or with dynabeads (TC-B), were challenged for 72 h with irradiated (2500 rads, XR) autologous PBMCs (10^5 per well) in the absence or presence of WT1-Pepmix. T cells only (none) were used as a negative control. Cells were pulsed with [^{3}H] thymidine for an additional 18 h and then harvested. Error bars (±SD) represent duplicate wells from one experiment.

4.3.3. Identification of a novel WT1-60 peptide recognized by TCL-57 T cells

The specificity of TCL-57 was further investigated as this T cell line showed no response towards the WT1-84 peptide. The TCL-57 (activated with dynabeads) was screened by proliferation assay against individual 110 WT1-Pepmix peptides using a microscale WT1 peptide set, a 96-well plate that contains a single WT1 peptide of the 110-Pepmix peptides in each well (Figure 4.8A). In this proliferation assay, five peptides designated WT1-$60_{237\text{-}251}$, WT1-$61_{241\text{-}255}$, WT1-$79_{313\text{-}327}$, WT1-$80_{317\text{-}331}$ and WT1-$96_{381\text{-}395}$ induced strong proliferative activity (ranging from 4.7 to 8.3 fold) of the TCL-57 cells (Figure 4.8B). The rest of the peptides did not induce any significant proliferative activity. These five peptides were ordered and specificity of the TCL-57 was again tested by challenging T cells against each one of the individual-test peptides in a proliferation assay. As shown in Figure 4.9, the peptide WT1-$60_{237\text{-}251}$ (TMNQMNLGATLKGVA) induced the strongest T cell proliferative response. WT1-60 peptide induced a 10 fold increase in proliferative activity of TCL-57. In addition, the proliferative response of T cells to WT1-60 was 3.3 times higher than that against the Pepmix.

A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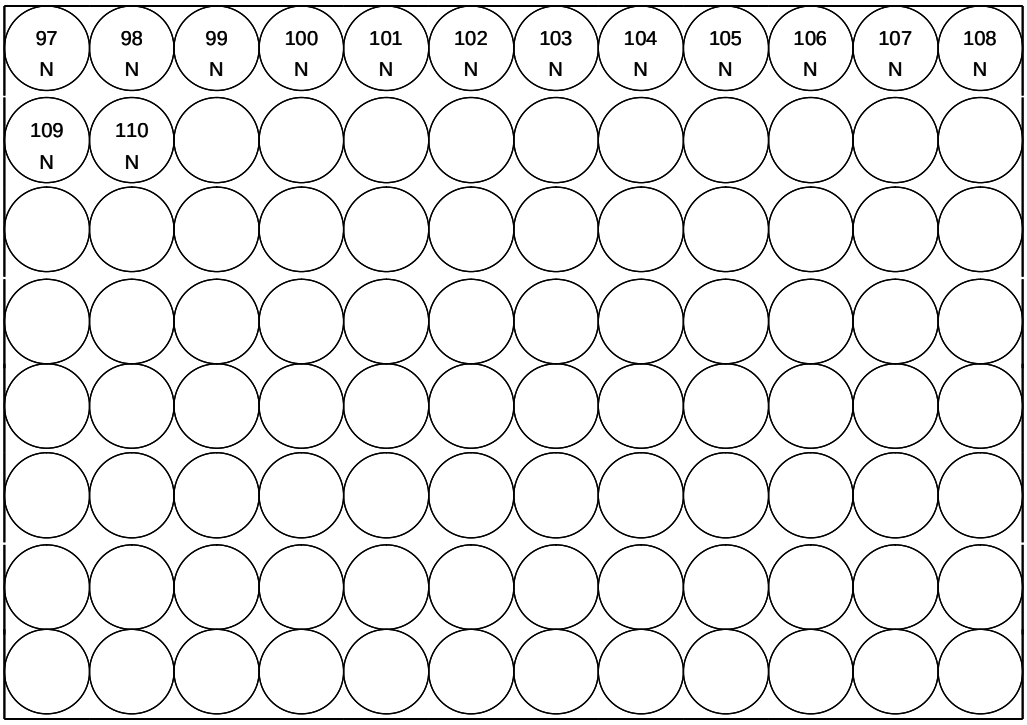
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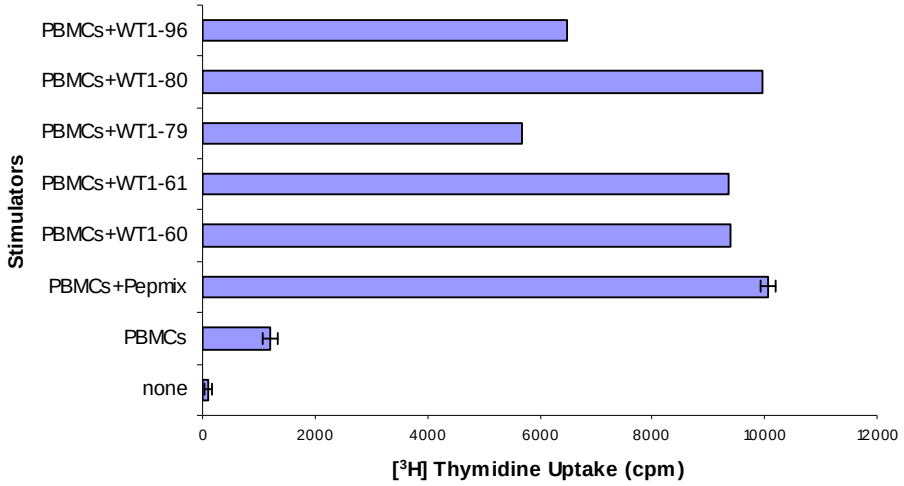

Figure 4.8. Screening of TCL-57 specificity using a microscale WT1 peptide set.

The proliferative activity of TCL-57 against PBMCs loaded with WT1-Pepmix or with individual peptide was tested to determine which single peptides induced responses to the WT1-Pepmix. T cells (15.000 cells/well) were challenged for 72 h with irradiated (2500 rads, XR) autologous PBMCs (500.000 cells/well) in a microscale plate. Cells were pulsed with [^{3}H] thymidine for an additional 18h and then harvested. (A) Shows the location of the WT1-Pepmix peptides in the 96-well plates. Each well of the plates contains a single peptide of the 110-peptide set. N, the peptide did not induce any proliferative activity of the TCL-57; P, the peptide induced a significant proliferative activity of the TCL-57. (B) Shows the proliferation of the TCL-57 from the same screening experiment against PBMCs loaded with WT1-Pepmix or with individual peptide that induced responses to the peptide pools. T cells only (none) were used as negative control. Error bars (±SD) represent duplicate wells from one experiment.

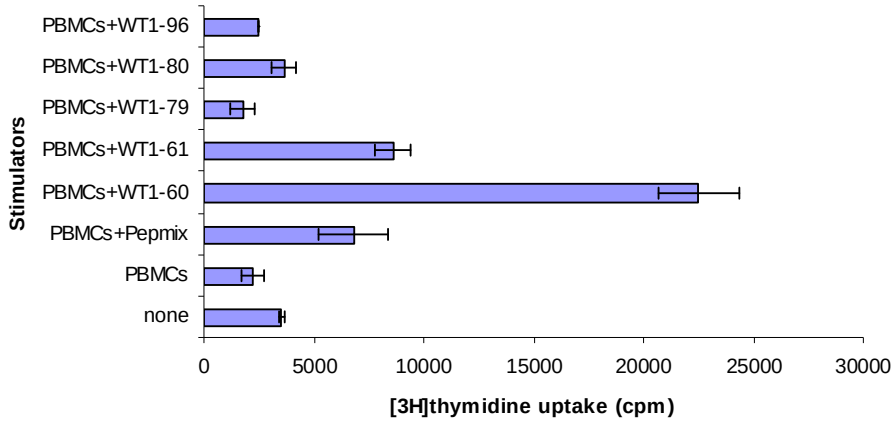

Figure 4.9. Proliferative activity of TCL-57 against the peptides WT1-60, WT1-61, WT1-79, WT1-80 and WT1-96 only.

This figure demonstrates that among the five peptides only one peptide (WT1-60) induced the highest proliferative activity of TCL 57. T cells ($25x10^4$ cells/well) were challenged for 72 h with irradiated (2500 rads, XR) autologous PBMCs (10^5 per well cells/well). Cells were pulsed with [^{3}H] thymidine for an additional 18 h and then harvested. T cells only (none) were used as negative control. Error bars (±SD) represent duplicate wells from two experiments.

4.3.4. Generation of TCL-57 derived T cell clones using the WT1-60 peptide and analysis of their HLA-restriction

Three attempts were performed to clone the TCL-57 T cells using a standard limiting dilution assay. T cells were seeded at 1, 2 and 5 cells/well against HLA-matched allogeneic LCLs loaded with the WT1-60 peptide in the presence of IL-2 and a pool of allogeneic PBMCs which was used as a feeder. Despite these three attempts to clone the T cells, no clones were obtained using this method. Therefore, another method was utilized to generate clones specific to the WT1-60 peptide. Because the dynabead CD3/CD28 T cell expander efficiently activated the poorly proliferating T cells, the beads were used to clone the T cells. The T cells were seeded at 5 cells/well in 96-well V-bottomed plates in the presence of the dynabeads and IL-2. The V-bottomed plates were used since no feeder cells were added. Using this method, 6 clones were obtained and were tested for their specificity using a proliferation assay against autologous PBMCs pulsed with or without the WT1-derived peptide. The attempts performed to clone the TCL-57 are summarized in Table 4.2. Figure 4.10 demonstrates that two clones, TCC-15 and TCC-19, were specific to WT1-60 peptide.

To define more precisely the HLA-DR restriction, HLA-DR–matched allogeneic LCLs sharing the DR-04 (LCL-11) or DRB1*0701 (LCL-18) with the TCC clones were used. Moreover, an HLA-DR*07–restricted epitope, $hTRT_{672-686}$ (RPGLLGASVLGLDDI), derived from human telomerase reverse transcriptase (Schroers et al., 2002) sequence was included as a negative control. Both clones, TCC-19 and TCC-15, did not show any proliferative response to LCL-18 loaded with WT1-60 peptide, ruling out the involvement of the DRB1*0701 in this presentation. However, LCL-11 sharing different HLA-DRB1*04 suballele (DRB1*0401) with the clones, induced a specific proliferative activity against the WT1-60 peptide, showing the requirement for DRB1*04 restriction in this process (Figure 4.11A and B). In addition, HLA-DR mAb blocked the T cell response showing the requirement for HLA-DR molecule in the presentation of WT1-60 peptide to both T cell clones (Figure 4.11A and B).

Table 4.2. Results of the attempts to clone the TCL-57.

Stimulators	*attempts*	*feeders*	*IL-2 (IU/mL)*	*Plates*	*Seeding density (cells/well)*		
					1	2	5
HLA-matched allogeneic LCLs	3	allogeneic PBMCs	130	U-bottomed	N	N	N
Dynabead CD3/CD28 T cell expander	1	Without feeders	130	V-bottomed	ND	ND	Y

N	No clones were obtained
ND	Not done
Y	Clones were obtained

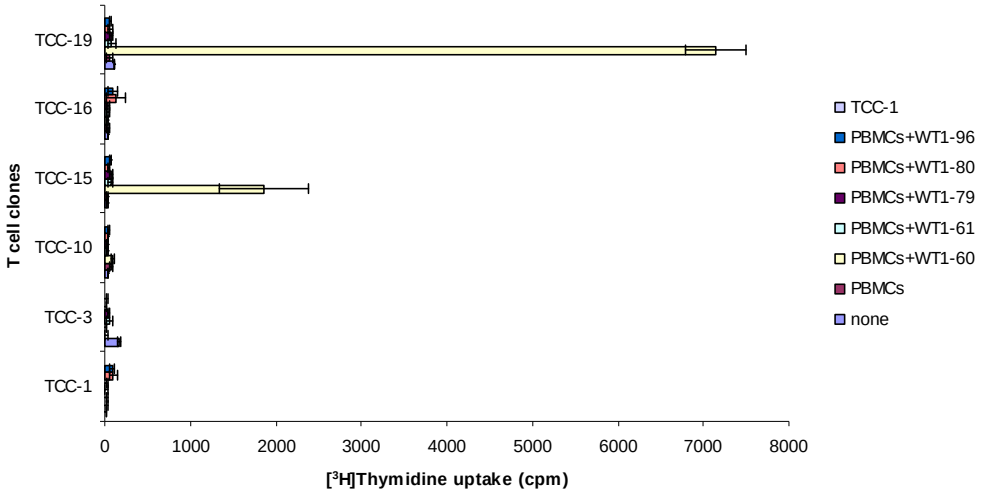

Figure 4.10. Proliferative activity of T cell clones generated from TCL-57.

The TCL-57 was cloned using the dynabead CD3/CD28 T cell expander. 6 clones were obtained and tested for their specificity against autologous PBMCs pulsed with (blue colour) or without WT1 derived peptide (green colour) in a proliferation assay.

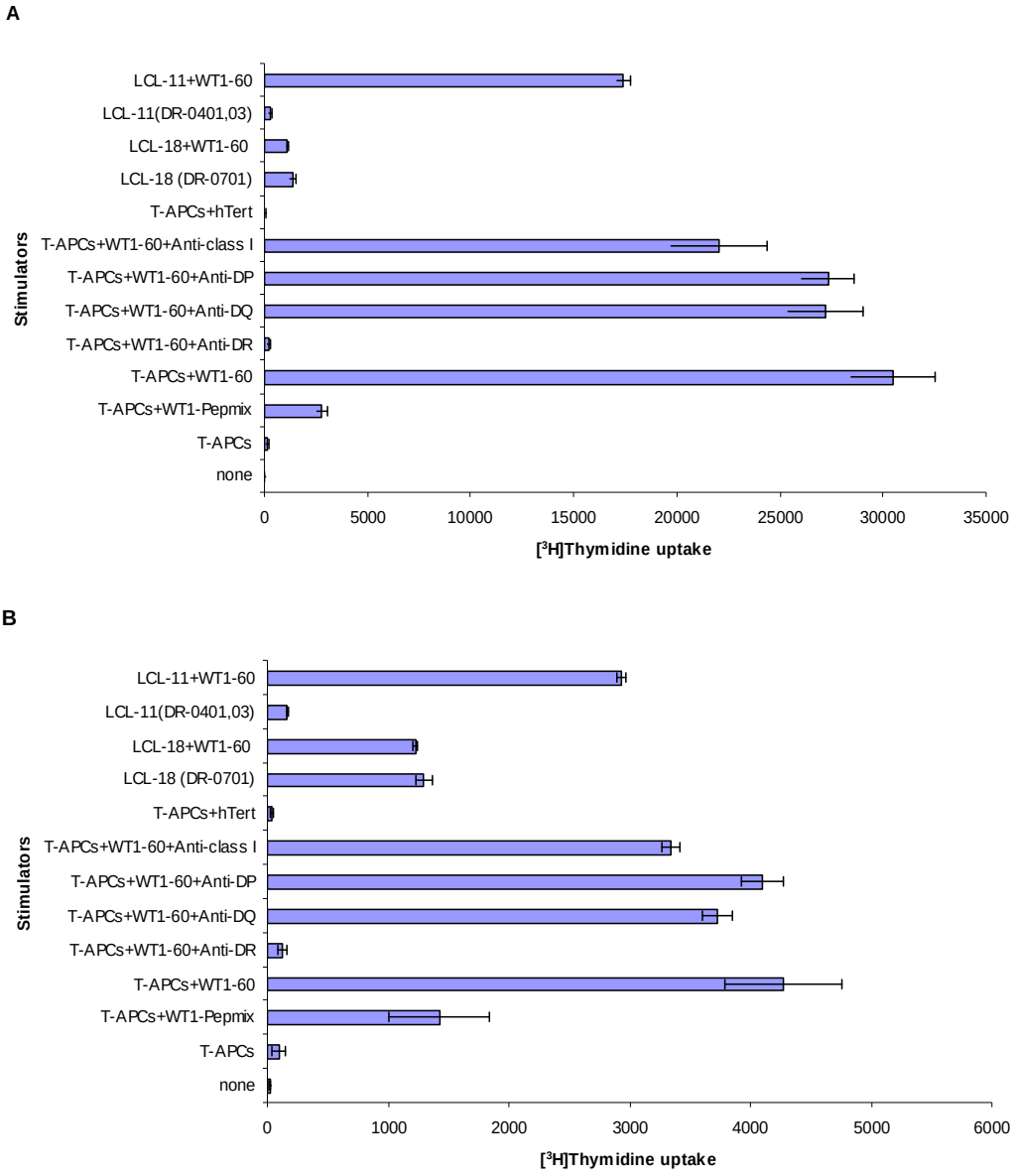

Figure 4.11. Proliferative activity of TCC-15 and TCC-19 T cell clones.

TCC-15 (A) or TCC-19 (B) T cells ($25x10^4$ per well) from a healthy donor were challenged for 72 h with irradiated (2500 rads, XR) autologous T-APCs (10^5 per well) or allogeneic LCLs in the absence or presence of either WT1-Pepmix or WT1-60. T-APCs pulsed with or without hTert peptide (HLA-DR7-restricted epitope) was included as a positive control. To assess for T cell restriction, HLA-ABC, HLA-DR, HLA-DQ and HLA-DP mAbs were added to the corresponding wells containing T-APCs 20 min before the addition of T cells. Cells were pulsed with [^{3}H] thymidine for an additional 18 h and then harvested. T cells only (none) were used as a negative control. Data represent the average of two different experiments performed in duplicate. Error bars indicate the SD.

4.3.5. Evaluation of the cytokine profile of the TCL-57 T cell line and its derivative clones

Cytokines generated during an immune response dictate the outcome of this response. Hence, the cytokine profile generated by the TCL-57 cell line and clones was evaluated by enzyme-linked immunosorbent assays (ELISAs). The released cytokines were determined from cell culture supernatants of T cell cultured for 48 hours with irradiated autologous PBMCs or T-APCs pulsed with WT1-Pepmix or WT1-60 peptide. Autologous PBMCs were used to test the cytokine released by the T cell line. Because no more autologous PBMCs were left, the T-APCs were used to test the cytokine released by the T cell clone. Before using the T-APCs in this assay, these cells were tested and compared with the PBMCs in a proliferation assay which showed that the T-APCs were highly effeicint in activating T cells.

A similar cytokine production pattern was found between the line and the two clones. However, the cytokines produced by the clones should identify the type of the immune response if a different pattern was seen. The T cell line secreted IL-4, IL-5 and GM-CSF specifically to WT1-60 and WT1-pepmix, and small amounts of TGF-β1, IL-10 and IFN-γ. This clearly indicates a T helper 2 (Th2) polarized immune response. For the clones, a similar cytokine production pattern was found although an increase in IFN-γ production was found upon stimulation with pepmix or WT1-60 which was more prominent in TCC-15. Figure 4.12 and Figure 4.13 show the cytokine profile of the T cell line and clones, respectively.

4.3.6. Proliferative activity of TCC-15 clone against HLA-DR-matched leukemic cells

The proliferative activity of the clone TCC-15 only was tested against PBMCs obtained from WT1-expressing AML and ALL patients because the TCC-19 cell number was too low. The expression of WT1 mRNA in leukaemia samples was examined using conventional and quantitative RT-PCR (Table 4.3). Because the HLA-DR was the restriction element for the clones, its expression was evaluated by FACS analysis. All the examined leukemic samples expressed HLA-DR, although at different levels (Table 4.3). In addition, Table 4.3 shows the percentage of leukemic blasts in the sample used. PBMCs from DR0701-1302 matched for DR0701 AML

(AML-28), DR0701-0406 fully matched ALL (ALL-21) and HLA-DR0301-0405 allelic positive ALL (ALL- 16) were used. The DR non-matched 1104-1605, but WT1-expressing AML (AML-27) was used as a negative control. As shown in Figure 4.14, the TCC-15 clone did not recognize any of the leukemic cells used.

The data indicates that WT1-60 peptide might be either not naturally processed by the leukemic cells or only very low levels of this peptide is presented but it is insufficient to be recognized by anti-WT1 specific T cells.

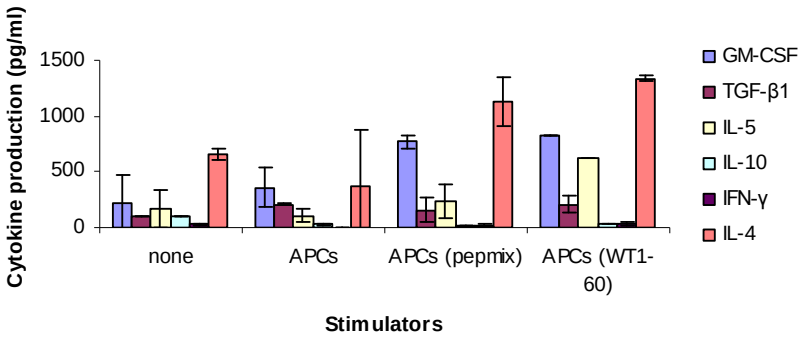

Figure 4.12. Cytokine productions by the TCL-57 T-cell line.

The cytokines were quantified by ELISAs using cytokine-specific capture antibodies. The released cytokines were determined from cell culture supernatants of T cell cultured for 48 hours with irradiated autologous PBMCs pulsed with WT1-Pepmix or WT1-60 peptide. The cytokines level was determined twice. Values represent the mean of the two experiments performed in duplicate. Error bars indicate the SD.

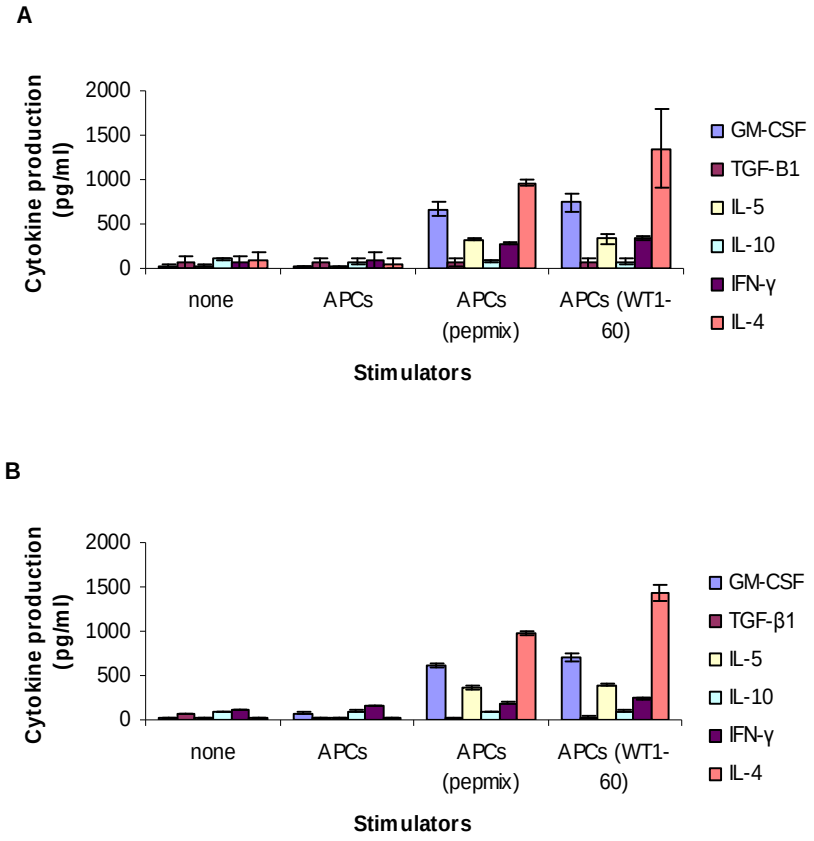

Figure 4.13. Cytokine production by the T cell clones TCC-15 and TCC-19.

The cytokines were quantified by ELISAs using cytokine-specific capture antibodies. The released cytokines were determined from cell culture supernatants of T cell cultured for 48 hours with irradiated autologous T-APCs pulsed with WT1-Pepmix or WT1-60 peptide. (A) Cytokine production by the T cell clone TCC-15. The cytokine levels were determined twice for TCC-15. Data represent the mean of two different experiments performed in duplicate. Error bars indicate the SD. (B) Cytokine production by the T cell clone TCC-19. The cytokine levels were determined only once for TCC-19. Data represent the mean of duplicate wells from one experiment. Error bars indicate the SD.

Table 4.3. Patients information.

Patients	*% Blasts*[1]	*HLA-DRB1**	*QRT-PCR*[2]	*RT-PCR*[3]	*% HLA-DR/MFI*[4]	*Status*[5]	*Subtypes*
AML-27	81	1104,1605	7950	+++	60/200	new	M4
AML-28	61	0701,1302	1800	+	ND	new	M4
ALL-16	NA	0301,0405	8780	++	20/211	new	T-cell
ALL-21	83	0701,0406	333	+	72/667	new	Pre B-cell

1 % blasts were determined routinely by the hematopathology laboratory

2 Quantitative real-time PCR data for the expression level of WT1 which normalized from K562 on 1000

3 Conventional RT-PCR data for the expression of WT1

4 % positive for HLA-DR molecule/mean of fluorescence intensity (MFA) as determined by FACS analysis

5 New indicates that samples were taken at first diagnosis.

NA=not available

ND= not done

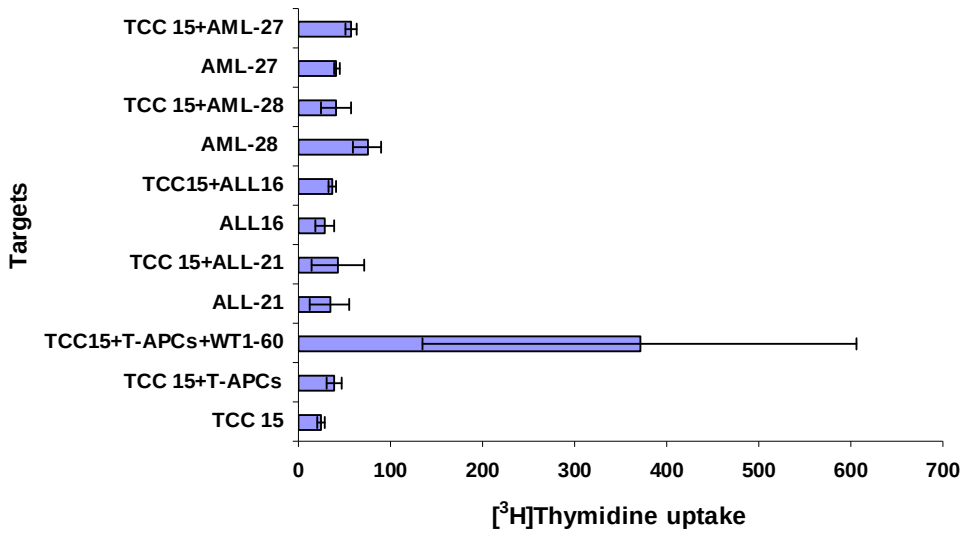

Figure 4.14. Evaluation of proliferation of TCC-15 against different HLA-matched leukemias. Proliferative activity of TCC-15 against PBMCs from DR0701 matched AML (AML-28), non-matched (AML-27), DR0701-0406 matched (ALL-21) and HLA-DR04 allelic positive (ALL- 16) patients. T cells ($25x10^4$ per well) were challenged for 72 h with irradiated (2500 rads, XR) PBMCs (10^5 per well) from AML and ALL patients. Cells were pulsed with [^{3}H] thymidine for an additional 18 h and then harvested. T cells only and T cells challenged with irradiated autologous T-APCs in the absence or presence of the WT1-60 peptide served as negative and positive controls, respectively. Data represent the mean of two different experiments performed in duplicate. Error bars indicate the SD.

4.4. Discussion

We have previously identified novel human anti-WT1 Tregs cell line and clones using a pool of overlapping peptides spanning the entire WT1 sequence. The T-cell line and clones specifically recognized a WT1-$_{333-347}$ (RYFKLSHLQMHSRKH) peptide designated as WT1-84 in an HLA-DRB1*0402–restricted-manner (Lehe et al., 2008). In this study, the same methodology was used to generate antigen-specific $CD4^+$ T cells with specific proliferative responses to the previously identified peptide in 2 out of 3 donors. In this, TCL-52 and TCL-62 generated T cell lines from two donors who share the same HLA-DR-allele (HLA-DR-*0402) with the donor used in the previous study recognized the WT1-84 epitope. However, the TCL-57 T cell line generated from a donor with an HLA-DR-*0406 allele failed to recognize the previously identified epitope but rather recognized another peptide WT1-60$_{237-251}$(TMNQMNLGATLKGVA) designated (WT1-60).

The WT1-60 peptide was able to induce a specific proliferative response in the generated T cell line and clones against peptide-pulsed autologous APCs. However, no significant proliferation of the T -cell clone was recorded against leukemic cell samples of three patients expressing the WT1 antigen as well as the HLA-DR molecule. Furthermore, the WT1-60 peptide was able to induce a specific proliferative cross-reactivity in the T cell clones after challenging with LCL-11 sharing the HLA-DR04 allele with the clones. No proliferative response of the T cell clones was recorded against LCL-18 (HLA-DR0701 homozygous donor). Therefore, the WT1-60 peptide seems to be presented through the HLA-DR04 molecule. Because AML-28 donor shares the HLA-DR*0701 with the clones, no proliferative response of the T cell clone was recorded against this leukemic cells. The failure of the T cell clone to recognize ALL-16, although expressing high levels of WT1 antigen and shares the HLA-DR04 allele with the clones might be due to the differences in the sub-allelic level or to low HLA-DR expression. However, the fully-matched HLA-DR ALL sample (ALL-21) did not induce any proliferative response in the T cell clone. In this, 72% of the ALL-21 cells express the HLA-DR molecule, however leukemic cells from this patient did not express high levels of the WT1 antigen and this may account for the T cell unresponsiveness. Another, explanation for non-recognition of this epitope by the leukemic cells is that the

peptide may not be naturally processed or may be below the threshold required to trigger T-cell recognition and effector function in addition to the inhibitory effect of the leukemic cells (Dermime et al., 2002).

The generated T cell line and clones exerted a Th2 cytokine profile (IL-5, IL-4) in response to the WT1-60 peptide. It has been shown that the affinity of the antigen for TCR and the antigen dose can influence the differentiation of Th1 and Th2 cells (Constant and Bottomly, 1997). Th2 cells are generated only in the presence of a low dose of an antigen, and a weak TCR signal is needed for their generation (Tao et al., 1997). In this study, only 10 μg/mL of the 110 Pepmix including the $WT1_{237-251}$ peptide were used to prime the T cells. Therefore, the antigen dose and peptide competition in the case of the WT1-Pepmix may contribute to the generation of a Th2 helper cell type. In addition, high concentration of IL-2 (260 IU/mL) was used for the T-cell generation. High dose of IL-2 has been shown to increase the frequency of Tregs in cancer patients (Ahmadzadeh and Rosenberg, 2006). It is possible that the presence of high dose of IL-2 in the T-cell cultures played a role in the polarization of T cells to Th2 phenotype as it has been described in other systems (Cote-Sierra et al., 2004).

A recent study by Durinovic-Bello and colleagues (Durinovic-Bello et al., 2006) showed that human Th2 cells that exert a down-regulatory Treg phenotype also express the Foxp3 molecule. However, Foxp3 has also been shown to be expressed transiently in activated non-Tregs, whereas it is stably expressed in Tregs (Wang et al., 2007). In the present study, 16.8% of the generated TCL-57 T cell line expressed Foxp3 and exerted a $CD4^+CD25^+Foxp3^+$ Treg phenotype. This percentage is higher than the percentage of native Treg isolated from peripheral blood, which is usually does not exceed 5% of $CD4^+$ T cells in normal adult individuals. A preliminary result showed that the T cell clones expressed higher Foxp3 molecule than the TCL-57 T cell line. However, the expression of Foxp3 and other Treg-related markers such as GITR, CTLA-4 and CD127 in the T cell clone (TCC-15) should be further examined to confirm the Treg phenotype. It is well known that Tregs suppress effector T cells by inhibiting activation, proliferation, differentiation, and effector function (Sakaguchi et al., 2009, Askenasy et al., 2008, Spadafora-Ferreira et al., 2007). Therefore, the generated clone should be tested for its ability to inhibit

immune responses in functional assays to confirm the suppressive effect of these T cells.

The use of anti-CD3 and anti-CD28 monoclonal antibodies to expand and clone antigen-specific T cells has been previously reported (Riddell and Greenberg, 1990, Trickett and Kwan, 2003). This method has also been described to rapidly expand antigen-specific T cells for potential clinical applications (Thompson et al., 2003, Garlie et al., 1999, Laport et al., 2003) . In the present work this methodology was used to activate and expand the exhausted T cell line (TCL-57) using beads coated with anti-CD3/anti-CD28 monoclonal antibodies. This method was very efficient not only in activating the T cells but also in enhancing the process of T cell cloning. In this, the T cell line and clones retained their specificity to both Pepmix and WT1-60 peptide following two rounds of re-stimulations with the beads. Therefore, this method allowed the activation and expansion of $CD4^+$ T cells without altering their antigenic specificity. In addition, this method dramatically reduces the number of antigen presenting cells required for re-stimulation.

In conclusion, antigen-specific $CD4^+$ T cells were detected using a pool of overlapping peptides spanning the entire sequence of the WT1 antigen. These cells showed very high and specific proliferation response to the WT1-$60_{237-251}$ peptide and exerted a Th2 cytokines (IL-5, IL-4) profile. Therefore, the generated $CD4^+$ T cells can be considered as a WT1-60-specific Th2 T cell type. Further investigations are needed to examine whether these cells are of theTh2 like-Treg type. If these cells were confirmed to be Tregs, leukemic PBMCs should be tested to verify whether this population exists in leukaemia patients as they may then contribute to the inhibition of specific T cell anti-WT1 responses in such patients similar to that described in our previous study (Lehe et al., 2008).

5. IMMUNOGENICITY OF M-PHASE PHOSPHOPROTEIN 11 AND PROTEINASE-3 IN LEUKAEMIAS

5.1. Introduction

Despite major advances in molecular biology and targeted therapy of leukaemia, current treatment strategies induce adverse side effects and fail to achieve and maintain remission in many patients. In acute myeloid leukaemia (AML), the most deadly form of leukaemia (Bozzone, 2009), the majority of adult patients younger than 60 years achieve a complete remission following consolidation therapy. However, only approximately 30 to 40% maintain durable remission (Tallman et al., 2005). Furthermore, the complete remission rate and duration attained by older AML patients is less, with an overall survival rate of 20 to 40% (Tallman et al., 2005). Therefore, there is an urgent need for the development of leukaemia-targeted immunotherapy designed to eliminate residual leukemic cells thus enhancing the graft-versus-leukaemia (GVL) effect observed after allogeneic hematopoietic stem cell transplantation (HSCT) and/or prolonging a complete remission achieved by chemotherapy (Guglielmelli et al., 2007, Boon and Old, 1997).

The WT1 antigen represents a crucial target for tumour immunotherapy. However, cancer cells frequently escape T-cell recognition by losing or down-regulating the expression of the targeted-tumour antigen (Begley and Ribas, 2008). Therefore, immunotherapeutic approaches based on targeting epitopes derived from more than one antigen may be more effective in inducing anti-tumour immune responses.

5.2. Aim

There is an urgent need for the development of leukaemia-targeted immunotherapeutic approaches using defined leukaemia-associated antigens (LAA) that are preferentially expressed by most leukaemia subtypes and absent or minimally expressed in vital tissues. Although various antigenic epitopes derived from leukaemia antigens have been identified, progress in targeting specific immunotherapeutic approaches depends in the recognition of a wider range of leukaemia antigens. The aim of the study was to identify potential $CD8^+$ cytotoxic T lymphocyte (CTL) epitopes in the leukaemia-associated antigens PR-3 and MPP11 that bind to the most common class I molecule, HLA-A*0201 molecule, and provoke specific cytotoxic T lymphocyte responses. This would allow the use of

these peptides in leukaemia immunotherapy by either expanding ex vivo tumour-reactive specific CTLs for adoptive T-cell immunotherapy or actively inducing leukaemia antigen-specific T–cell immunity in vivo by vaccinating patients with well defined leukaemia-associated antigens. Forty samples from AML and ALL patients were also screened for MPP11 positivity.

5.3. Results

5.3.1. MPP11 antigen screening in leukaemia patients and normal donors

The mRNA expression pattern of the MPP11 antigen was evaluated in PBMCs of patients with AML (n=20), ALL (n=20) and in PBMCs of healthy volunteers (n=28) using conventional RT-PCR. This screening was performed for two purposes. The first purpose was to compare the MPP11 gene expression level in acute leukaemia patients and in normal donors. The second purpose was to be able to select the HLA-A*0201^{+} leukaemia donors expressing the MPP11 gene to be used (if needed) as targets for T cells generated against the MPP11 peptides.

Total RNA was extracted from the PBMCs of patients and healthy donors and reverse transcriptized into cDNA using Moloney murine leukaemia virus. The extraction and reverse transcription methods are described in Chapter 2 section 2.13 and 2.14, respectively. The conventional RT-PCR was performed as described in Chapter 2 section 2.15. The amplification product of the house keeping gene β-actin served as control of cDNA quality. RNA isolated from K562 cell line, which expresses MPP11, served as a positive control. Distilled water served as a negative control. The expression of the MPP11 gene was classified into one of the following categories: - , no detectable mRNA expression; +, low expression; ++, moderate expression; +++, high expression. The intensity of the positive bands determined the level of antigen expression.

The results show that 16/20 (80%) of AML patients and 17/20 (85%) of ALL patients expressed the MPP11 gene. In total, the MPP11 was expressed in 33 out of 40 (82.5%) acute leukaemia patients and in 9/28 (32%) normal donors. The level of the expression of the MPP11 antigen was generally higher in acute leukaemia patients compared to low levels of expression in normal donors. Results are shown

in Figure 5.1 and Table 5.1. As shown in Table 5.1, there was no clear relation between MPP11 expression levels and the blast number (the percentage of blast varies between 1 and 99%).

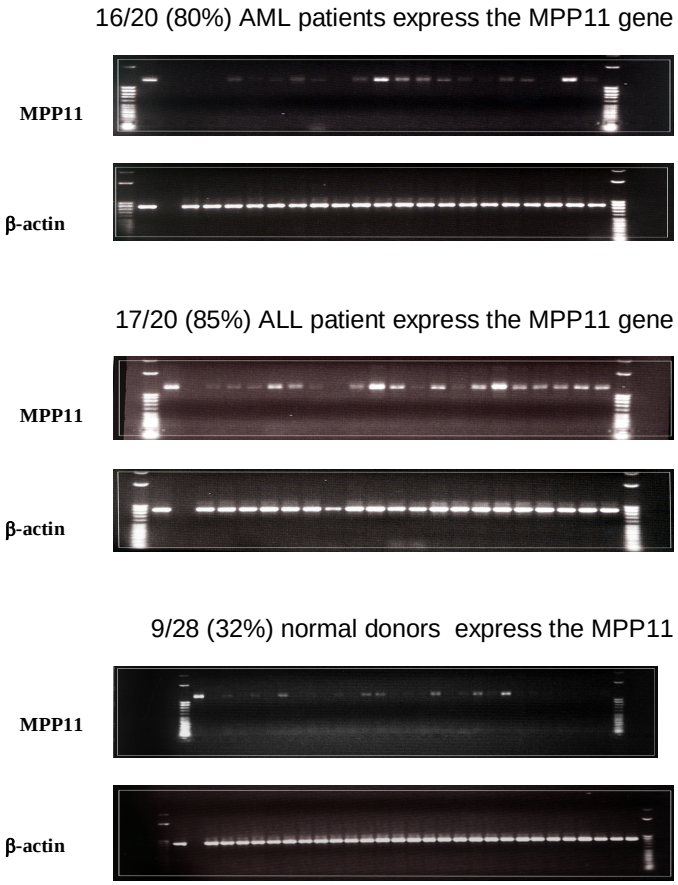

Figure 5.1. RT-PCR analysis of MPP11 gene expression in PBMCs from AML, ALL patients and normal donors.

80% of AML patients and 85% of ALL patients expressed the MPP11 antigen, whereas only 32% of the normal donors expressed the MPP11 antigen.

Table 5.1. Clinical features of leukaemia patients with MPP11 gene expression

Subtypes[1]	***Patients #***	***Status[2]***	***% Blast[3]***	***MPP11 expression***
M0	AML 3	New	84	++
M1	AML 1	New	61	-
M2	AML 5	New	1	+
	AML 7	New	61	+
	AML 8	New	20	++
	AML 19	New	23	+++
	AML 29	Rel Post chemo	81	++
M4 E0	AML 13	2nd Rel post allo BMT	10	++
	AML 36	New	12	+
M4	AML 25	New	52	+
	AML 27	New	81	+
	AML 28	New	61	++
M5a	AML 2	New	NR	-
	AML 12	New	79	-
M5	AML 11	New	80	+
	AML 16	New	4	+++
	AML 24	New	50	++
	AML 31	New	13	-
	AML 34	Rel Post allo BMT	80	+++
M6	AML 20	New	65	+++
Pre T-cell	ALL 26	New	99	++
T-cell	ALL 1	Rel	6	+
	ALL 2	Rel Post-BMT	87	+
	ALL 3	Rel	87	+
	ALL 13	New	75	+
	ALL 16	New	NR	+++
	ALL 18	New	94	++
	ALL 22	New	47	-
	ALL 23	New	84	++
Pre B-cell	ALL 4	New	55	++
	ALL 5	New	56	++
	ALL 20	New	5	-
	ALL 21	New	83	++
	ALL 27	New	59	++
	ALL 28	New	88	++
	ALL 29	New	2	++
	ALL 36	Rel on chemo	NR	++
B-cell	ALL 10	New	59	-
BP / Biclonal	ALL 7	New	89	+
NR	ALL 24	Rel	61	+++

New, newly diagnosed; Rel, relapsed; - , no detectable mRNA expression; +, low expression; ++, moderate expression; +++, high expression

5.3.2. Selection of potential CTL epitopes derived from MPP11 and PR3 proteins

The candidate epitopes derived from MPP11 and PR-3 were obtained using internet-based prediction programs. The selected peptides were synthesized and tested for their capacity to bind to the HLA-A*0201 molecule and/or generate cytotoxic T lymphocyte responses. Potential CTL epitopes derived from MPP11 antigen were predicted using the SYFPEITHI and BIMAS epitope prediction programs available at www.syfpeithi.de and www-bimas.cit.nih.gov, respectively. In each of the programs website, the amino acid sequences of the MPP11 (Figure 5.2) were entered. The HLA-A*0201 was selected for the MHC type and 9-mer was selected for the length of the peptides. Subsequently, the results from the database displayed numerous 9-mer peptides along with their positions and their predicted binding scores. Peptides qualified as positive if the score ranked ≥22 using the SYFPEITHI software or ≥100 using the BIMAS software. Four native peptides derived from MPP11 protein were selected. Although these peptides recorded a theoretically low binding affinity to the HLA-A*0201 molecule using the BIMAS software, they showed high binding affinity to the same molecule by the SYFPEITHI software. Peptides binding scores were ranging from 22 to 25 according to SYFPEITHI and from 79 to 4 according to BIMAS. In addition to the predicted peptides, tyrosine amino acid substitution in the P1 position of the first predicted epitope MPPN-1 (QLLIKAVNL) was also carried out. The binding score of the modified epitope, MPPY-1 (YLLIKAVNL), was enhanced from 25 to 27 as assessed by the SYFPEITHI server and from 79 to 364 as evaluated by the BIMAS server. The sequences of the peptides as well as their binding scores are presented in Table 5.2.

Potential PR3-derived candidate sequences predicted to bind to the HLA-A*0201 molecule were obtained using three different epitope prediction programs (SYFPEITHI, BIMAS and RANKPEP). The amino acid sequences of PR-3 (Figure 5.3) were entered into the computer programs, the MHC type and the length of the peptides were selected and potential candidate epitopes were displayed on the database. Two 9-mer peptides (PR-9 and PR-129) were selected as potential epitopes for the generation of cytotoxic T lymphocyte responses. The PR-9 peptide was selected as having the highest probability of binding based on the proposed SYFPEITHI algorithm. On the other hand, PR-129 had the highest score using

BIMAS and RANKPEP algorithms. In addition to the two selected peptides, PR-169 peptide-the previously described PR-1 epitope (Molldrem et al., 1996) was selected to serve as a positive control. The predicted binding scores by different computer programs for the 3 different PR3 peptides are listed in Table 5.3.

The standard T2 binding assay, illustrated in Figure 2.1 was used to evaluate the ability of MPP11 and PR-3 candidate peptides to bind and stabilize HLA-A*0201 molecule using the well-characterized A2-binding peptides WT1-187 and PR-169 as positive controls. The human processing-defective T2 cell line express empty HLA class I molecules. The binding assay is based on the stabilization of HLA class I molecules on the cell surface by the addition of peptides exogenously. The T2 cells are incubated with peptides overnight and if the peptide binds to the HLA-A*0201, the surface expression will be stabilized and there will be an upregulation of this molecule. Changes in its expression were assessed by staining HLA-A*0201 and quantifying the fluorescence intensity by flow cytometry analysis. The peptides are assumed to bind if their fluorescence ratio is greater than 1. All T2 binding assays were done in duplicate. Figure 5.4 and Figure 5.5 show the flow cytometric analyses of T2 cells following overnight incubation with 30 µM of either MPP-11 or PR-3 derived peptides, respectively.

As shown in Figure 5.6, MPP-3 and MPP-4 peptides (Fluorescence Index [FI] =1.1 and 2.5, respectively) stably bind to the HLA*A-0201molecule. The affinity of MPP-4 peptide to the molecule was comparable with that observed for the positive control WT1-187 epitope (FI =2.6). 187N is a WT1 derivative peptide known to highly bind to the HLA-A*0201 molecule (Oka et al., 2000). Because of their high binding affinities, MPP-3 and MPP-4 peptides were selected for the generation of peptide-specific T lymphocyte lines. On the other hand, the peptides MPP-1N, MPP1-Y, and MPP-2 were excluded from testing for immunogenicity since they did not show any binding or only very weak binding to the HLA-A*0201 molecule.

Regarding PR3 peptides, the PR-129 peptide up-regulated the HLA-A*0201 molecule and showed high affinity to this molecule (FI=1.9) compared to the positive control PR-169 peptide (FI=2.5). However, the PR-9 peptide had a low binding affinity to the HLA-A*0201 molecule (FI=0.6). The results of the T2 binding assay for the PR3 peptides are shown in Figure 5.7.

DVSRCAHRAR PGAIMLLLPS AADGRGTAIT HALTSASTLC QVEPVGRWFE
AFVKRRNRNA SASFQELEDK KELSEESEDE ELQLEEFPML KTLDPKDWKN
QDHYAVLGLG HVRYKATQRQ IKAAHKAMVL KHHPDKRKAA GEPIKEGDND
YFTCITKAYE MLSDPVKRRA FNSVDPTFDN SVPSKSEAKD NFFEVFTPVF
ERNSRWSNKK NVPKLGDMNS SFEDVDIFYS FWYNFDSWRE FSYLDEEEKE
KAECRDERRW IEKQNGATRA QRKKEEMNRI RTLVDNAYSC DPRIKKFKEE
EKAKKEAEKK AKAEAKRKEQ EAKEKQRQAE LEAARLAKEK EEEEVRQQAL
LAKKEKDIQK KAIKKERQKL RNSCKIEEIN EQIRKEKEEA EARMRQASKN
TEKSTGGGGN GSKNWSEDDL QLLIKAVNLF PARTNSRWEV IANYMNIHSS
SGVKRTAKDV IGKAKSLQKL DPHQKDDINK KAFDKFKKEH GVVPQADNAT
PSERFEGPYT DFTPWTTEEQ KLLEQALKTY PVNTPERWEK IAEAVPGRTK
KDCMKRYKEL VEMVKAKKAA QEQVLNASRA KK

Figure 5.2. MPP11 Protein sequence.

MAHRPPSPAL ASVLLALLLS GAARAAEIVG GHEAQPHSRP YMASLQMRGN
IHPSFVLTAA HCLRDIPQRL VNVVLGAHNV RTQEPTQQHF SVAQVFLNNY
PGSHFCGGTL DAENKLNDIL LIQLSSPANL QCLAMGWGRV QELNVTVVTF
FCRPHNICTF VPRRKAGICF GDSGGPLICD GIIQGIDSFV IWGCATRLFP
DFFTRVALYV DWIRSTLRRV EAKGRP

Figure 5.3. PR-3 protein sequence.

Table 5.2. Peptide Sequences derived from MPP11 and their binding prediction to HLA- A*0201 molecule

Given name	*Start position*	*Sequence*	*BIMAS* Score*	*SYFPEITHI** Score*
MPP-1N	421	QLLIKAVNL	79	25
MPP-1Y	Y-422	YLLIKAVNL	364	27
MPP-2	419	DLQLLIKAV	12	24
MPP-3	13	AIMLLLPSA	6	23
MPP-4	37	STLCQVEPV	4	22

Table 5.3. Peptide Sequences derived from PR3 and their binding prediction to HLA- A*0201 molecule

Given name	*start position*	*Sequence*	*BIMAS* Score*	*SYFPEITHI** Score*	*RANKPEP*** Score*
PR-169	169	VLQELNVTV	484.7	28	72.8%
PR-129	129	NLSASVATV	159.9	28	90.35%
PR-9	9	ALASVLLAL	49.1	30	86.84%

* The BIMAS scoring system is based on the calculation of the theoretical half-life of the MHC class I/peptide complex, which is a measure peptide-binding affinity. Peptides qualified as positive if the score was ≥100.

** The SYFPEITHI scoring system is based on the presence of certain amino acids in certain position along the MHC-binding groove. Optimal anchor residues are valued at 10 points, unusual anchors are given 6-8 points and preferred residues are worth 1-4 points. Amino acids with negative effect on the bining affinity score between -1 and -3. The maximum score for HLA-A*0201 is 36. Peptides qualified as positive if the score ranked ≥22.

*** The RANKPEP scoring system includes information on the score of the peptide and the % optimum score of the predicted peptide relative to that of a sequence that yields the maximum score with selected profile. Peptides qualified as positive if the % optimum was ≥50%.

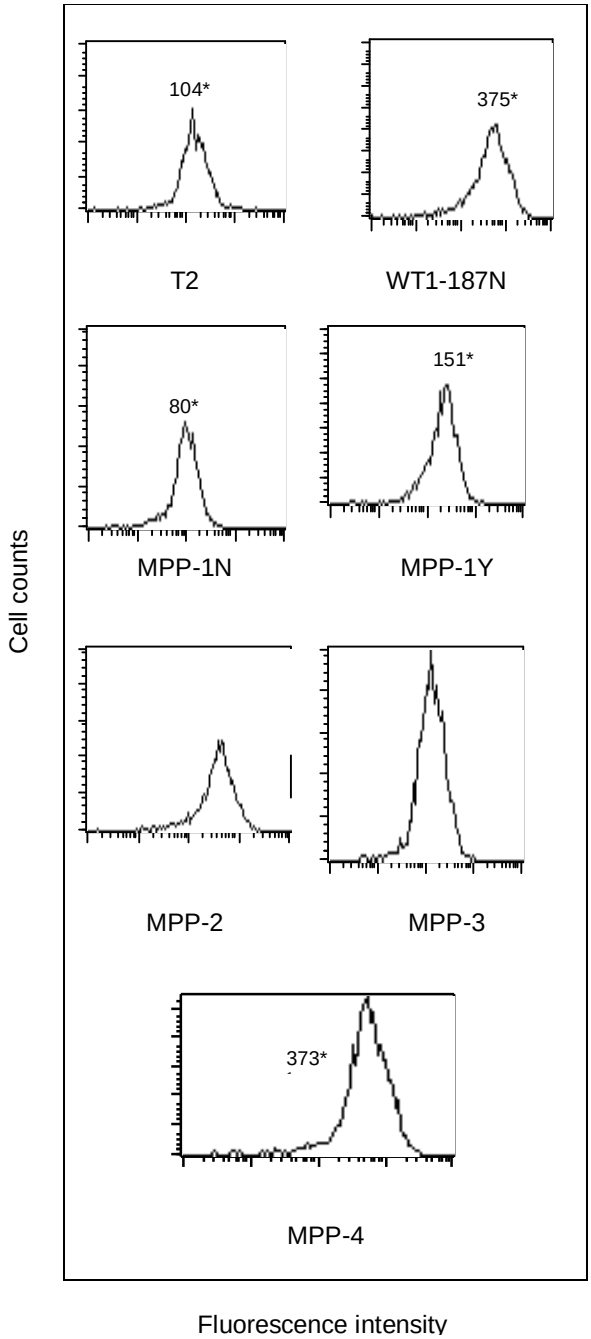

Figure 5.4. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 30 μM of WT1-187N or each MPP11-derived peptide. The WT1-187N peptide was used as a positive control. Following incubation with the peptides, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Average of median fluorescence intensities of duplicate experiments of T2 binding assay.

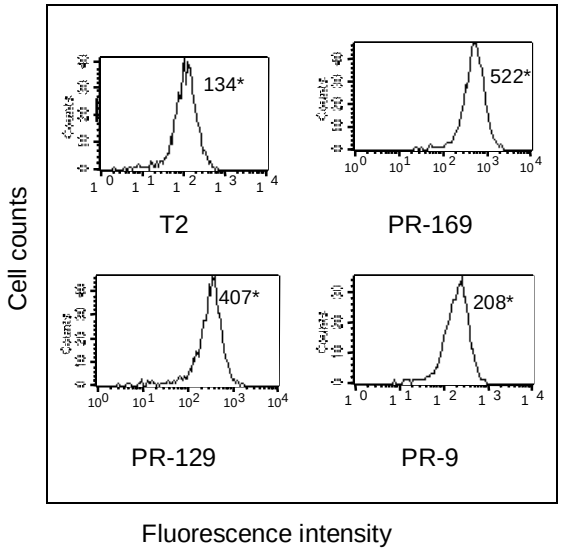

Figure 5.5. Flow cytometric analysis of HLA-A2 molecules expressed by T2 cells cultured overnight in the absence or in the presence of 30 μM of each PR-3-derived peptide.

The PR-169 peptide was used as a positive control. Following incubation with the peptides, the T2 cells were stained with HLA-A2 specific monoclonal antibody (BB7.2) and analyzed by flow cytometry. *Average of median fluorescence intensities of duplicate experiments of T2 binding assay.

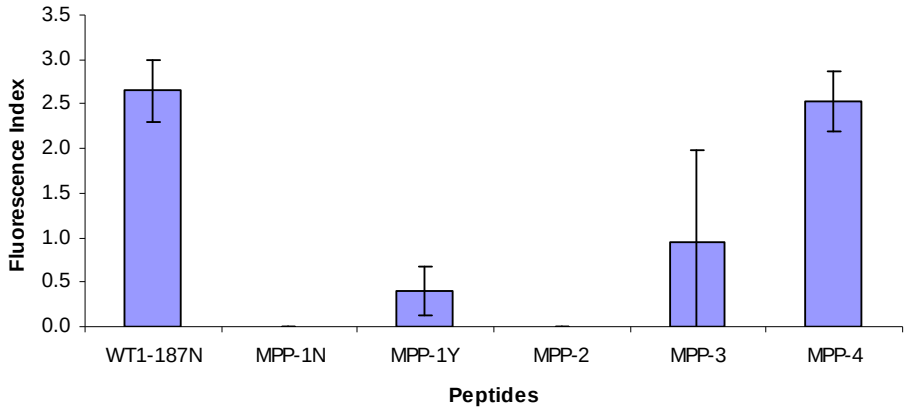

Figure 5.6. The binding ability of MPP11-derived peptides to the HLA-A*0201 molecule as measured by the T2 peptide-binding assay.

WT1-187N, a well-characterized A2-binding peptide, served as a positive control. MPP-3 and MPP-4 peptides stably bind to the HLA-A*0201 as demonstrated by stabilization of surface HLA-A2. The peptides MPP-1N, MPP-2 and the modified peptide MPP-1Y showed no binding to HLA-A*0201 and thereby were not used to generate CTLs. Each value represents the average of duplicate assays and error bars indicate the SD.

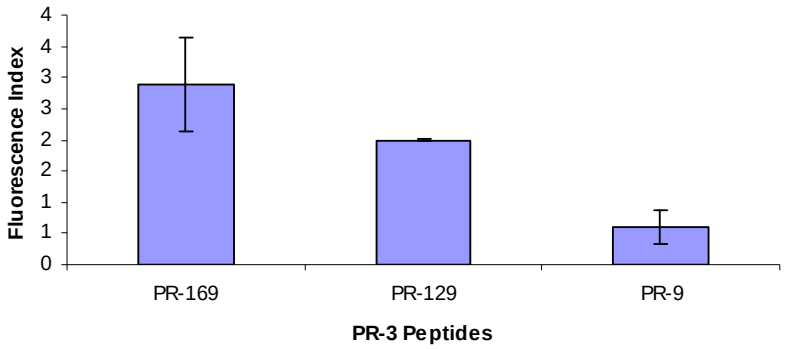

Figure 5.7. The binding ability of PR-3 derived peptides to the HLA-A*0201 molecules as measured by the T2 binding assay.

PR-169 is a well-characterized A2-binding peptide that is used as a positive control. PR-129 peptide has a high binding affinity whereas PR-9 has a very low binding affinity to the HLA-A*0201 molecule. Each value represents the average of duplicate assays and error bars indicate the SD.

5.3.3. Generation of MPP11 specific T lymphocyte lines by T2 cells

The ability of the MPP11-derived peptides to induce specific cytotoxic T lymphocytes (CTLs) was tested. PBMCs obtained from two HLA-A*0201 positive healthy volunteers designated as BC-21 and BC-32 were utilized to generate CTLs specific to the selected peptides. The responder cells were $CD8^+$ T cells purified by MACS negative selection as described in Materials and Methods section 2.6.1. The purified $CD8^+$ T cells were then stimulated *in vitro* with T2 cells. The choice of T2 cells as APCs was made to reduce the number of PBMCs used and was based on previous studies (Houbiers et al., 1993, Molldrem et al., 1996, Oka et al., 2000). The T2 cells were pulsed with MPP-4, MPP-3 or WT1-126N peptide and used to prime and activate the purified $CD8^+$ T cells every 7-10 days. The T cell line generated against the WT1-126N peptide was used as a control. The generated T cell lines were tested against different HLA-A*0201^+ targets in the presence or absence of autologous peptide using a Chromium Release Assay and an ELISPOT assay after 3 to 8 rounds of stimulations.

The specificity of *in vitro* primed CTLs was first tested for specific IFN-γ release to stimulation with the corresponding peptides using an ELISPOT assay. The T cell lines (MPP-3, MPP-4 and WT1-126N) generated from both donors (BC-21 and BC-32) did not produce antigen specific IFN-γ spots when stimulated with T2-loaded peptide (Figure 5.8 and Figure 5.9). In addition, autologous PBMCs un-pulsed or peptide-pulsed, K562 and K562-A2.1 were used to test the specificity of the T cell lines generated from BC-21 donor. K562 is a leukaemia cell line expressing MPP11 antigen and K562-A2.1 is the same cell line but transfected with the HLA-A*0201 molecule. As shown in Figure 5.8, no antigen specific IFN-γ spots were formed when the three BC-21-T cell lines were challenged with targets other than T2 cells.

The next step in monitoring $CD8^+$ T cell responses to MPP11-derived peptides was to test the cytotoxic activity of the generated T cell lines in a standard Cr^{51}-release assay. Consistent with the results obtained with the ELISPOT assay; the T cell lines generated from the two different donors (BC-21 and BC-32) did not elicit any peptide-specific cytotoxic responses. The T cell lines generated from BC-21 donor were challenged with T2 cells or LCL-21 pulsed with no peptide or with each

peptide at different E:T ratio starting from 50:1. As shown in Figure 5.10, Figure 5.11 and Figure 5.12, the MPP-3, the MPP-4 and the WT1-126N T cell lines did not recognize peptide-pulsed T2 or LCL-21 targets cells. The T cell lines generated from the other donor (BC-32) were challenged with T2 cells or LCL-32 pulsed with no peptide or with each peptide at different E:T ratio starting from 100:1. In these cytotoxic experiments, K562 and K562-A2.1 were used as targets too. The three tested T cell lines did not show any peptide-specific cytotoxic responses toward peptide-pulsed T2, LCL-32 as well as K562-A2.1 targets cells (Figure 5.13, Figure 5.14 and Figure 5.15).

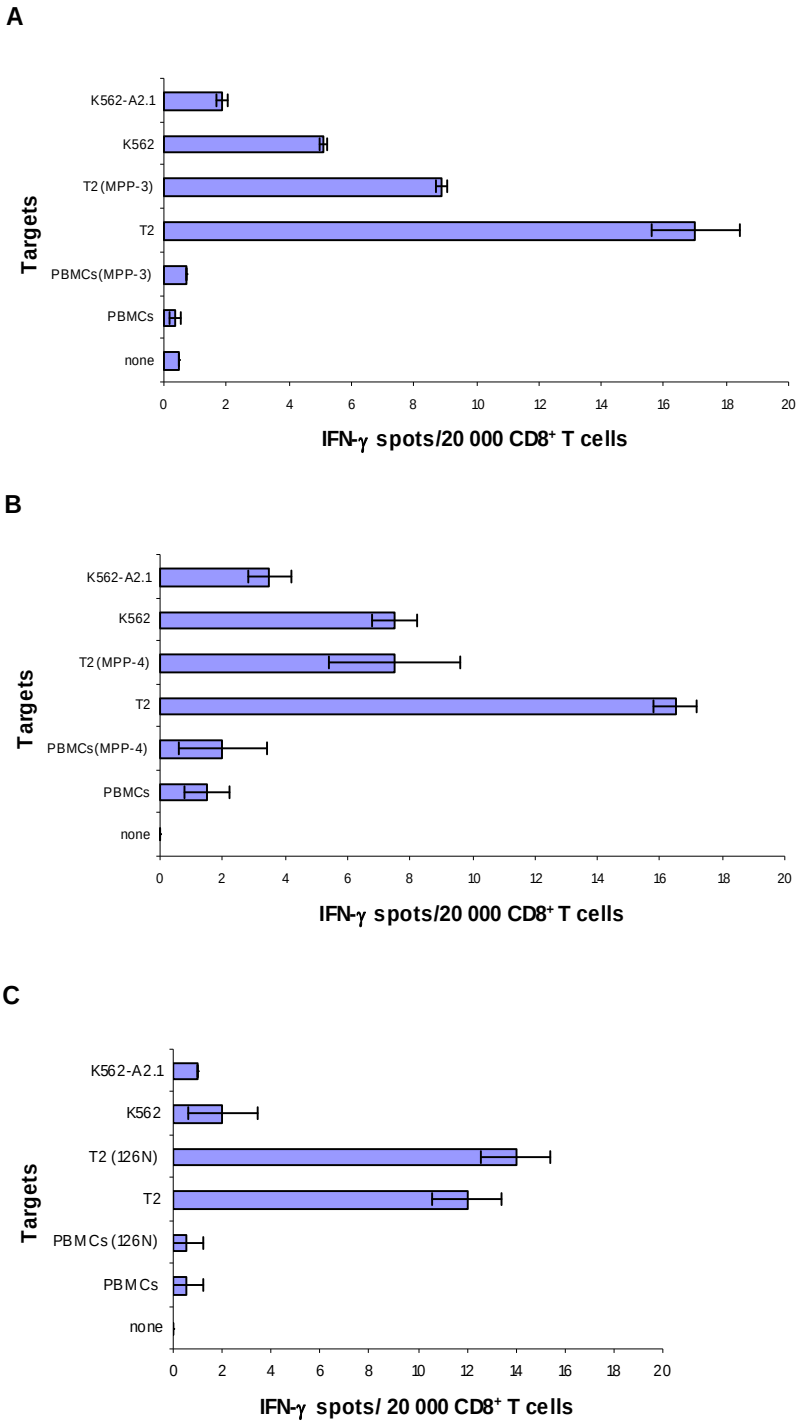

Figure 5.8. Non-specific IFN-γ release of BC-21 CTLs generated by T2 cells against the MPP-3, MPP-4 and WT1-126N peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-21, an HLA-A0201^{+} donor, and tested following three to eight rounds of *in vitro* T cell stimulations. Targets were autologous PBMCs T2 cells, K562 and K562-A2.1. The PBMCs and T2 cells were pulsed with either no peptide or with peptide.

Figure 5.8. Continued

The background observed when T cells alone were used is shown. Non-specific IFN-γ release is shown for T cell line generated against the MPP-3 peptide (A). Non-specific IFN-γ release is shown for T cell line generated against the MPP-4 peptide (B). Non-specific IFN-γ release is shown for T cell line generated against the WT1-126N peptide (C). Results are expressed as IFN- γ positive cells per 20.000 $CD8^+$ T cells and are the mean of triplicate assays (±SD).

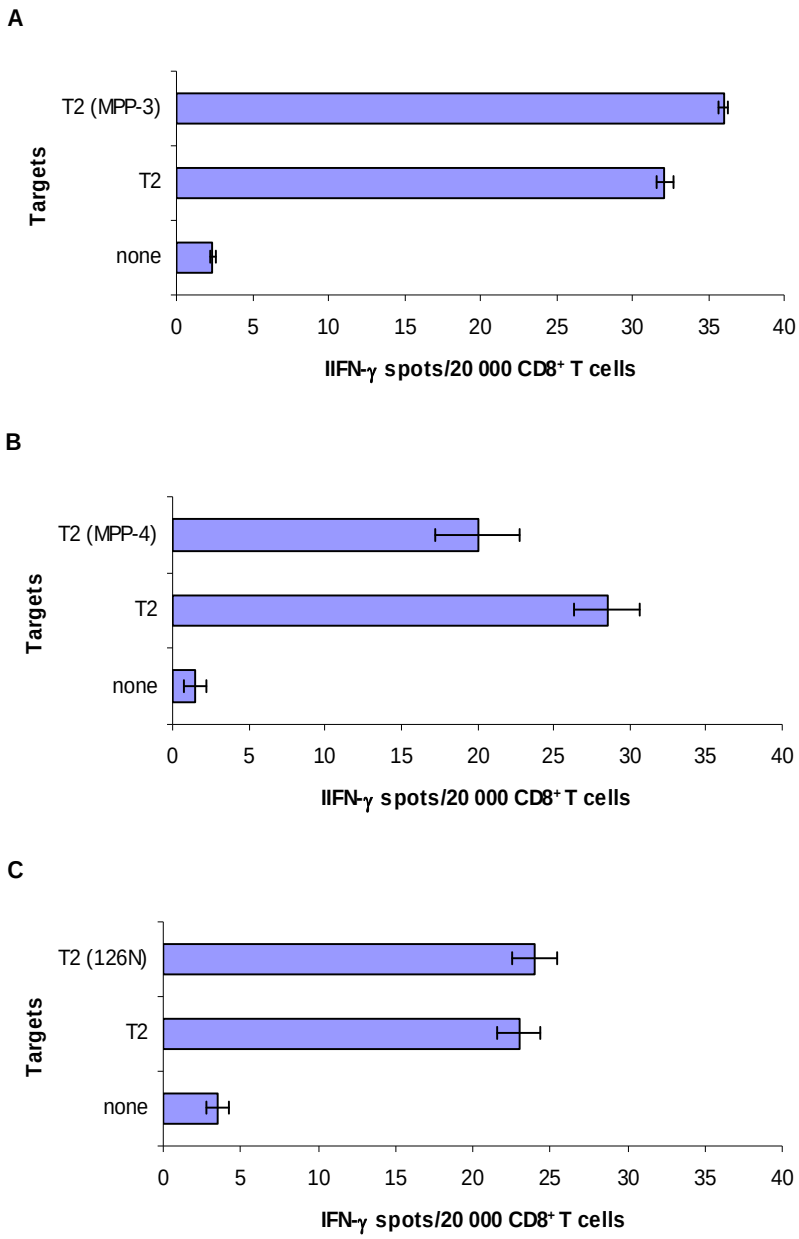

Figure 5.9. Non-specific IFN-γ release of BC-32 CTLs generated by T2 cells against the MPP-3, MPP-4 and WT1-126N peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-32, an HLA-A0201^{+} donor, and tested following three to eight rounds of *in vitro* T cell stimulations. Targets were T2 cells pulsed with either no peptide or with peptide. The background observed when T cells alone were used is shown. Non-specific IFN-γ release is shown for T cell line generated against the MPP-3 peptide (A). Non-specific IFN-γ release is shown for T cell line generated against the MPP-4 peptide (B). Non-specific IFN-γ release is shown for T cell line generated against the WT1-126N peptide (C). Results are expressed as IFN- γ positive cells per 20.000 CD8^{+} T cells and are the mean of triplicate assays (±SD).

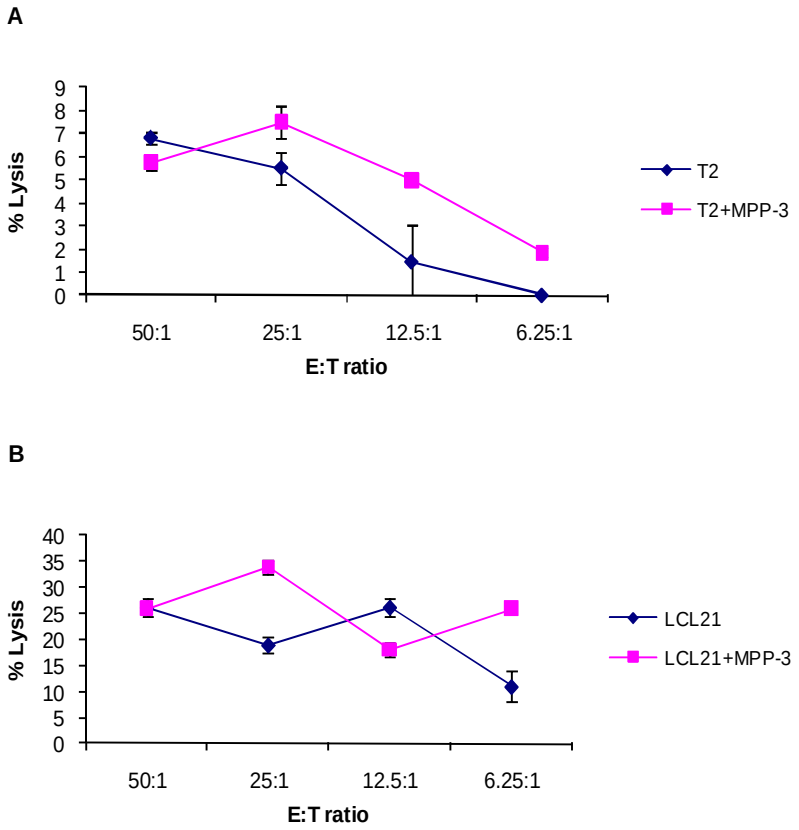

Figure 5.10. Non-specific lysis by BC-21 CTLs generated by T2 cells against MPP-3 peptide as tested using a standard chromium release assay.

The effector cells were MPP-3 T cell line generated from an HLA-A0201^{+} donor, BC-21, by T2 cells. The T cell line was tested following three to eight rounds of in vitro T cell stimulations. Target cells were pulsed with either no peptide (dark blue symbol) or with MPP-3 peptide (pink symbol). The MPP-3 T cell line was stimulated with T2 cells (A), and with autologous LCL-21 (B). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of three experiments. Error bars indicate the SD.

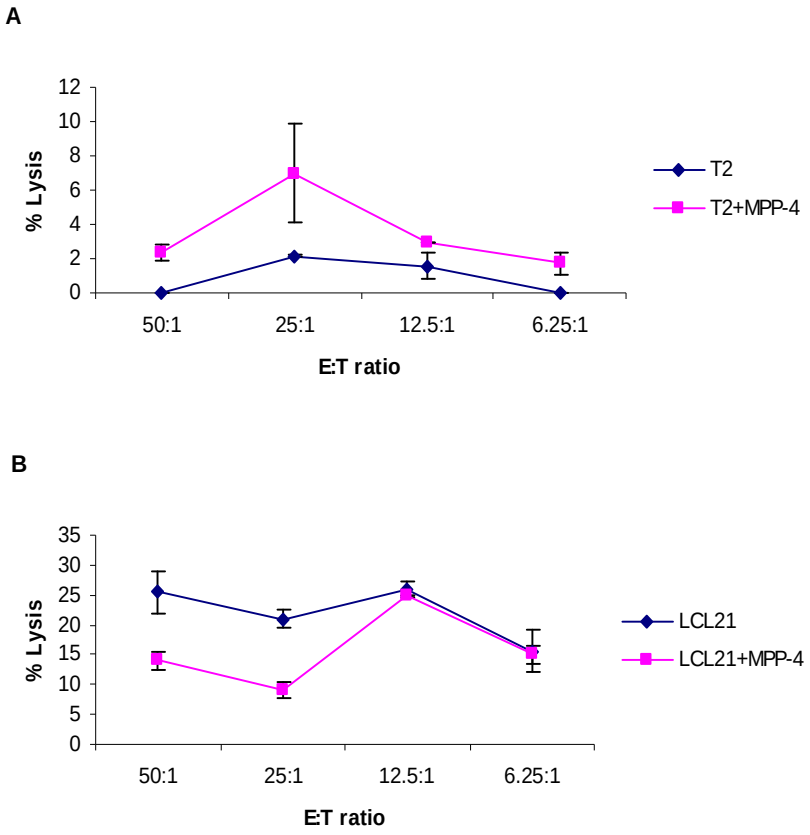

Figure 5.11. Non-specific lysis by BC-21 CTLs generated by T2 cells against MPP-4 peptide as tested using a standard chromium release assay.

The effector cells were MPP-4 T cell line generated from an HLA-A0201^{+} donor, BC-21, by T2 cells. The T cell line was tested following three to eight rounds of in vitro T cell stimulations. Target cells were pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). The MPP-4 T cell line was stimulated with T2 cells (A), and with autologous LCL-21 (B). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of three experiments. Error bars indicate the SD.

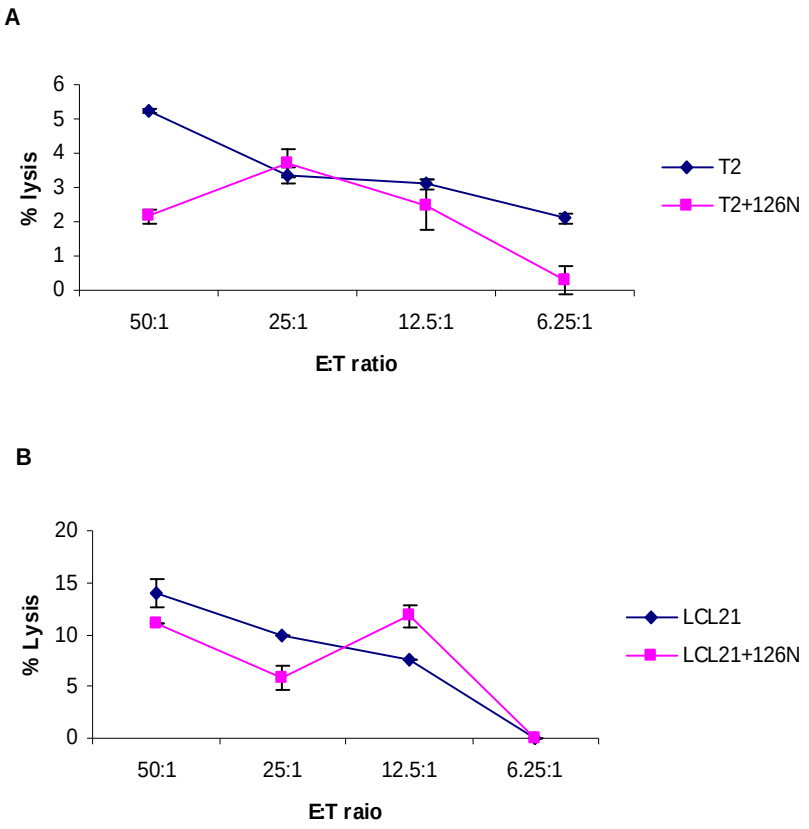

Figure 5.12. Non-specific lysis by BC-21 CTLs generated by T2 cells against WT1-126N peptide as tested using a standard chromium release assay.

The effector cells were WT1-126N T cell line generated from an HLA-A0201^{+} donor, BC-21, by T2 cells. The T cell line was tested following three to eight rounds of *in vitro* T cell stimulations. Target cells were pulsed with either no peptide (dark blue symbol) or with WT1-126N peptide (pink symbol). The WT1-126N T cell line was stimulated with T2 cells (A), and with autologous LCL-21 (B). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of three experiments. Error bars indicate the SD.

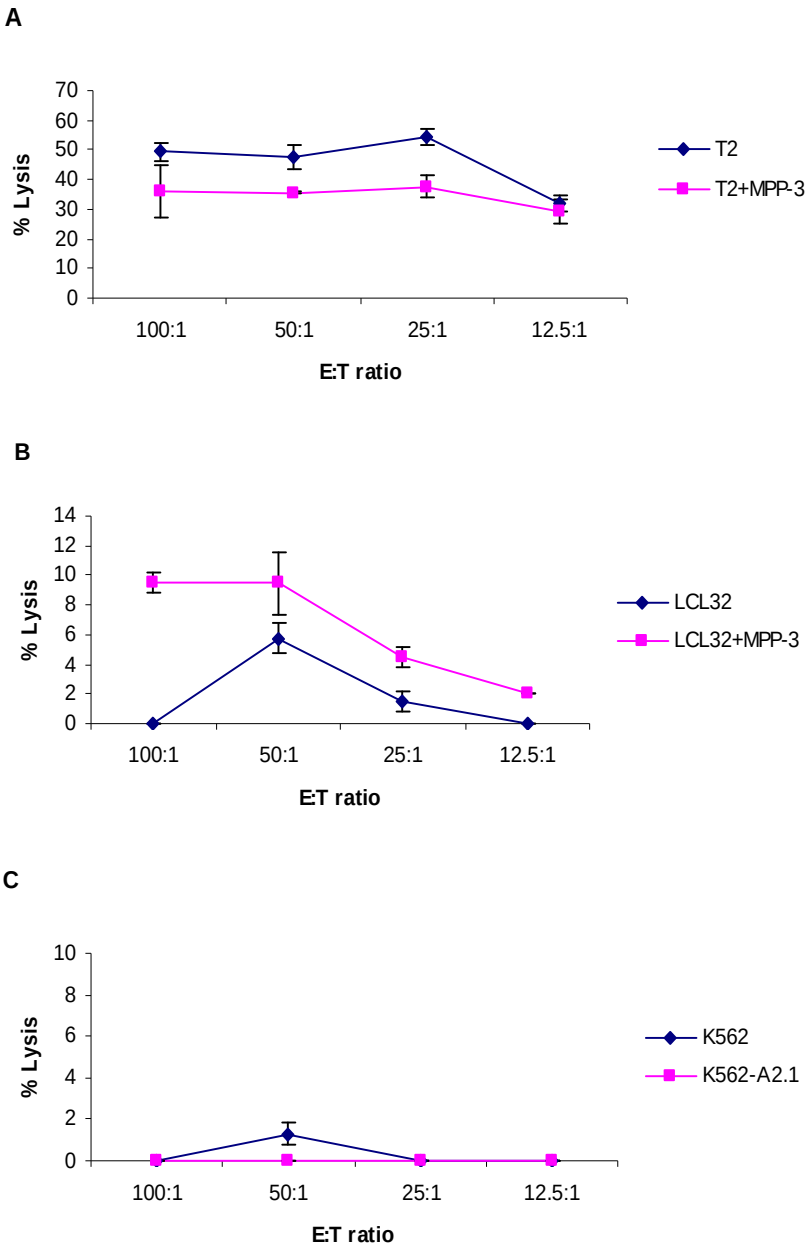

Figure 5.13. Non-specific lysis by BC-32 CTLs generated by T2 cells against MPP-3 peptide as tested using a standard chromium release assay.

The effector cells were MPP-3 T cell line generated from an HLA-A0201^{+} donor, BC-32, by T2 cells. The T cell line was tested following three to eight rounds of in vitro T cell stimulations. Target cells were pulsed with either no peptide (dark blue symbol) or with MPP-3 peptide (pink symbol). The MPP-3 T cell line was stimulated with T2 cells (A), with autologous LCL-32 (B) and with K562-A2.1. (C). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of three experiments. Error bars indicate the SD.

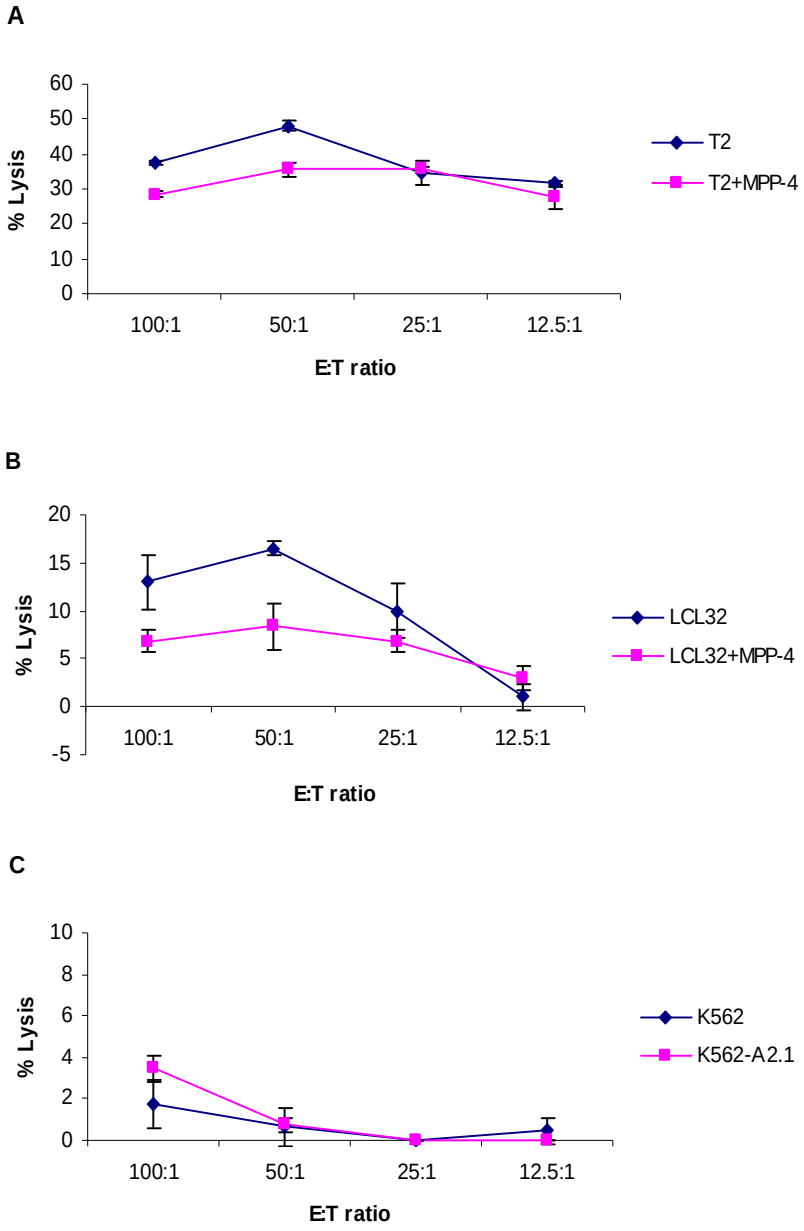

Figure 5.14. Non-specific lysis by BC-32 CTLs generated by T2 cells against MPP-4 peptide as tested using a standard chromium release assay.

The effector cells were MPP-4 T cell line generated from an HLA-A0201^{+} donor, BC-32, by T2 cells. The T cell line was tested following three to eight rounds of in vitro T cell stimulations. Target cells were pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). The MPP-4 T cell line was stimulated with T2 cells (A), with autologous LCL-32 (B) and with K562-A2.1. (C). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of three experiments. Error bars indicate the SD.

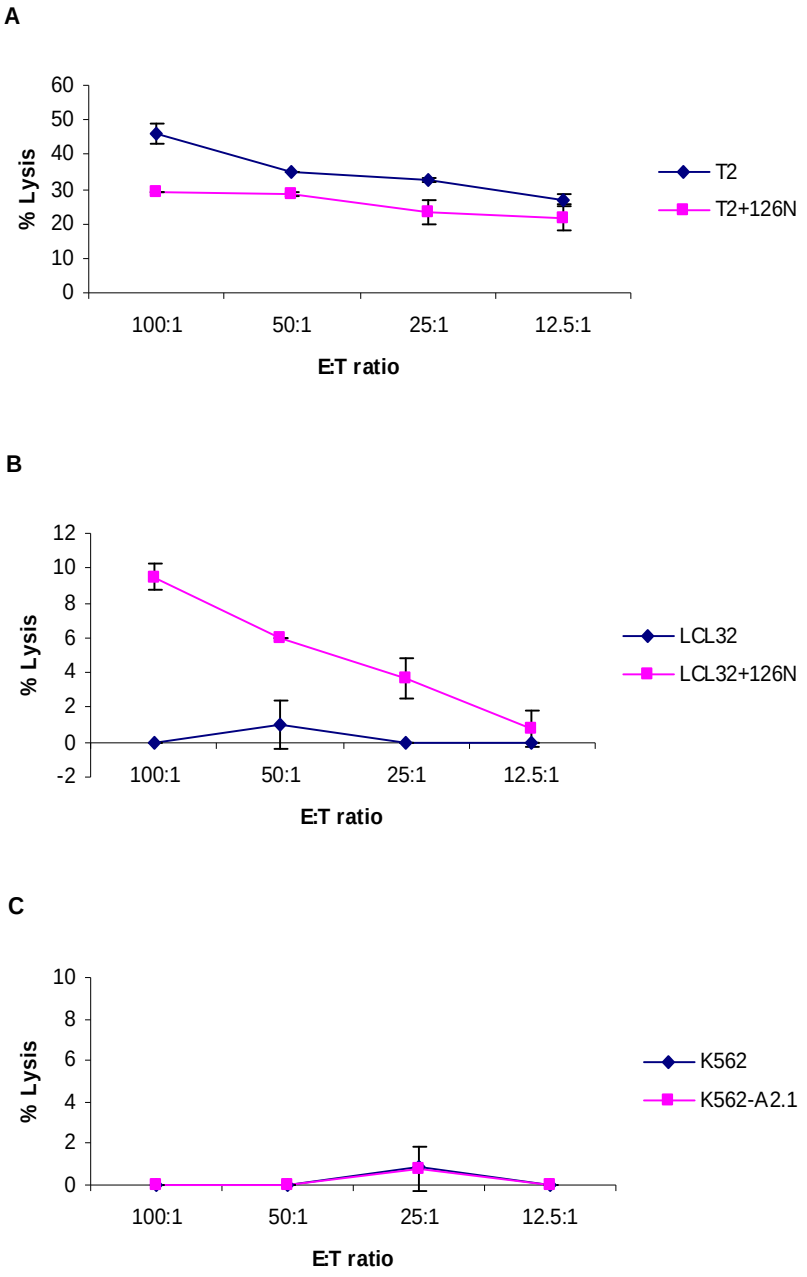

Figure 5.15. Non-specific lysis by BC-32 CTLs generated by T2 cells against WT1-126N peptide as tested using a standard chromium release assay.

The effector cells were WT1-126N T cell line generated from an HLA-A0201^{+} donor, BC-32, by T2 cells. The T cell line was tested following three to eight rounds of in vitro T cell stimulations. Target cells were pulsed with either no peptide (dark blue symbol) or with WT1-126N peptide (pink symbol). The WT1-126N T cell line was stimulated with T2 cells (A), with autologous LCL-32 (B) and with K562-A2.1. (C). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of three experiments. Error bars indicate the SD.

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5.3.4. Generation of MPP11 specific T lymphocyte lines by DC2d

The T cell lines generated form the two different donors (BC-21 and BC-32) by T2 cells showed no specific $CD8^+$ T cell responses to any of the peptides tested including the positive control peptide WT1-126N. Therefore, a second protocol for the generation of $CD8^+$ T cell lines was then used where peptide-pulsed DC2d (fast DC) were used to prime the purified $CD8^+$ T cells and peptide-pulsed monocytes were used for subsequent T cell stimulations. The DC2d were generated as described in Materials and Methods section 2.5.4.3. PBMCs obtained from four different HLA-A*0201 positive healthy volunteers were utilized to generate CTLs specific to the selected peptides. The four donors were designated as BC-21, BC-32, BC-37 and BC-41. The PBMCs from each donor were used as the source of the responder cells and antigen presenting cells. The responder cells were $CD8^+$ T cells purified by MACS negative selection and stimulated *in vitro* with antigen presenting cells-loaded peptide for 7-10 days.

To investigate whether the MPP11 synthetic peptides could stimulate peptide-specific CTLs, the generated T cell lines were tested against different HLA-$A*0201^+$ targets in the presence or absence of autologous peptide using a Chromium Release Assay and an ELISPOT assay after 3 to 8 rounds of stimulations.

5.3.5. $CD8^+$ T cell responses to MPP11- derived peptides

5.3.5.1. MPP-4 CTL s produced specific IFN-γ

After the third round of stimulation, the specificity of in vitro primed CTLs was first tested for specific IFN-γ release to stimulation with the corresponding peptides using an ELISPOT assay. The BC-21 CTLs generated against the MPP-4 peptide produced specific IFN-γ spots when stimulated with T2-loaded peptide whereas non-specific production was seen in case of CTLs generated from the same donor against MPP-3 as shown in Figure 5.16A and B. The anti-MPP-3 and anti-MPP-4 T cell lines generated from the three other donors (BC-32, BC-37 and BC-41) were also tested. However, these T cell lines did not produce any specific IFN-γ spots when challenged with either MPP-3 or MPP-4 peptides (Figure 5.17, Figure 5.18 and Figure 5.19). The specificity of BC-32 and BC-37 T cell lines was tested by challenging the T cells from each donor with T2 cells and autologous LCLs

unpulsed or pulsed with their corresponding peptide (Figure 5.17A and B and Figure 5.18A and B). The specificity of BC-41 T cell lines was tested using unloaded or peptide-loaded T2 cells Figure 5.19A and B.

Because $CD8^+$ T cells are known to utilize cytotoxic factors such as granzyme B and/or perforin to induce killing of their target cells, the BC-21 MPP-4-CTL was therefore tested for the release of granzyme B and/or perforin. The test was performed after 5 rounds of stimulation. MPP-4 pulsed T2 and autologous LCLs were used as stimulators. No specific production of granzyme B and/or perforin to the peptide MPP-4 was observed due to very high background responses seen when BC-21 T cells alone were used in the assay (Figure 5.20 A and B). Although specific IFN-γ release was seen with the MPP-4 T cell line generated from BC-21 donor only, the MPP-4 T cell lines generated from BC-32, BC-37 and BC-41 were also tested for the release of granzyme B and/or perforin. MPP-4 pulsed T2 or K562-A2.1 cells were used as stimulators. As shown in Figure 5.21, Figure 5.22 and Figure 5.23, the three T cell lines did not produce any specific granzyme B or perforin spots when challenging with APCs loaded with MPP-4 peptide.

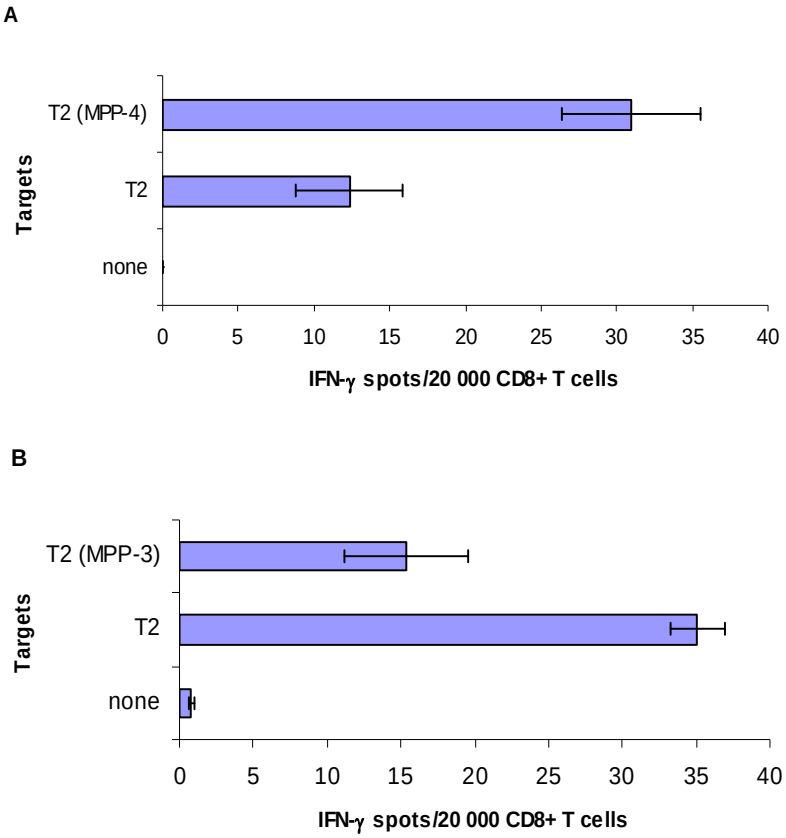

Figure 5.16. Specificity of BC-21 CTLs generated by DC2d against the MPP-3 and MPP-4 peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-21, an HLA-A0201^{+} donor, and tested following three to eight rounds of in vitro T cell stimulations. Targets were T2 cells pulsed with either no peptide or with peptide. The background observed when T cells alone were used is shown. Results are expressed as IFN- γ positive cells per 20.000 CD8^{+} T cells and are the mean of triplicate assays (±SD). MPP-4-CTLs released IFN-γ upon re-stimulation with T2 cells pulsed with MPP-4 peptide (A). T cell line generated against the MPP-3 peptide released more IFN-γ upon re-stimulation with T2 cells alone (B).

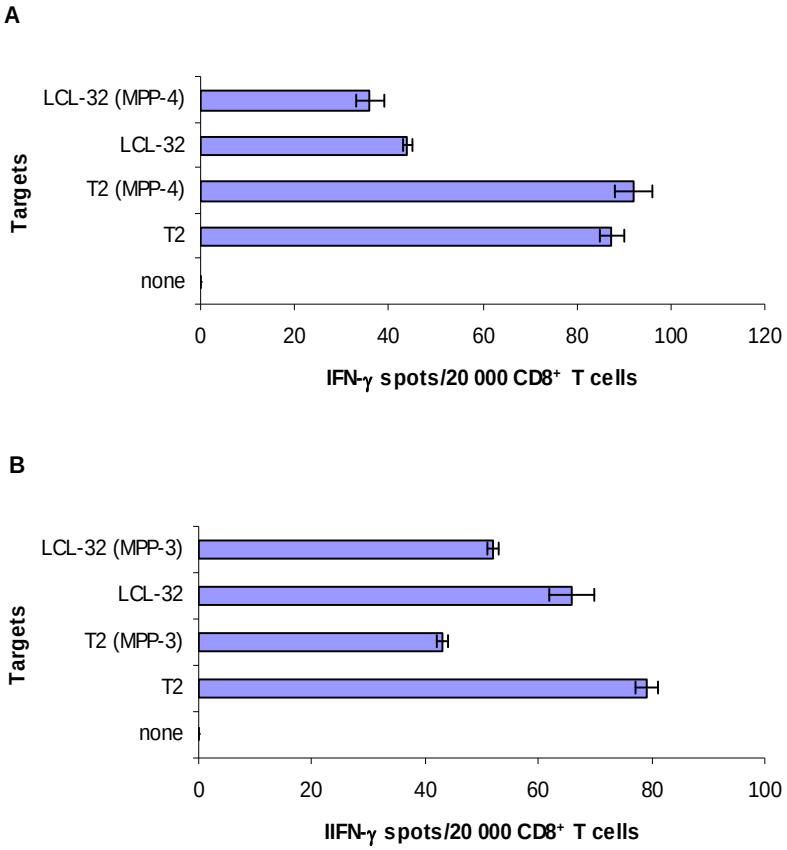

Figure 5.17. Non-specific IFN-γ release of BC-32 CTLs generated by DC2d cells against the MPP-4 and the MPP-3 peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-32, an HLA-A0201^{+} donor, and tested following three to eight rounds of *in vitro* T cell stimulations. Targets were T2 cells and LCL-32 pulsed with either no peptide or with peptide. The background observed when T cells alone were used is shown. Non-specific IFN-γ release is shown for T cell line generated against the MPP-4 peptide (A). Non-specific IFN-γ release is shown for T cell line generated against the MPP-3 peptide (B). Results are expressed as IFN- γ positive cells per 20.000 CD8^{+} T cells and are the mean of triplicate assays (±SD).

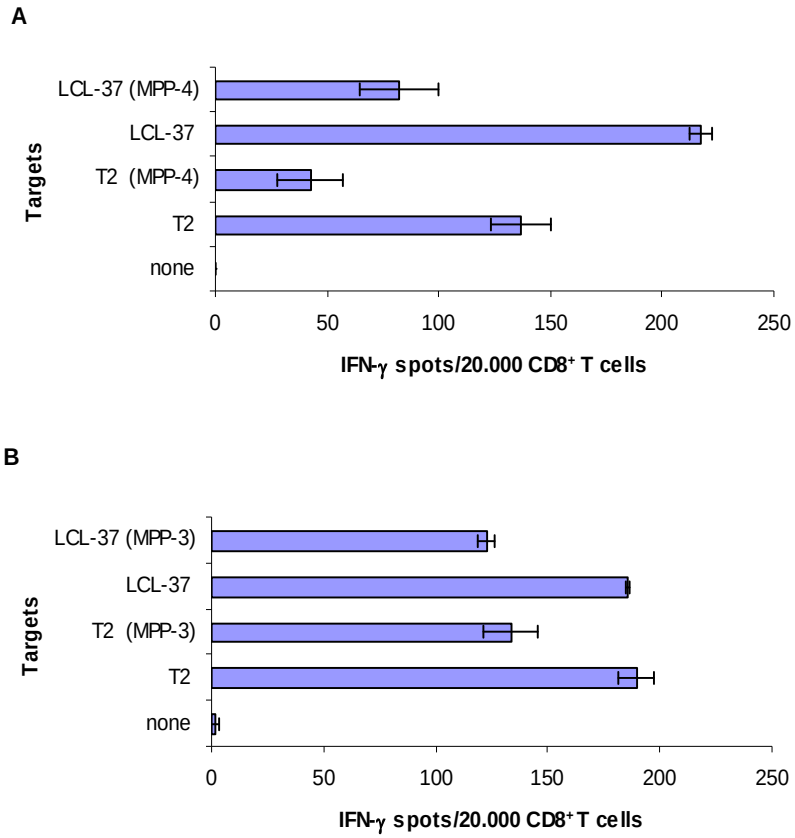

Figure 5.18. Non-specific IFN-γ release of BC-37 CTLs generated by DC2d cells against the MPP-4 and the MPP-3 peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-37, an HLA-A0201^{+} donor, and tested following three to eight rounds of *in vitro* T cell stimulations. Targets were T2 cells and LCL-37 pulsed with either no peptide or with peptide. The background observed when T cells alone were used is shown. Non-specific IFN-γ release is shown for T cell line generated against the MPP-4 peptide (A). Non-specific IFN-γ release is shown for T cell line generated against the MPP-3 peptide (B). Results are expressed as IFN- γ positive cells per 20.000 CD8^{+} T cells and are the mean of triplicate assays (±SD).

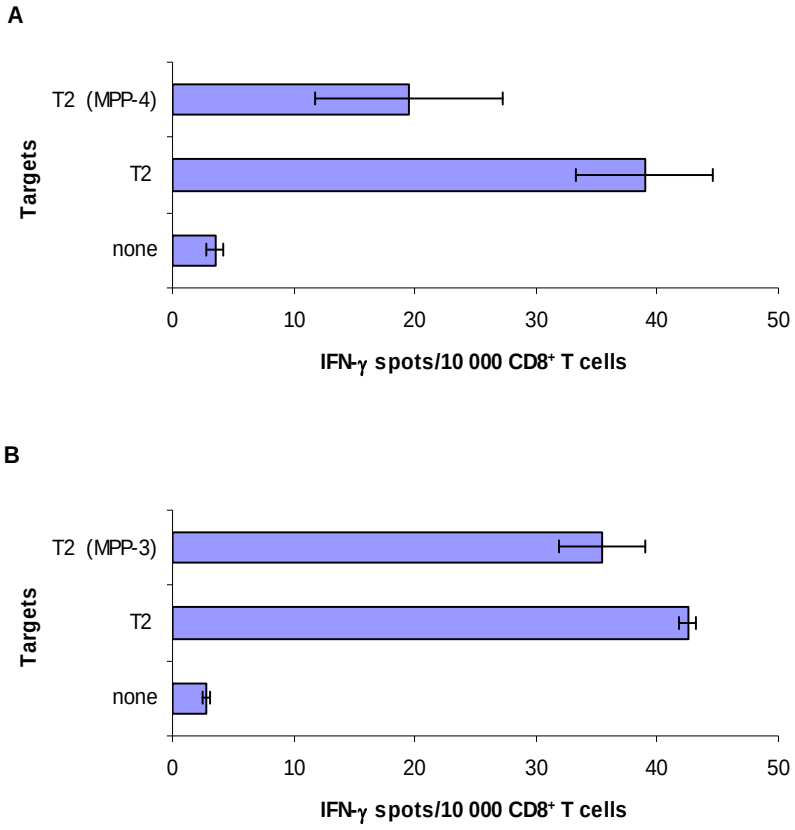

Figure 5.19. Non-specific IFN-γ release of BC-41 CTLs generated by DC2d cells against the MPP-4 and the MPP-3 peptides using an IFN-γ- ELISPOT assay.

The T cell lines were established from BC-41, an HLA-A0201^{+} donor, and tested following three to eight rounds of *in vitro* T cell stimulations. Targets were T2 cells pulsed with either no peptide or with peptide. The background observed when T cells alone were used is shown. Non-specific IFN-γ release is shown for T cell line generated against the MPP-4 peptide (A). Non-specific IFN-γ release is shown for T cell line generated against the MPP-3 peptide (B). Results are expressed as IFN- γ positive cells per 20.000 CD8^{+} T cells and are the mean of duplicate assays (±SD).

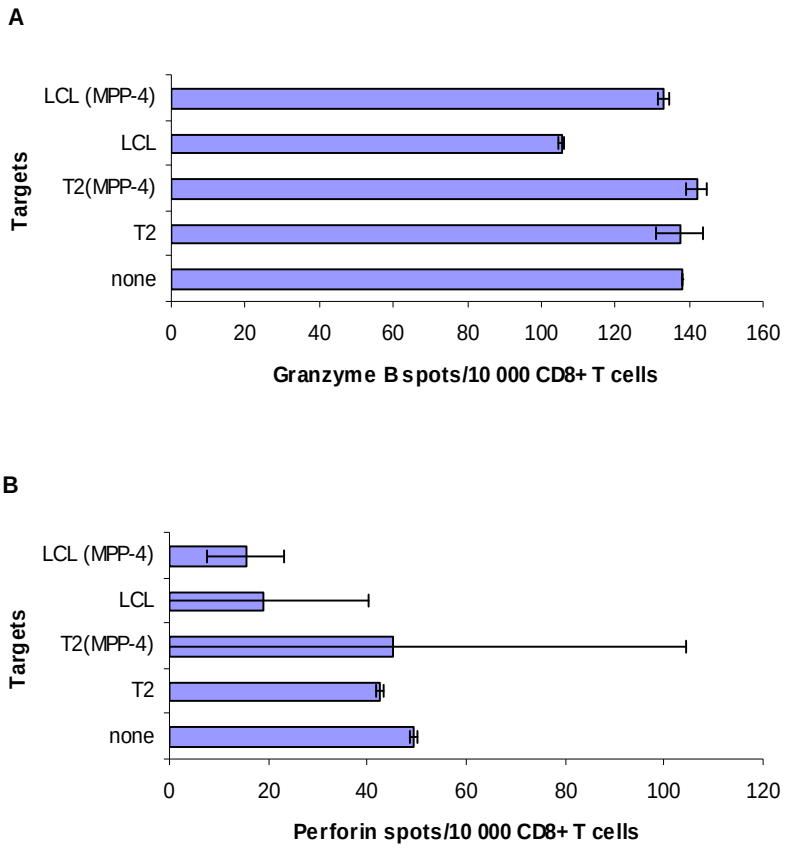

Figure 5.20. Specificity of BC-21 CTLs generated by DC2d against the MPP-4 peptides using granzyme B and perforin ELISPOT assay.

The T cell lines were established from BC-21, an HLA-A0201^{+} donor, and tested following five to eight rounds of in vitro T cell stimulations. Targets were T2 cells and autologous LCL-21 cells pulsed with either no peptide or with MPP-4 peptide. Results are expressed as granzyme B (A) or perforin (B) positive cells per 10.000 CD8^{+} T cells and are the mean of duplicate assays (±SD). T cell line MPP-4 released high granzyme B and perforin when T cells alone were used and when no peptide was used.

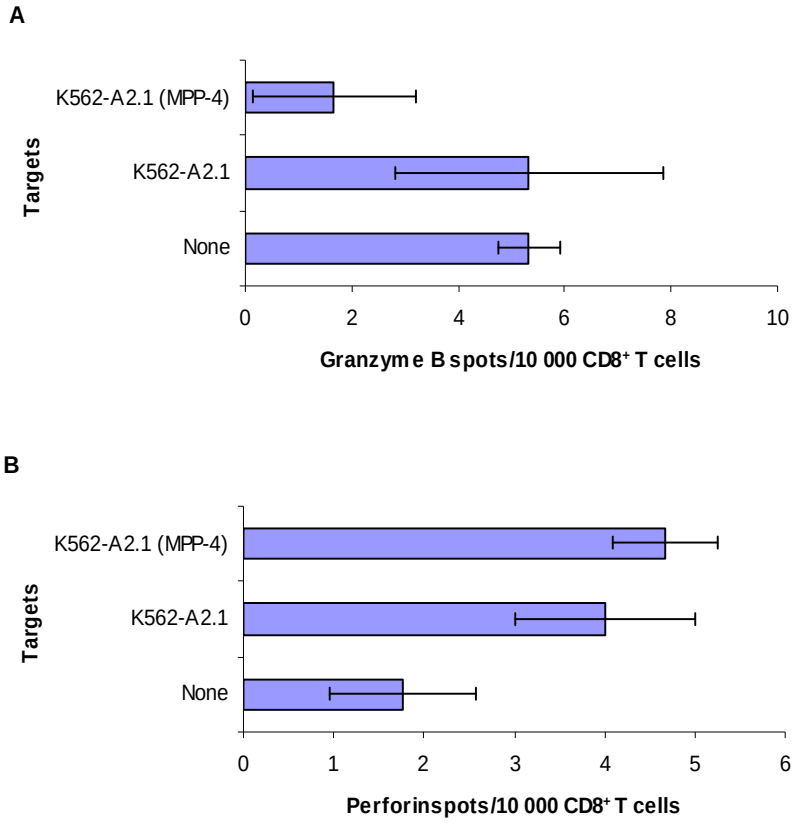

Figure 5.21. Non-specific granzyme B and perforin release by BC-32 CTLs generated by DC2d cells against the MPP-4 peptides using an ELISPOT assay.

The anti-MPP-4 T cell line was established from BC-32, an HLA-A0201$^+$ donor, and tested following five to eight rounds of *in vitro* T cell stimulations. Targets were K562-A2.1 cells pulsed with either no MPP-4 peptide or with the peptide. The background observed when T cells alone were used is shown. (A) Shows non-specific granzyme B release for BC-32 MPP-4 T cell line. (B) Shows non-specific perforin release for BC-32 MPP-4 T cell line. Results are expressed as granzyme B or perforin positive cells per 10.000 CD8$^+$ T cells and are the mean of duplicate assays (±SD).

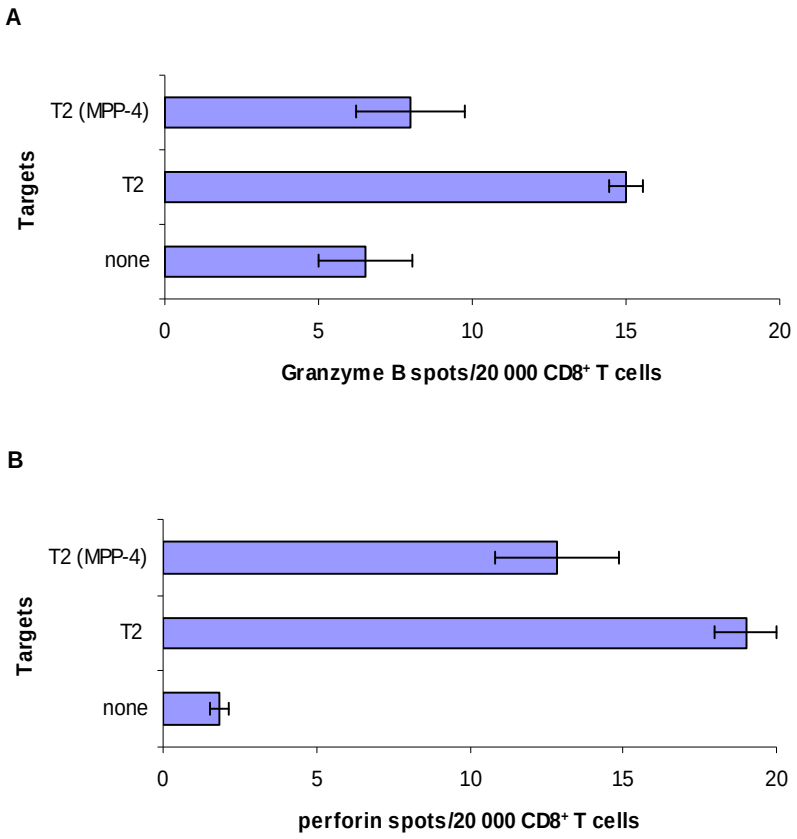

Figure 5.22. Non-specific granzyme B and perforin release of BC-37 CTLs generated by DC2d cells against the MPP-4 peptides using an ELISPOT assay.

The anti-MPP-4 T cell line was established from BC-37, an HLA-A0201$^+$ donor, and tested following five to eight rounds of *in vitro* T cell stimulations. Targets were T2 cells pulsed with either no MPP-4 peptide or with the peptide. The background observed when T cells alone were used is shown. (A) Shows non-specific granzyme B release for BC-37 MPP-4 T cell line. (B) Shows non-specific perforin release for BC-37 MPP-4 T cell line. Results are expressed as granzyme B or perforin positive cells per 20.000 CD8$^+$ T cells and are the mean of duplicate assays (±SD).

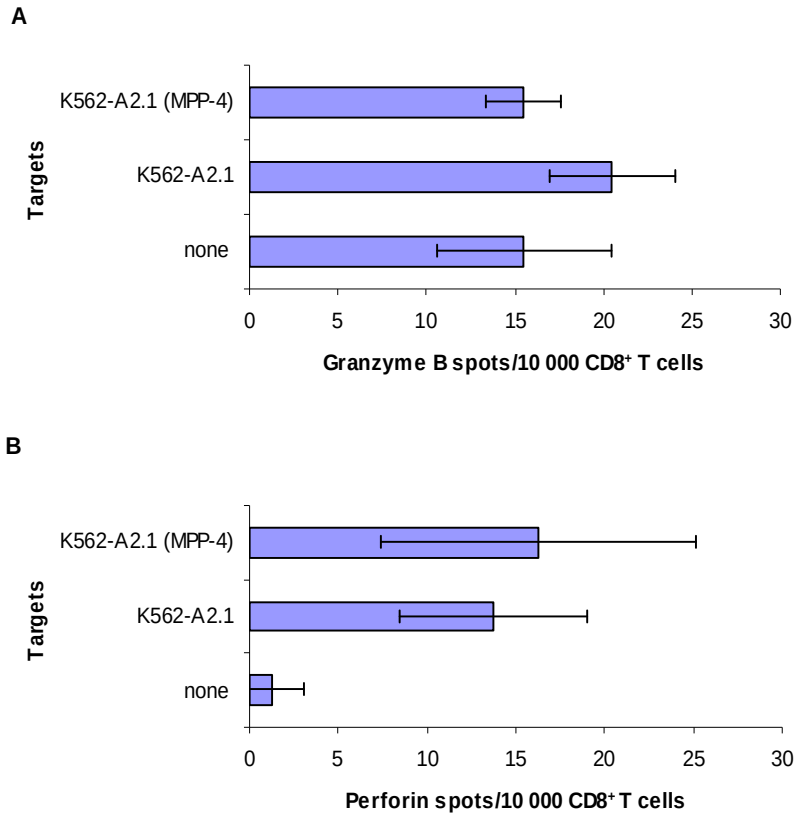

Figure 5.23. Non-specific granzyme B and perforin release of BC-41 CTLs generated by DC2d cells against the MPP-4 peptides using an ELISPOT assay.

The anti-MPP-4 T cell line was established from BC-41, an HLA-A0201$^+$ donor, and tested following five to eight rounds of *in vitro* T cell stimulations. Targets were K562-A2.1 cells pulsed with either no MPP-4 peptide or with the peptide. The background observed when T cells alone were used is shown. (A) Shows non-specific granzyme B release for BC-41 MPP-4 T cell line. (B) Shows non-specific perforin release for BC-41 MPP-4 T cell line. Results are expressed as granzyme B or perforin positive cells per 10.000 CD8$^+$ T cells and are the mean of duplicate assays (±SD).

5.3.5.2. MPP-4 CTLs specifically lysed MPP-4-pulsed target cells

The expanded anti-MPP-3 and anti-MPP-4 T cell lines generated from the four HLA-A*0201 donors were tested for their specificity against T2 cells and autologous LCL using a chromium release assay. Specific lysis of 16.4% against the T2 cells loaded with MPP-4 peptide compared to 0.25% in the absence of the peptide was recorded for TCL-21 MPP-4 at an effector to target ratio (E:T) of 50:1 (Figure 5.24A). 34.3% specific lysis for the same T cell line was recorded against autologous LCL (LCL-21) loaded with the MPP-4 peptide compared to 6.5% against autologous LCL in the absence of the peptide at E: T ratio of 50:1 (Figure 5.24B). The cytotoxic activity of MPP-4 T cell line against K562-A2.1 was also measured. K562-A2.1 is a leukaemia cell line expressing MPP11 and transfected with the HLA-A*0201 molecule. At an E:T ratio of 50:1, MPP-4 CTLs exerted a lytic activity of 13% against this leukaemia cell line. Whereas only 3.65% lysis towards the wild type K-562 (lacking the HLA-A2 expression) was recorded at the same E: T ratio (Figure 5.24C).

In accordance with the ELISPOT results, only the MPP-4 T cell line generated from BC-21 showed peptide specificity while T cell lines generated from the other three donors (BC-32, BC-37 and BC-41) could not lyse different HLA-A2.1^{+} target cells loaded with the MPP-4 peptide (Figure 5.25, Figure 5.26 and Figure 5.27). Non-specific lysis was recorded for the TCL-32 MPP-4 challenged with unpulsed or peptide pulsed T2 cells (Figure 5.25A) and LCL-32 (Figure 5.25B). Non-specific lysis was recoreded by the same T cell line against K-562 and K562-A2.1 (Figure 5.25C). Very low level of lysis (2.8% only) by TCL-37 MPP-4 of peptide-loaded LCL-37 was recorded (Figure 5.26). The TCL-41 MPP-4 failed to recognize peptide-loaded LCL-41 (Figure 5.27A) as well as K562-A2.1 (Figure 5.27B).

Once more the results of the ELISPOT are consistent with the cytotoxic findings. Herein, all of the four T cell lines generated against the MPP-3 peptide did not show any specific cytotoxic activity (Figure 5.28, Figure 5.29, Figure 5.30 and Figure 5.31). The TCL-21 MPP-3 failed to recognize peptide-loaded T2 cells (Figure 5.28A) as well as peptide-loaded autologous LCL (Figure 5.28B). The TCL-32

MPP-3 failed to recognize the peptide-loaded T2 cells (Figure 5.29A) and showed no lytic activity against unpulsed or peptide-pulsed autologous LCL (Figure 5.29B). The same thing was recorded for the TCL-37 MPP-3 when challenged with autologous LCL (Figure 5.30). The TCL-41 MPP-3 did not recognize peptide-pulsed LCL-41 (Figure 5.31A) as well as K562-A2.1 (Figure 5.31B).

5.3.5.3. Cloning of MPP-4 CTLs

The TCL-21 MPP-4 that showed specificity to the MPP-4 peptide was cloned at 1, 2 and 5 cells/well against the T2 loaded peptide and a pool of allogeneic PBMCs in the presence of IL-2 and IL-15 using a standard limiting dilution assay. In order to obtain an optimal expansion, anti-CD3 antibody (OKT3) was added. 200 T cell clones were generated. These clones were screened for their specificity against the MPP-4 peptide using the cytotoxic chromium release assay. The T2 cells were either unpulsed or pulsed with MPP-4 peptide. Unfortunately, none of the clones showed specific lytic activity for the MPP-4 peptide. Therefore, another attempt was done to clone the T cell line in the presence of IL-2 only (without the addition of either IL-15 or OKT3), however, the generated T cell clones (9 clones) died at an early stage. Moreover, the specific cytotoxicity of the generated MPP-4 T cell lines was only seen with the line generated from the BC-21 donor. We were not successful in generating specific T cell lines against the MPP-4 peptide using a further three HLA-A*0201 positive donors.

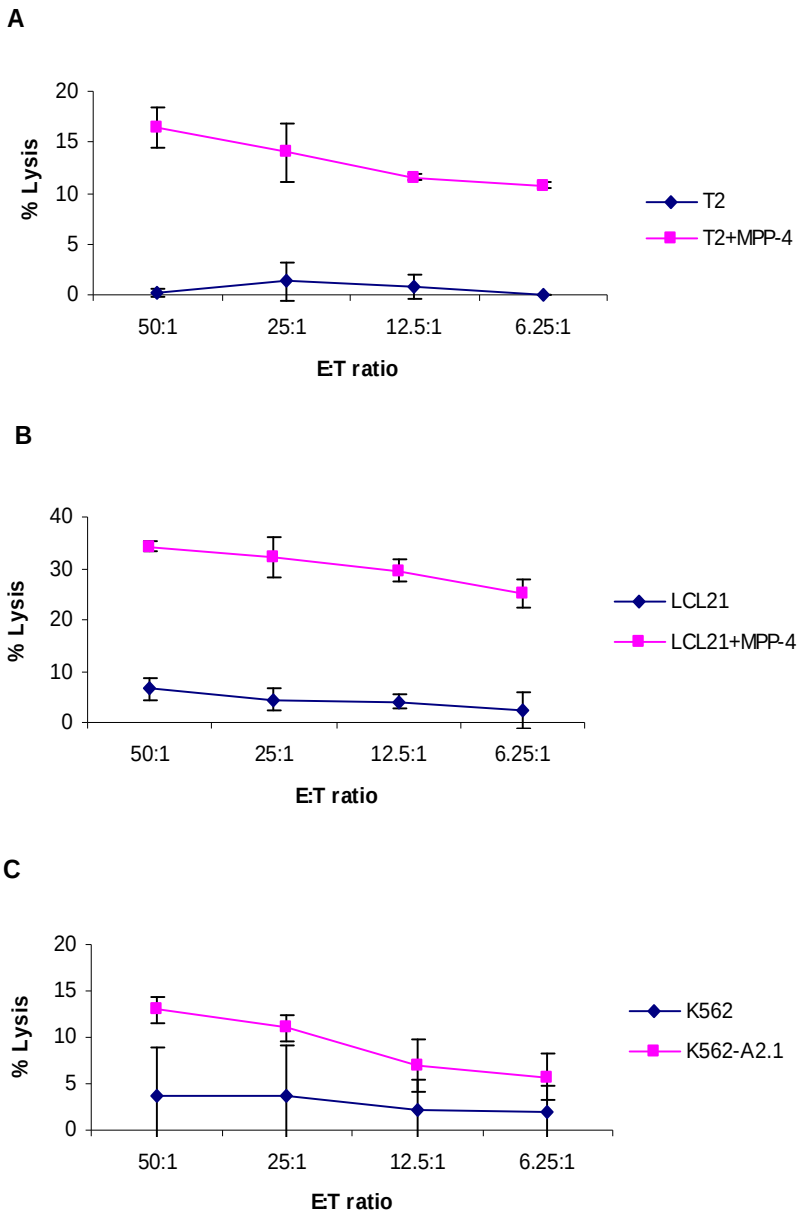

Figure 5.24. Recognition of HLA-A0201^{+} target cells by BC-21 T cell line generated by DC2d against MPP-4 peptide as tested using a standard chromium release assay.

The MPP-4 T cell line generated from normal HLA-A0201^{+} donor, BC-21, was used as effector cells. (A) Target cells were T2 cells pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). (B) Target cells were autologous LCL-21 pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). (C) Target cells were K562 (dark blue symbol) and K562-A2.1 (pink symbol). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiment and error bars indicate the SD.

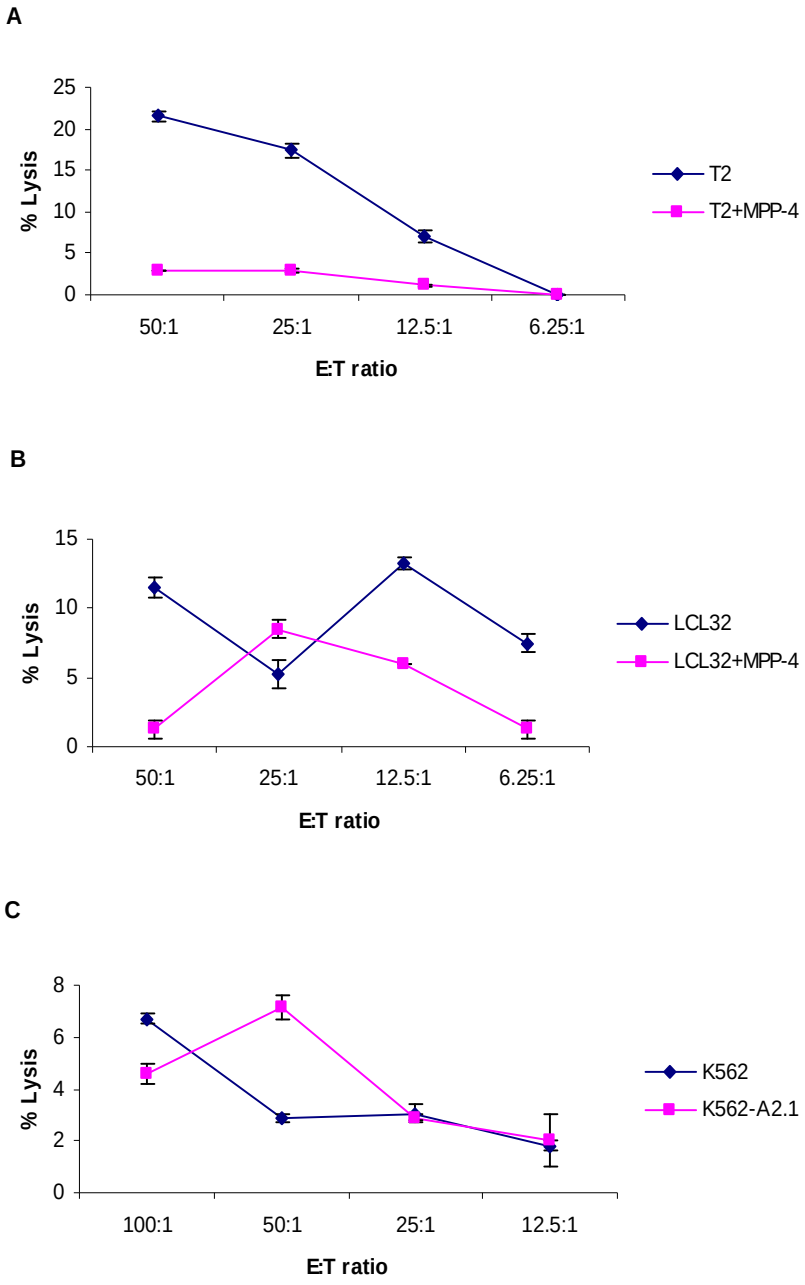

Figure 5.25. Non-specific lysis by BC-32 T cell line generated by DC2d against MPP-4 peptide as tested using a standard chromium release assay.

The MPP-4 T cell line generated from normal HLA-A0201^{+} donor, BC-32, were used as effector cells. (A) Target cells were T2 cells pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). (B) Target cells were autologous LCL-32 pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). (C) Target cells were K562 (dark blue symbol) and K562-A2.1 (pink symbol). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiment and error bars indicate the SD.

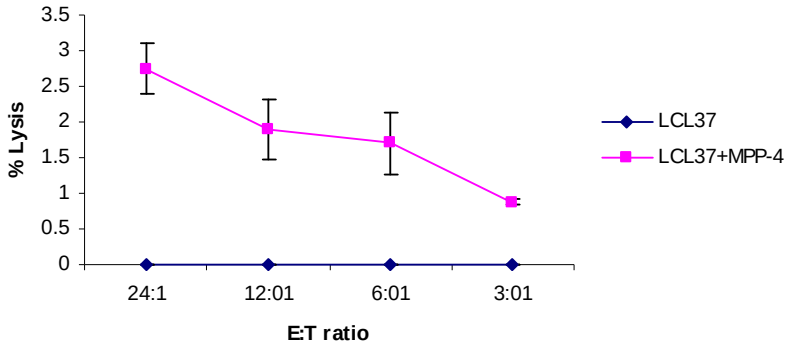

Figure 5.26. Non-specific lysis by BC-37 T cell line generated by DC2d against MPP-4 peptide as tested using a standard chromium release assay.

The MPP-4 T cell line generated from normal HLA-A0201^{+} donor, BC-37, were used as effector cells. Target cells were autologous LCL-37 pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiment and error bars indicate the SD.

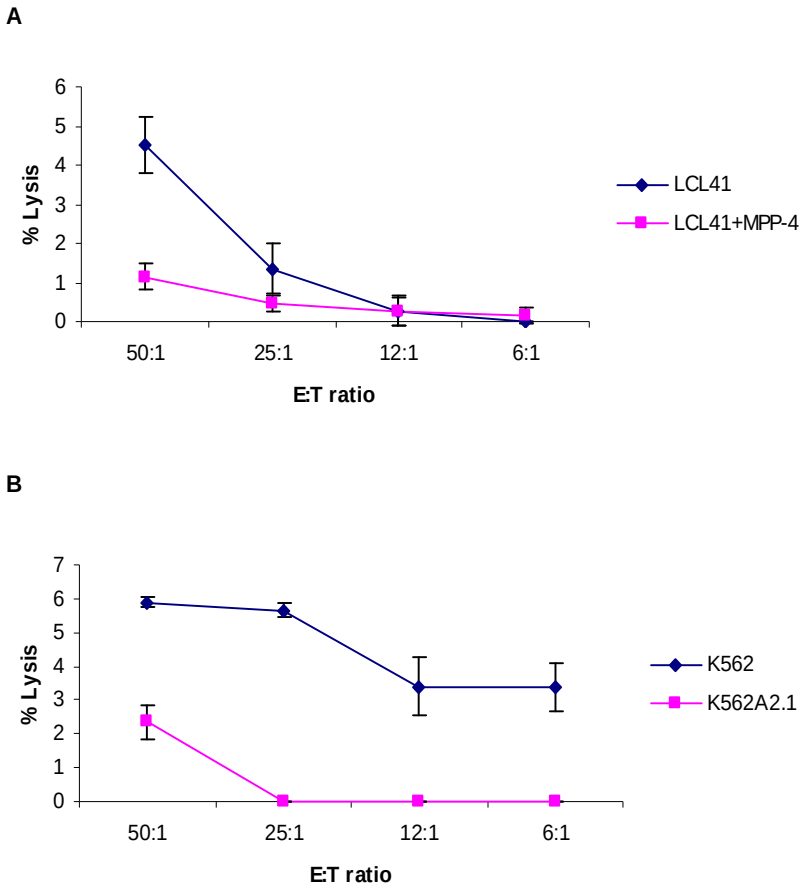

Figure 5.27. Non-specific lysis by BC-41 T cell line generated by DC2d against MPP-4 peptide as tested using a standard chromium release assay.

The MPP-4 T cell line generated from normal HLA-A0201^{+} donor, BC-41, were used as effector cells. (A) Target cells were autologous LCL-41 pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). (B) Target cells were K562 (dark blue symbol) and K562-A2.1 (pink symbol). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiment and error bars indicate the SD.

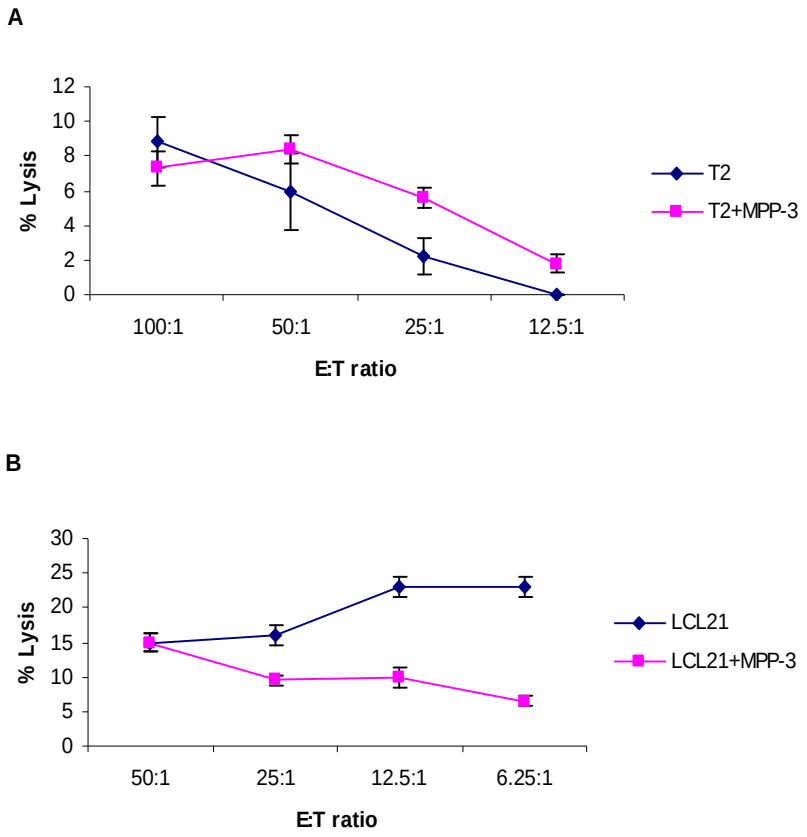

Figure 5.28. Non-specific lysis by BC-21 T cell line generated by DC2d against MPP-3 peptide as tested using a standard chromium release assay.

The MPP-3 T cell line generated from a normal HLA-A0201^{+} donor, BC-21, were used as effector cells. (A) Target cells were T2 cells pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). (B) Target cells were autologous LCL-21 pulsed with either no peptide (dark blue symbol) or with MPP-4 peptide (pink symbol). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

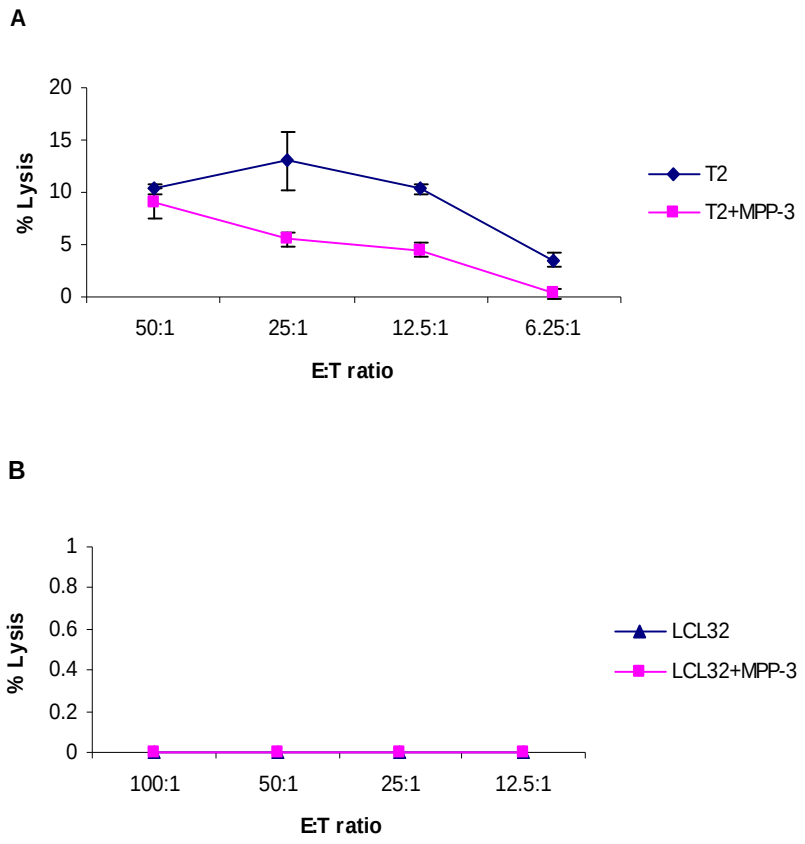

Figure 5.29. Non-specific lysis by BC-32 T cell line generated by DC2d against MPP-3 peptide as tested using a standard chromium release assay.

The MPP-3 T cell line generated from a normal HLA-A0201^{+} donor, BC-32 was used as effector cells. The T cells were stimulated with T2 cells (A) and with autologous LCL-32 (B). Target cells pulsed with no peptide are represented by dark blue symbol, whereas target cells pulsed with MPP-3 peptide are represented by pink symbol. The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

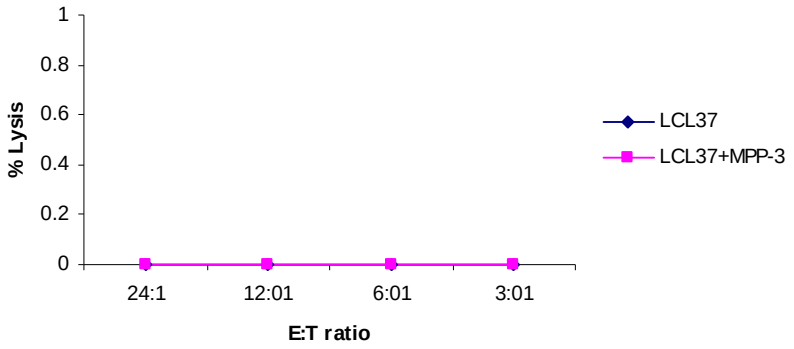

Figure 5.30. Non-specific lysis by BC-37 T cell line generated by DC2d against MPP-3 peptide as tested using a standard chromium release assay.

The MPP-3 T cell line generated from a normal HLA-A0201^{+} donor was used as effector cells. The T cells were stimulated with autologous LCL-37. LCL-37 pulsed with no peptide are represented by dark blue symbol, whereas LCL-37 pulsed with MPP-3 peptide are represented by pink symbol. The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

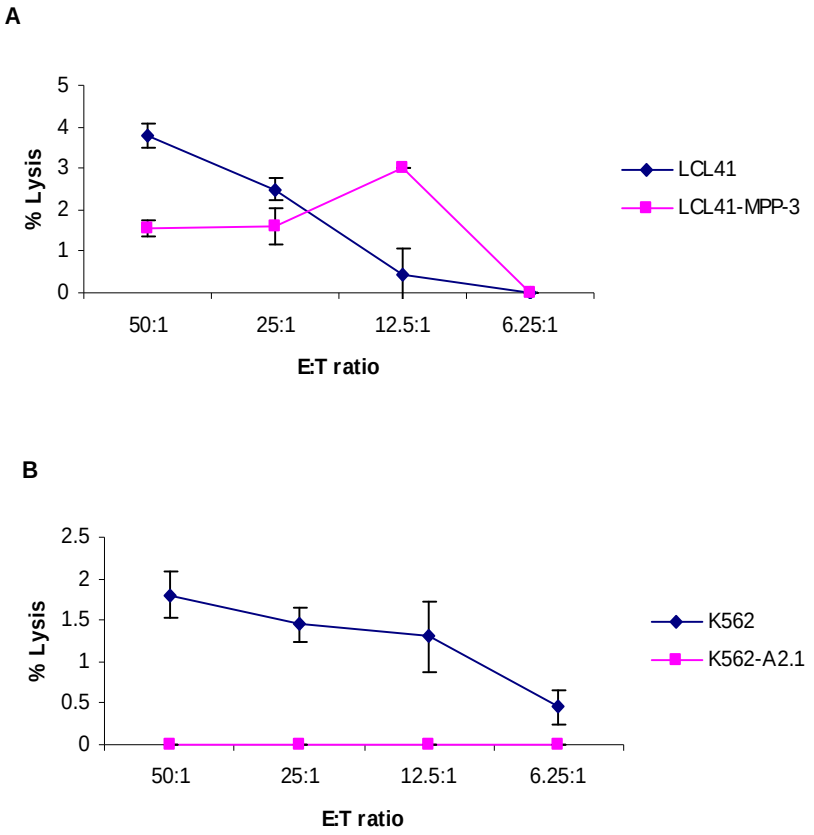

Figure 5.31. Non-specific lysis by BC-41 T cell line generated by DC2d against MPP-3 peptide as tested using a standard chromium release assay.

The MPP-3 T cell line generated from a normal HLA-A0201^{+} donor, BC-41, was used as effector cells. (A) Target cells were T2 cells pulsed with either no peptide (dark blue symbol) or with MPP-3 peptide (pink symbol). (B) Target cells were K562 (dark blue symbol) and K562-A2.1 (pink symbol). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

5.3.6. Generation of PR3 specific T lymphocyte lines

The ability of the PR3-derived peptides to induce specific cytotoxic T lymphocytes (CTLs) was tested. PBMCs obtained from three different HLA-A*0201 positive healthy volunteers were utilized to generate CTLs specific to the selected peptides. The three donors were designated as BC-32, BC-37 and BC-41. The PBMCs from each donor were used as the source of the responder cells and antigen presenting cells. The responder cells were $CD8^+$ T cells purified by MACS negative selection and stimulated in vitro with antigen presenting cells-loaded peptide for 7-10 days. The protocol used for the generation of $CD8^+$ T cell lines utilized peptide-pulsed DC2d (fast DC) to prime the purified $CD8^+$ T cells and peptide-pulsed monocytes for subsequent T cell stimulations. The DC2d were generated as described in Materials and Methods section 2.5.4.3.

To investigate whether the PR3 synthetic peptides could stimulate peptide-specific CTLs, the generated T cell lines were tested against different HLA-A*0201^+ targets in the presence or absence of autologous peptide using a Chromium Release Assay and an ELISPOT assay after 3 to 8 rounds of stimulations.

5.3.7. $CD8^+$ T cell responses to PR3-derived peptides

5.3.7.1. IFN-γ and granzyme B production by PR3 T cell lines

After the fourth round of stimulation, the specificity of the in vitro primed CTLs was tested first for IFN-γ release to stimulation with the corresponding peptides using an ELISPOT assay. All the generated BC-32-CTL lines were able to produce IFN-γ against autologous targets regardless of the presence or absence of the corresponding peptide. No specific IFN-γ production was recorded for the two CTLs generated against the PR-129 and PR-9 test peptides (Figure 5.32A and B). However, the CTL line generated with the PR-169 positive control peptide produced IFN-γ after stimulation with autologous LCLs loaded with the PR-169 peptide (Figure 5.32C). Interestingly, when monocytes were used as stimulators, IFN- γ specific production by the anti-PR169 CTL were observed in the presence of peptide and only when IL-2 (10 ng/ml) was added (Figure 5.32C).

The specificity of the T cell lines generated from the two other donors, BC-37 and BC-41, was also tested in IFN-γ–ELISPOT assays. Targets used to test BC-37 T cell lines were T2 cells and autologous LCL pulsed with either no peptide or with corresponding PR-3-peptide. K562-A2.1 cells, loaded with either no peptide or with corresponding PR-3-peptide, were used to test BC-41 T cell lines. As shown in Figure 5.33 and Figure 5.34, none of the generated PR-3-T cell lines produced specific IFN-γ spots when challenged with peptide-loaded target cells including the positive control PR-169.

The next ELISPOT assay was to test whether the generated PR3-T cell lines produce specific granzyme B. K562-A2.1 and T2 cells loaded with each peptide were used as stimulators in this ELISPOT assay. No granzyme B production was recorded for the BC-37-CTLs, BC-32-CTLs as well as BC-41-CTLs generated against the PR-9 and PR-129 test peptides (Figure 5.35A and B), (Figure 5.36A and B) and (Figure 5.37A and B). However, the control peptide PR-169-CTL generated from BC-37, BC-32 and BC-41 produced granzyme B after stimulation with T2 or K562-A2.1 loaded with the PR-169 peptide (Figure 5.35C, Figure 5.36C and Figure 5.37C).

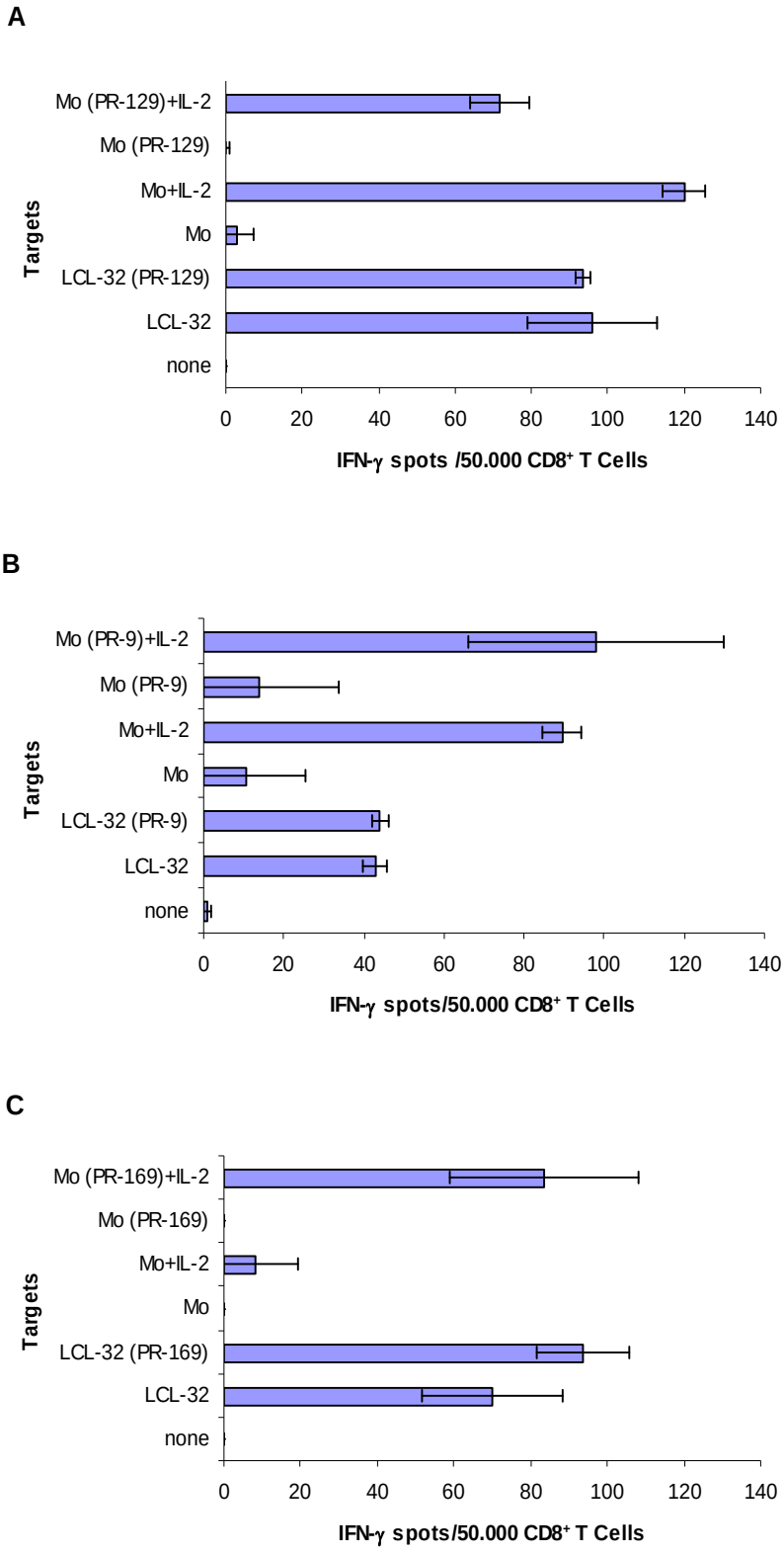

Figure 5.32. ELISPOT assay for IFN-γ production by BC-32 T cell lines generated by DC2d cells against peptides derived from the PR-3 antigen.

Figure 5.32. Continued

The T cell lines were established from a healthy HLA-A*0201^{+} donor, BC-32, and tested following four to eight rounds of in vitro T cell stimulations. T cell line generated against PR-169 peptide was included as a positive control. Targets were LCL-32 and autologous monocytes pulsed with either no peptide or with peptide. Monocytes were pulsed with each peptide in the presence or absence of IL-2. T cell lines generated against PR-129 (A) and PR-9 (B) did not produce more spots of IFN-γ when stimulated with target cells pulsed with the corresponding peptide. The positive control PR-169 produces more IFN-γ spots when stimulated with LCL-pulsed peptide and monocyte-pulsed peptide in the presence of IL-2 (C). The background observed when T cells alone were used is shown. Results are expressed as IFN- γ positive cells per 50.000 CD8^{+} T cells and are the mean of duplicate assays (±SD). Mo= Monocytes.

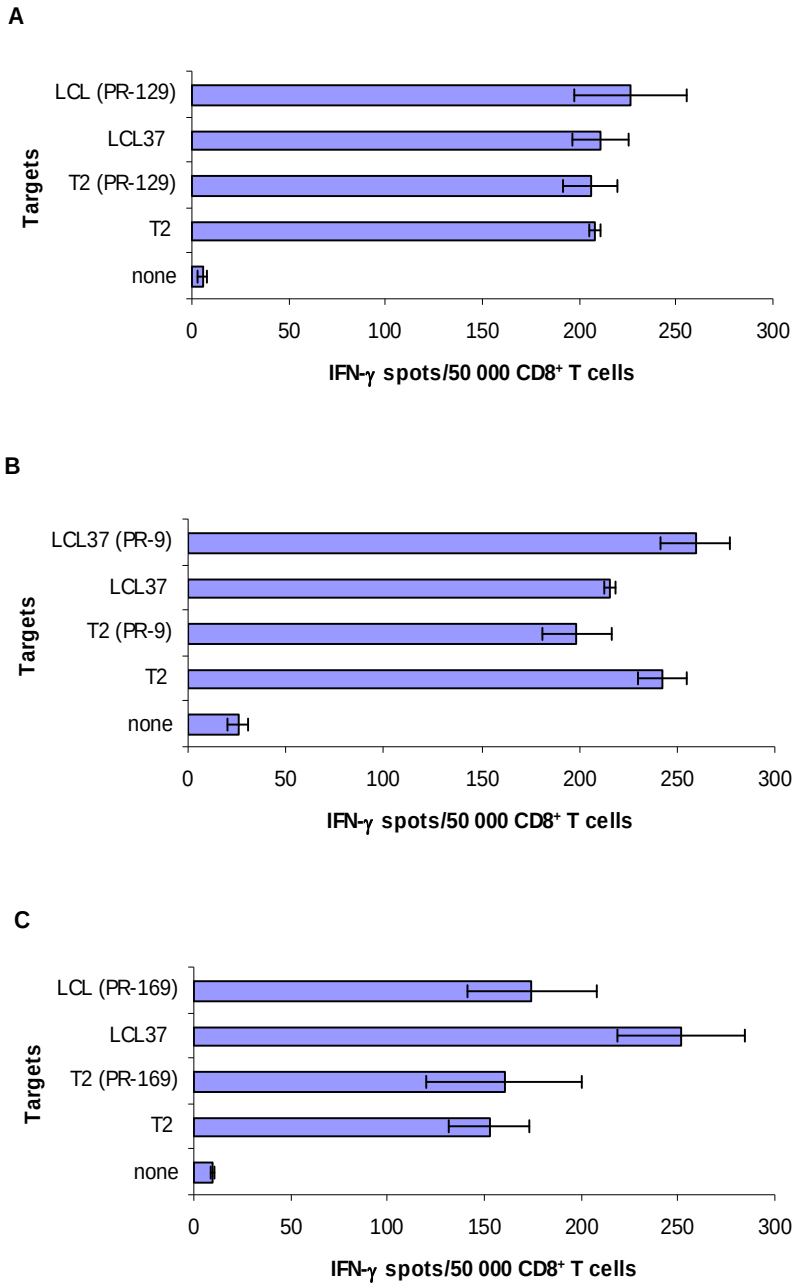

Figure 5.33. ELISPOT assay for IFN-γ production by BC-37 T cell lines generated by DC2d cells against peptides derived from the PR-3 antigen.

The T cell lines were established from a healthy HLA-A*0201^{+} donor, BC-37, and tested following four to eight rounds of in vitro T cell stimulations. T cell line generated against PR-169 peptide was included as a positive control. Targets were T2 cells and LCL-37 pulsed with either no peptide or with peptide. The T cell lines generated against PR-129 (A), PR-9 (B) and PR-169 (C) did not produce more spots of IFN-γ when stimulated with target cells pulsed with the corresponding peptide. The background observed when T cells alone were used is shown. Results are expressed as IFN- γ positive cells per 50.000 CD8^{+} T cells and are the mean of duplicate assays (±SD).

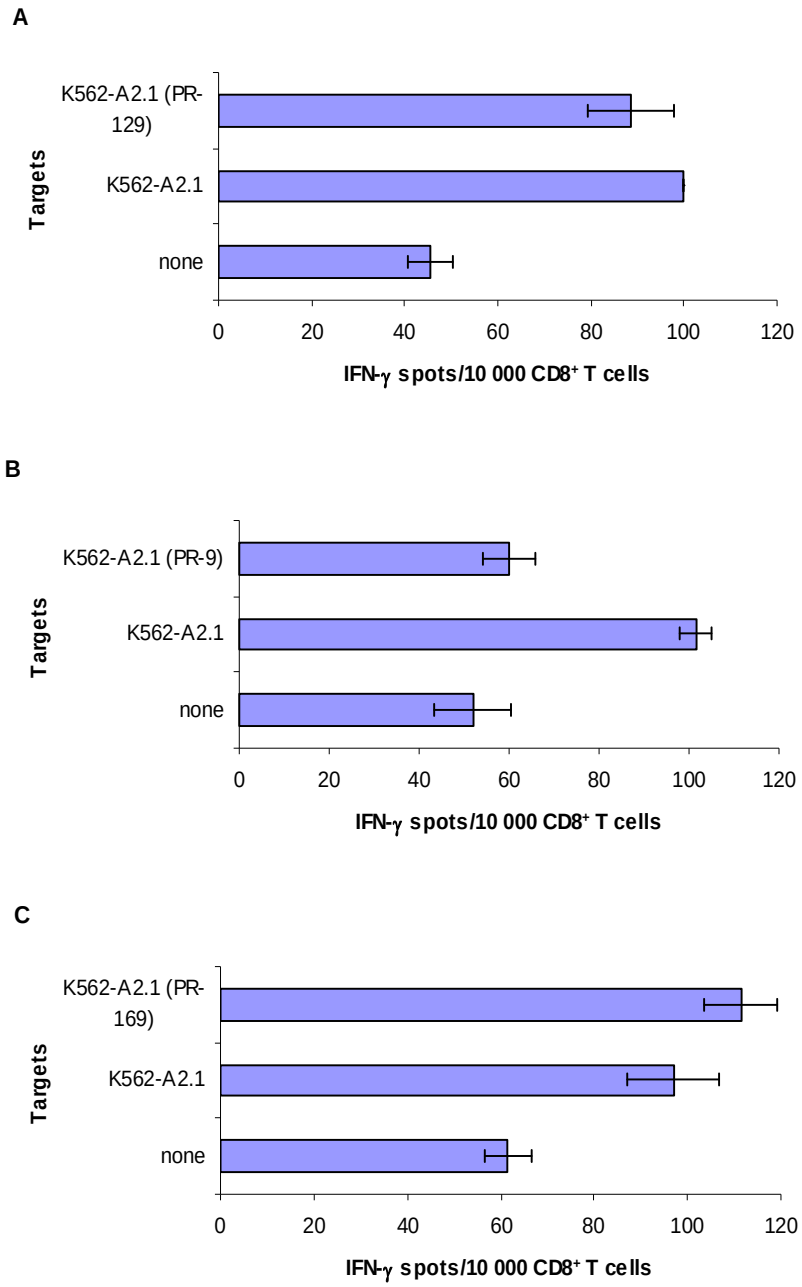

Figure 5.34. ELISPOT assay for IFN-γ production by BC-41 T cell lines generated by DC2d cells against peptides derived from the PR-3 antigen.

The T cell lines were established from a healthy HLA-A*0201$^+$ donor, BC-41, and tested following four to eight rounds of in vitro T cell stimulations. T cell line generated against PR-169 peptide was included as a positive control. Targets were T2 cells and LCL-37 pulsed with either no peptide or with peptide. The T cell lines generated against PR-129 (A), PR-9 (B) and PR-169 (C) did not produce more spots of IFN-γ when stimulated with target cells pulsed with the corresponding peptide. The background observed when T cells alone were used is shown. Results are expressed as IFN- γ positive cells per 10.000 CD8$^+$ T cells and are the mean of duplicate assays (±SD).

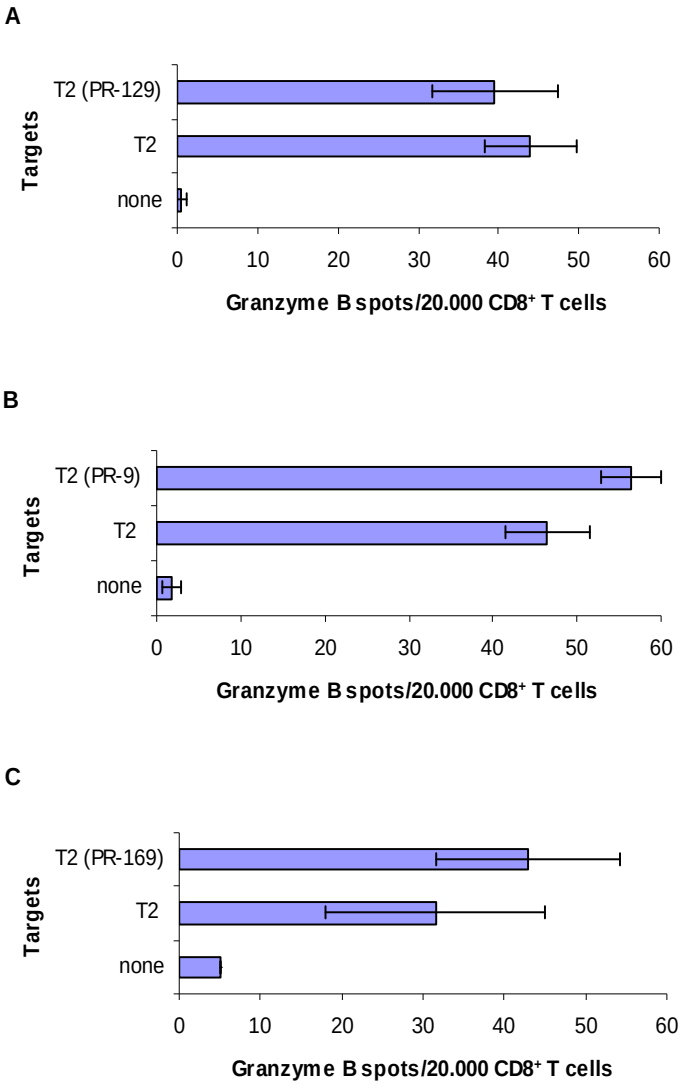

Figure 5.35. ELISPOT assay for granzyme B production by BC-37 T cell lines generated by DC2d cells against the peptides derived from PR-3 antigen.

The T cell lines were established from a healthy HLA-A*0201$^+$ donor, BC-37, and tested following three to eight rounds of in vitro T cell stimulations. T cell line generated against PR-169 peptide was included as a positive control. Targets were T2 pulsed with either no peptide or with peptide. T cell lines generated against PR-129 (A) and PR-9 (B) did not produce more spots of granzyme B when stimulated with T2 cells pulsed with the corresponding peptide. The positive control PR-169 produces more granzyme B spots when stimulated with T2-pulsed peptide (C). The background observed when T cells alone were used is shown. Results are expressed as granzyme B positive cells per 20.000 CD8$^+$ T cells and are the mean of duplicate assays (±SD).

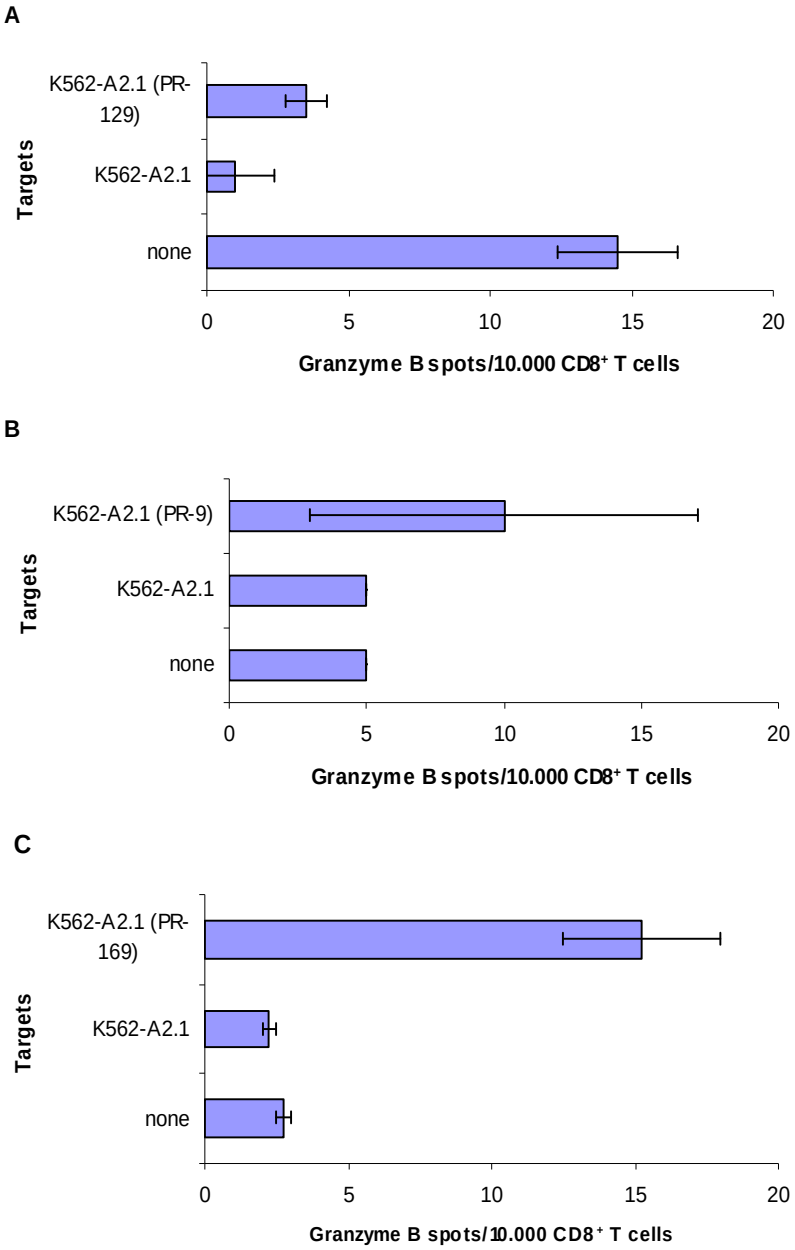

Figure 5.36. ELISPOT assay for granzyme B production by BC-32 T cell lines generated by DC2d cells against the peptides derived from PR-3 antigen.

The T cell lines were established from a healthy HLA-A*0201^{+} donor, BC-32, and tested following three to eight rounds of in vitro T cell stimulations. T cell line generated against PR-169 peptide was included as a positive control. Targets were K562-A2.1 cells pulsed with either no peptide or with peptide. T cell lines generated against PR-129 (A) and PR-9 (B) did not produce more spots of granzyme B when stimulated with K562-A2.1 cells pulsed with the corresponding peptide. The positive control PR-169 produces more granzyme B spots when stimulated with K562-A2.1-pulsed peptide (C). The background observed when T cells alone were used is shown. Results are expressed as granzyme B positive cells per 10.000 CD8^{+} T cells and are the mean of duplicate assays (±SD).

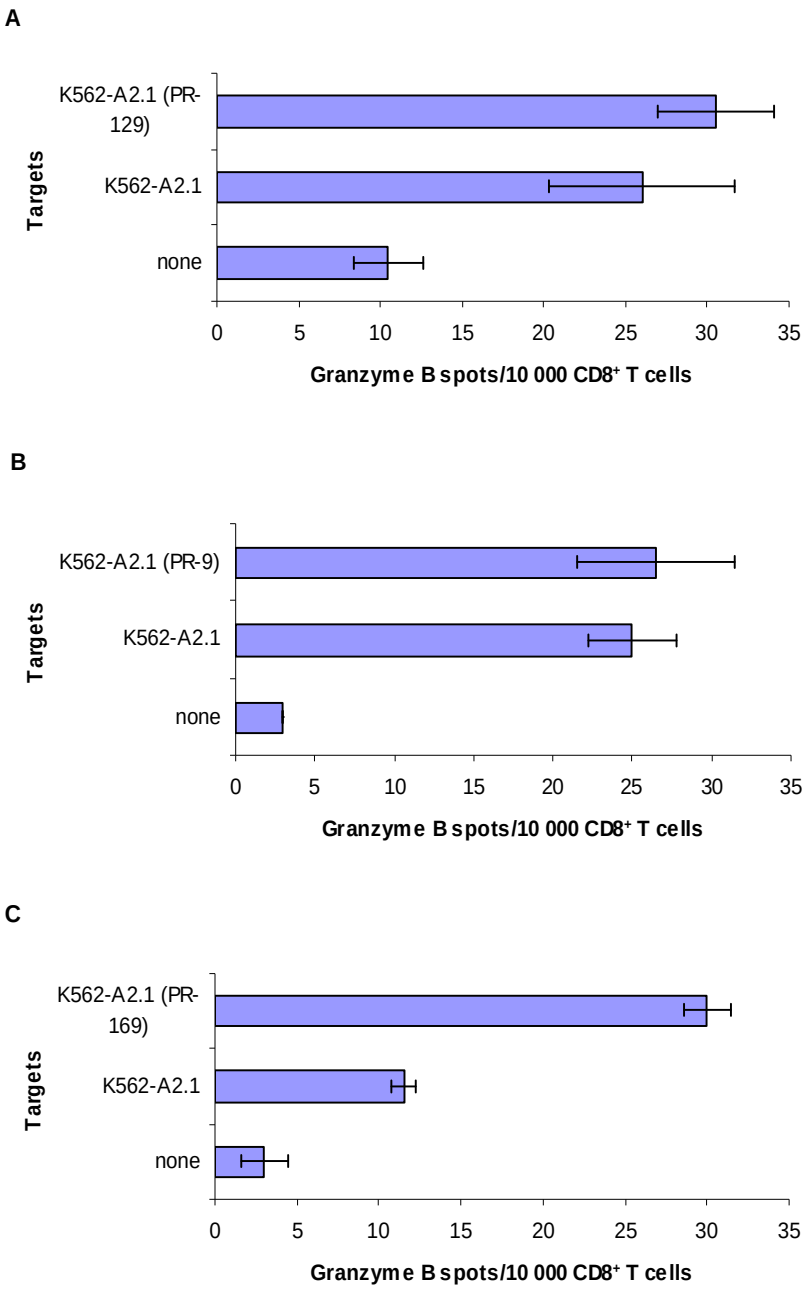

Figure 5.37. ELISPOT assay for granzyme B production by BC-41 T cell lines generated by DC2d cells against the peptides derived from PR-3 antigen.

The T cell lines were established from a healthy HLA-A*0201^{+} donor, BC-41, and tested following three to eight rounds of in vitro T cell stimulations. T cell line generated against PR-169 peptide was included as a positive control. Targets were K562-A2.1 cells pulsed with either no peptide or with peptide. T cell lines generated against PR-129 (A) and PR-9 (B) did not produce more spots of granzyme B when stimulated with K562-A2.1 cells pulsed with the corresponding peptide.

Figure 5.37. Continued

The positive control PR-169 produces more granzyme B spots when stimulated with K562-A2.1-pulsed peptide (C). The background observed when T cells alone were used is shown. Results are expressed as granzyme B positive cells per 10.000 $CD8^+$ T cells and are the mean of duplicate assays (±SD).

5.3.7.2. Cytotoxic activity of anti-PR3 CTLs

The next step in monitoring $CD8^+$ T cell responses to PR3-derived peptides was to test the cytotoxic activity of the generated T cell lines in a standard Cr^{51}-release assay. T2 cells or autologous LCLs were pulsed with no peptide or with each peptide and used as targets at different E:T ratio. Among the three HLA-A*0201 donors used to generate anti-PR-3 CTLs, peptide-specific cytotoxic responses were only demonstrated with a T cell line generated from one donor i.e. BC-32. Consistent with the results obtained with the ELISPOT assay; the T cell line generated against the previously identified epitope PR-169 showed peptide-specific cytotoxic responses, whereas the T cell lines generated from the same donor against the PR-9 and PR-129 test peptides did not elicit any peptide-specific cytotoxic responses. The PR-129-T cell line peptide did not recognize peptide-pulsed T2 and LCL-32 targets cells (Figure 5.17A and B). Similarly, the PR-9-T cell line peptide tested against T2 cells and LCL-32 did not recognize peptide-pulsed target cells (Figure 5.39A and B). The PR-169-CTL line recorded a specific lysis of 51% against T2 cells loaded with PR-169 peptide compared to 6% in the absence of the peptide at E:T ratio 25:1 (Figure 5.40A). In addition, 10% specific lysis was demonstrated for the same CTL line against LCL-32 pulsed-peptide and only 4% lysis against LCL-32 in the absence of the peptide (Figure 5.40 A &B).

The specificity of the T cell lines generated from BC-37 donor was also examined using cytotoxicity assays. However, none of the generated CTL lines showed a specific activity against peptide-loaded LCL-37 including the positive control PR-169 peptide (Figure 5.41A, B and C). The cytotoxic activity of the T cell lines generated from BC-41 could not be tested due to low cell number.

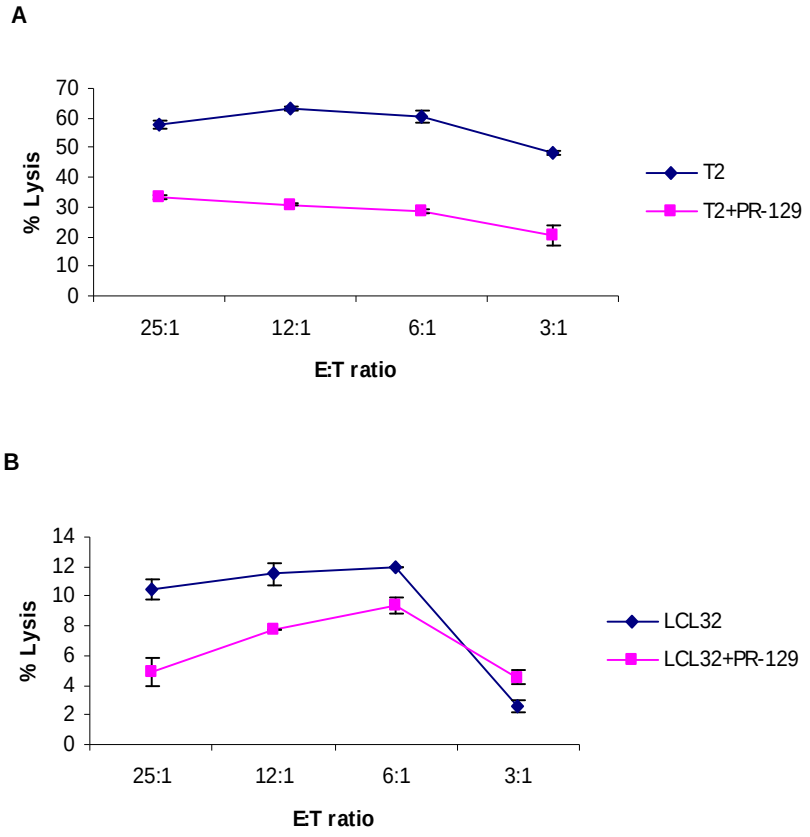

Figure 5.38. Non-specific lysis by BC-32 T cell line generated by DC2d against PR-129 peptide as tested using a standard chromium release assay.

The PR-129 T cell line generated from normal HLA-A0201^{+} donor, BC-32, was used as effector cells. Target cells were T2 cells and LCL-32 pulsed with either no peptide (dark blue symbol) or with PR-129 peptide (pink symbol). The PR-129 T cell line was stimulated with T2 cells (A) and with LCL-32 (B). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

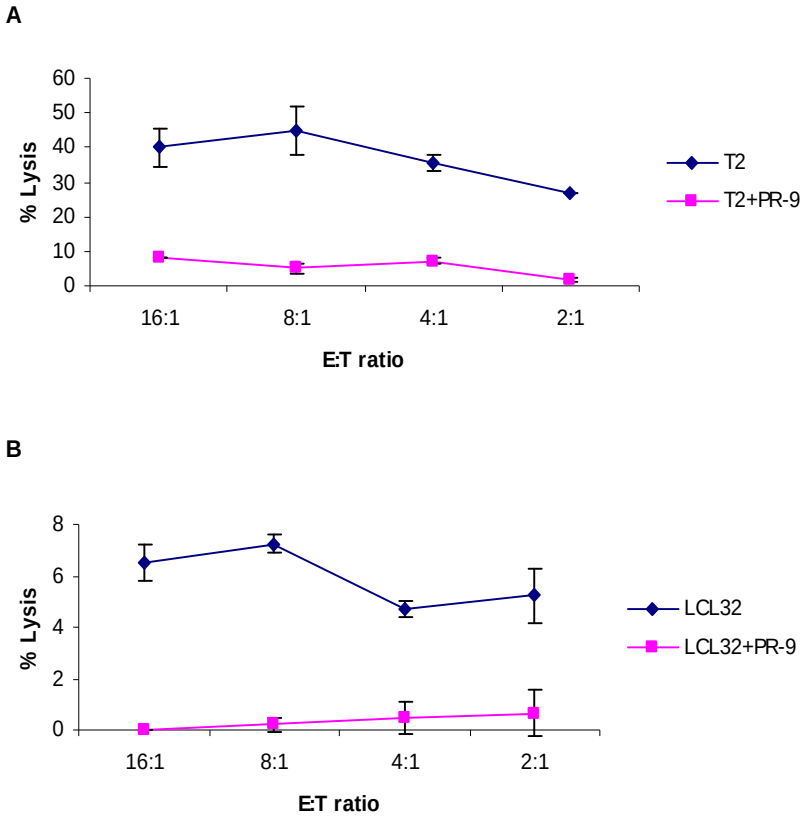

Figure 5.39. Non-specific lysis by BC-32 T cell line generated by DC2d against PR-9 peptide as tested using a standard chromium release assay.

The PR-9 T cell line generated from normal HLA-A0201^{+} donor, BC-32, was used as effector cells. Target cells were T2 cells and LCL-32 pulsed with either no peptide (dark blue symbol) or with PR-9 peptide (pink symbol). The PR-9 T cell line was stimulated with T2 cells (A) and with LCL-32 (B). The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

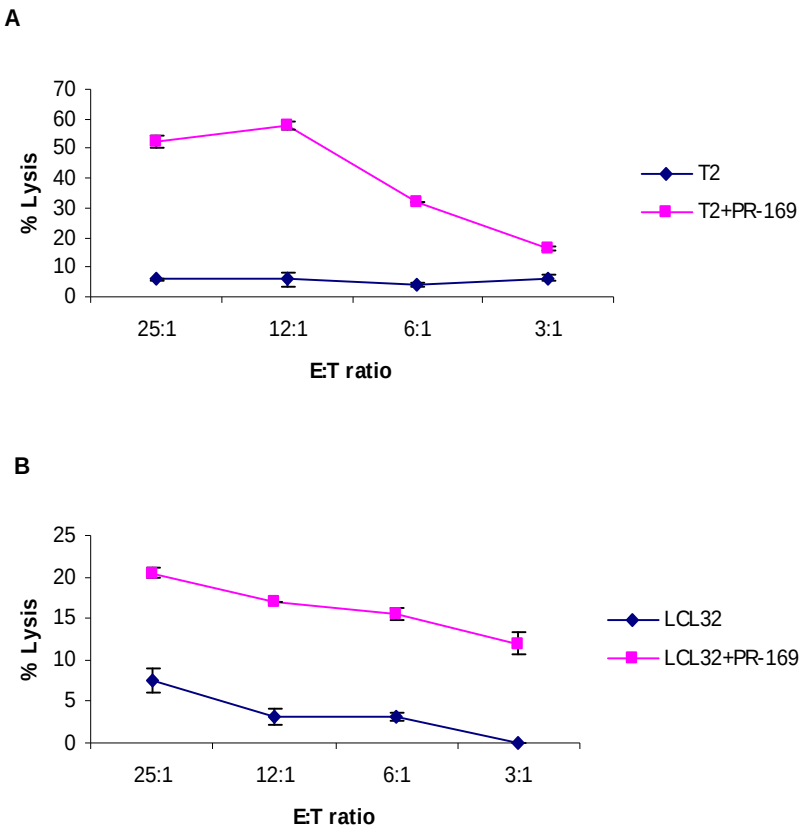

Figure 5.40. Recognition of peptide pulse target cells by BC-32 T cell line generated by DC2d against PR-169 peptide as tested using a standard chromium release assay.

The PR-169 T cell line generated from normal HLA-A0201^{+} donor, BC-32, was used as effector cells. Target cells were autologous T2 cells and LCL-32 pulsed with either no peptide (dark blue symbol) or with PR-169 peptide (pink symbol). (A) Shows the cytotoxic activity of PR-169 cell line against peptide-pulsed T2. (B) Shows the cytotoxic activity of PR-169 cell line against peptide-pulsed LCL-32. The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

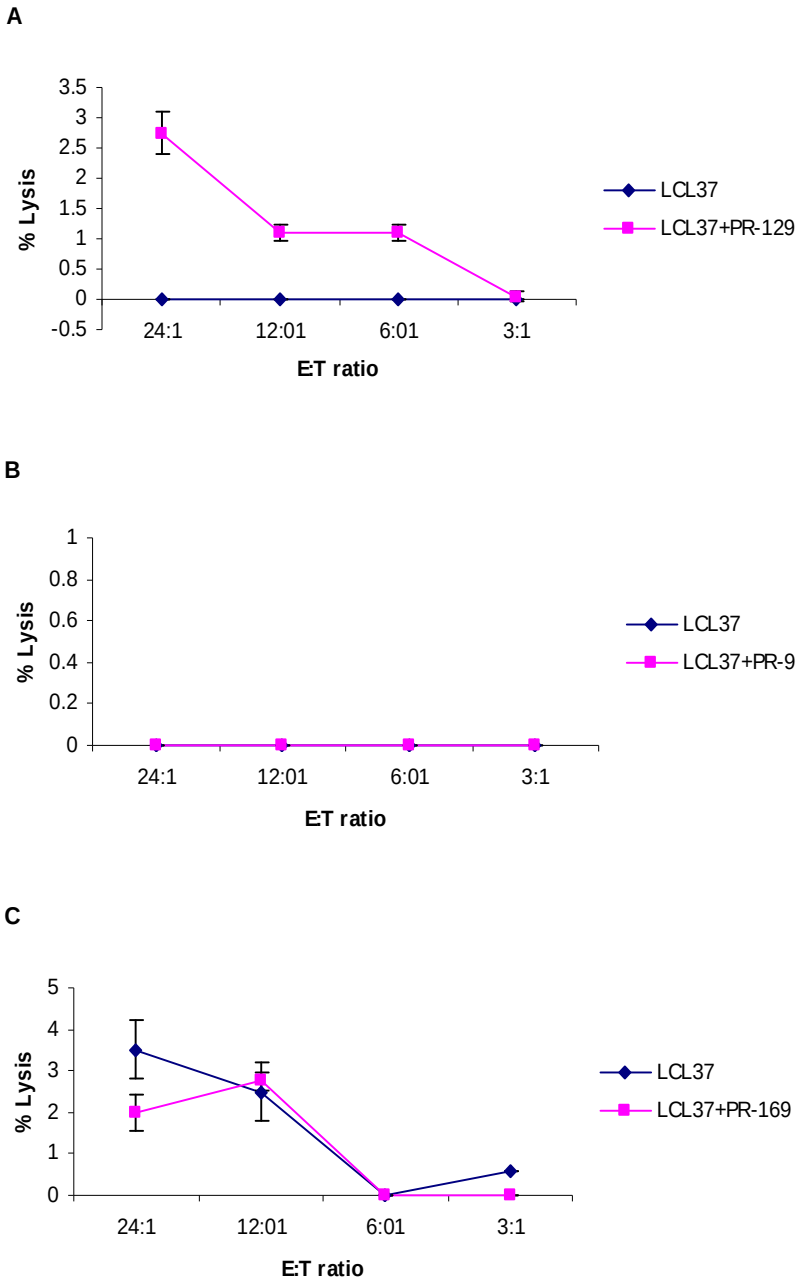

Figure 5.41. Non-specific lysis by BC-37 T cell lines generated by DC2d against the PR-3 peptides as tested using a standard chromium release assay.

The three T cell lines, PR-129, PR-9 and PR-169, were generated from a normal HLA-A0201^{+} donor, BC-37. The PR-169 T cell line was included as a positive control. The T cells were used as effector cells. Target cells were LCL-37 pulsed with either no peptide (dark blue symbol) or with corresponding peptide (pink symbol). (A) Shows non-specific lysis of the T cell line generated against the PR-129 peptide. (B) Shows non-specific lysis of the T cell line generated against the PR-9 peptide. (C) Shows non-specific lysis of the T cell line generated against the PR-169 peptide.

Figure 5.41. Continued

The data are presented as the percentage specific lysis at the indicated E:T ratios and represents the average of the two experiments and error bars indicate the SD.

5.4. Discussion

$CD8^+$ cytotoxic T lymphocytes (CTLs) recognize endogenously processed peptides consisting of 8-11 amino acids, most commonly 9 amino acids long, presented on the cell surface of target cells in the context of major histocompatibility complex (MHC) class I molecules. These cells are believed to play an important role in identifying and eliminating tumour cells (Melief and Kast, 1995). To date, several $CD8^+$ T cell epitopes derived from leukaemia antigens have been identified, some of them have been investigated in clinical trials (Heslop et al., 2003, Qazilbash et al., 2004, Schmitt et al., 2008). Importantly, the graft versus leukaemia (GVL) effect often seen after allogeneic HSCT has been found to be mediated mostly by donor-derived $CD8^+$ T cells recognizing peptides derived from leukaemia antigens and/or minor histocompatibility antigens (mHAs) presented on recipient cells (Schetelig et al., 2005, Wu and Ritz, 2006, Appelbaum, 2001). Overexpressed antigens such as WT1, PR-3 and MUC1 represent possible targets of specific GVL reactions (Kapp et al., 2009, Molldrem et al., 2000, Rezvani et al., 2003).

This study investigated the potential of $CD8^+$ T cell epitopes derived from the leukaemia associated antigens MPP11 and PR-3 using a reverse immunological approach. Previous studies have reported the existence of a humoral response against MPP11 protein as well as the overexpression of this protein in patients with myeloid leukaemias (Greiner et al., 2003, Greiner et al., 2004, Schmitt et al., 2006). However, the identification of $CD8^+$ T-cell epitopes derived from the MPP11 protein is a crucial contribution to the field of leukaemia immunotherapy. Although the antigenic epitope PR-1 has already been identified (Molldrem et al., 1996); PR-3 seems to be a rich source of peptide epitopes that can elicit a specific CTL response. Therefore, additional A*0201-restricted epitopes capable of inducing CTL responses were searched for within known databases, and tested.

The reverse immunological approach permitted the identification of class I as well as class II candidate epitopes derived from known tumour antigens on the basis of HLA-binding motifs (Stevanovic, 2002, Paschen et al., 2004). Using this approach, numerous epitopes could be identified in leukaemia (Molldrem et al., 1996, Oka et al., 2000, Greiner et al., 2005, Asemissen et al., 2006) as well as in solid tumours (Alves et al., 2003, Ayyoub et al., 2002, Fujie et al., 1999, Kawashima et al., 1998,

Kessler et al., 2001, Neumann et al., 2005). The amino acid sequences derived from MPP11 and PR-3 proteins were examined for the presence of peptides containing binding motifs for one of the most frequent class I alleles, A*0201 allele using computer-based epitope prediction programs. Four MPP11-derived peptides were identified which were predicted to bind with high affinity to HLA-A*0201 molecule. The peptides were named MPP-1N, MPP-2, MPP-3 and MPP-4. The actual binding capacity of the selected peptides was tested using T2 binding assay. The MPP11- derived peptides MPP-3 and MPP-4 recorded binding scores lower than MPP-1N, and MPP2 scores as determined by the SYFPEITHI software. However, in the actual T2 binding assay, MPP-3 and MPP-4 were found to have higher binding affinities compared to the two other peptides. This was probably due to a very poor dissolution of the MPP-1N and MPP-2 peptides since more hydrophobic amino acids are contained within their sequence. In addition to the four predicted peptides, the amino acid sequence of the highest scoring peptide i.e., MPP-1N (QLLIKAVNL) was modified. Studies (Tourdot et al., 2000, Valmori et al., 1998, Al Qudaihi et al., 2009) have shown that introducing a tyrosine amino acid residue at the first position (P1Y) of a peptide enhances epitope immunogenicity. Therefore, a tyrosine amino acid residue was introduced in P1 of the MPP-1N peptide in order to boost its binding affinity and /or immunogenicity. The modification of the MPP-1N peptide enhanced the binding affinity of the modified MPP-1Y peptide using the algorithm prediction but in the actual binding assay, the analogue peptide could not stabilize the HLA-A*0201 molecule which again may be due to its poor dissolution.

Inconsistent with other groups (Molldrem et al., 1996, Oka et al., 2000, Houbiers et al., 1993) who have succeeded in identifying $CD8^+$ T-cell epitopes using a simple method that employs T2 cells as antigen-presenting cells, specific immune responses could not be generated using this method to both MPP11-derived peptides as well as the WT1-126N peptide which was included as a positive control. Since MPP11 and WT1 are self-proteins, the majority of high avidity $CD8^+$ T cells might have been deleted during thymic maturation (Starr et al., 2003). Thus it is more difficult to generate cytotoxic T cell responses from the remaining low avidity-naïve precursors because their activation required more stringent costimulatory requirements (Janeway and Bottomly, 1994, Swain et al., 1996). DCs as antigen-presenting cells

were therefore used for the generation of anti-MPP11 CTL immune responses. DCs are known as the professional antigen presenting cells because of their unique ability to stimulate naïve T-cell responses (Banchereau and Steinman, 1998). However, following priming of naïve T cells (usually two rounds of T cell stimulation) other good APCs such as monocytes and T-APCs could be used for T cell expaniosn. The conventional protocol for generating monocyte-derived DCs include 7-9 days of culture, however, Dauer et al., (2003) described a rapid protocol for the generation of mature DCs from monocytes within only two days of in vitro culture. Such cells, termed as FastDC or DC2d, were found to be capable of inducing tumour-specific $CD8^+$ T cell responses as effectively as DCs generated according to the conventional 7-day method (Dauer et al., 2005, Ho et al., 2006). Moreover, CTL lines primed with DC2d expanded more effectively and showed greater lytic activity than lines stimulated with DC7d (Dauer et al., 2005, Ho et al., 2006). Using DC2d as antigen presenting cells to prime T cell responses, antigen-specific responses by CTL-21 generated against MPP-4 peptide were detected via cytokine production and cytotoxic assays. MPP-4-CTLs selectively recognized peptide-loaded T2 cells and responded by releasing IFN-γ in an ELISPOT assay (Fig. 5.3A). These T cells specifically killed T2 cells and autologous LCLs when loaded with the MPP-4 peptide suggesting recognition was selective to the MPP-4 peptide (Fig. 5.4 A&B). Moreover, they were capable of recognizing the leukaemia cell lines K562-A2.1 expressing MPP11 protein (Fig. 5.4 C). Even though, T2 cells were not efficient at priming and generating specific-$CD8^+$ T cell immunity, however these cells were very useful as targets when conducting functional assays such as cytotoxicity and ELISPOT.

Four healthy HLA-A*0201 positive individuals were used to generate MPP-4-specific CTLs; however, specific responses could only be observed in one CTL line established from one donor, i.e., BC 21. The failure to obtain specific CTL responses to tumour antigens in all four donors tested has been observed previously and it has been suggested to be due to the low frequency of peptide-specific $CD8^+$ T cell precursors in healthy individuals (Ohminami et al., 2000). The MPP-4 CTL line was cloned before testing its capability of recognizing fresh A*0201-positive leukemic cells that overexpressed MPP11 protein. Unfortunately, no MPP-4-specific clones could be generated. Although leukaemia samples were screened for the

MPP11 gene expression, the results were not used as no specific clones against MPP-4 were generated. Standard techniques for cloning T cells employ stimulation with antigen loaded-APCs in the presence of IL-2 and allogeneic feeder cells in limiting dilution cultures followed by repeated re-stimulations to maintain growth and specificity. In the first attempt, the MPP-4 T cell line was cloned by stimulation with peptide pulsed-T2 cells in the presence of allogeneic feeder cells, IL-2, IL-15 and anti-CD3 monoclonal antibody (OKT3). Interleukin-2 is the cytokine frequently used to promote the survival and expansion of cultured T cells (Smith, 1988). IL-2 and IL-15 share the β-chain and the common γ chain receptor (IL-2/IL-15 Rβγc), but each cytokine has its unique α-chain receptor (Carson et al., 1994, Grabstein et al., 1994). IL-15 was initially identified by its capability to promote the development of NK cells and the survival of $CD8^+$ memory T cells in vivo (Colucci et al., 2003, Zhang et al., 1998). However, it has been reported that anti-tumour-CTLs expanded and survived for relatively long time periods in vitro in the presence of IL-15 whereas CTLs maintained in IL-2 died at earlier stages (Lu et al., 2002). Furthermore, IL-15-maintained CTLs retained their effector function as cytotoxic cells and did not switch into memory cells (Lu et al., 2002). For TCR triggering, OKT3 was used as reported previously (Ho et al., 2006). Because all of the generated clones were not specific, another attempt to clone the line was conducted but without the addition of either IL-15 or anti-CD3 monoclonal antibody. This time the generated clones were few and died at an early stage perhaps because of old T cell culture.

Two PR-3 peptides (PR-129 and PR-9) that contained anchor motifs required for the binding to the class I-A*0201 molecule were identified. However, both peptides were incapable of inducing specific $CD8^+$ T cell responses. Although the PR-129 peptide displayed high binding affinity to the HLA-A*0201 molecule, there were no precursor T cells with appropriate T cell receptors (TCRs) reactive to these peptides. This result implied again that peptide binding capacity to the HLA molecule was not the only factor that determines its immunogenicity. The positive control PR-169 peptide was effective in inducing specific responses as measured by the ELISPOT as well as in cytotoxicity assays in two (BC 32 and BC 37) out of four HLA-A*0201 positive donors. The demonstration that the positive control PR-169 peptide was effective in inducing specific cytotoxic activity and specific cytokine production

indicated that the stimulation system used in the present study was optimum for generation of cytotoxic T cells. Previous studies have reported the overexpression of PR-3 protein in patients with myeloid leukaemias (Muller-Berat et al., 1994, Yong et al., 2008). In this study, the leukaemic samples were not screened for the PR-3 expression. There was no need to select leukaemia samples that express PR-3 antigen since no specific T cell responses were recorded against PR-9 and PR-129 peptides.

In summary, the data suggested that $CD8^+$ T cells reactive with the MPP-4 peptide could be generated from the T cell repertoire of HLA-$A0201^+$ healthy individuals. More importantly, these CTLs were able to specifically lyse the K562-A2.1 leukemic cells naturally expressing the MPP11 antigen. The study also demonstrated that both PR-129 and PR-9 peptides were non-immunogenic.

6. GENERAL DISCUSSION

A major obstacle facing cancer vaccines is tolerance to self-tumour antigens which can prevent the development of effective anti-tumour CTL responses. Different approaches have been tested to circumvent this problem (Bellantuono et al., 2002, Gao et al., 2000, Xue et al., 2005). Among these, modified peptide ligands known as heteroclitic peptides have been used to improve the reactivity of T cells specific for tumour antigens by enhancing their affinity to HLA molecules or TCR. These peptide ligands are analogues of native tumour antigens generated by single or double amino acid substitutions at certain MHC anchor positions. A number of peptide analogues derived from melanoma associated antigens and leukaemia antigens capable of recognizing native epitopes with enhanced immunogenicity have been described (Tsuboi et al., 2002, Pinilla-Ibarz et al., 2005, Valmori et al., 1998, Bae et al., 2004a, Bae et al., 2004b, Parkhurst et al., 1996). Tsuboi et al (2002) reported that a modified HLA-A*2402-restricted WT1 peptide exhibited a higher binding affinity and elicited WT1-specific CTLs more effectively than the natural WT1 peptide. Bae *et al* (2004a, , 2004b) identified a novel HLA-A*0201-restricted epitope derived from CD33 and showed that analogues to the native sequence displayed enhanced affinity and immunogenicity. Indeed, the modification approach has been used to enhance immunogenicity of epitopes not only derived from self antigens but also derived from the leukaemia-specific antigen BCR-ABL. In this, HLA-A*0201-restricted epitopes derived from BCR-ABL junctional region have been improved in terms of their binding capacity and immunogenicity by modifying their sequences (Pinilla-Ibarz et al., 2005). Based on these findings, analogues derived from WT1 and BCR-ABL have been tested in vaccination trials in which clinical responses to the vaccines have been reported (Maslak et al., 2008, Oka et al., 2004).

In this study, an analogue to a previously identified epitope, Db126 (RMFPNAPYL), derived from the WT1 antigen has been identified. The analogue 126Y (**Y**MFPNAPYL) was designed using a single amino acid substitution strategy initially described by Tourdot et al (Tourdot et al., 2000). This strategy consists of replacing the amino acid at position 1 of HLA-A*0201-associated peptides with a tyrosine residue (Y). Although vaccination trials using the native Db126 peptide have demonstrated some immunological effect (Rezvani et al., 2008, Mailander et al., 2004, Keilholz et al., 2006), the use of an analogue peptide might enhance the

vaccine potency as it may induce biological responses superior to that induced by the native sequence. Therefore, the purpose of developing this analogue was to test whether it would elicit potent $CD8^+$ CTL responses more efficiently than the native peptide and whether it would be capable of recognizing its native sequence. Data presented in chapter 3 showed that modification in the sequence of the wild type epitope improved its binding to the HLA-A*0201 molecule and enhanced its immunogenicity. Importantly, CTLs generated with the modified 126Y peptide lysed HLA-matched $WT1^+$ AML cells more efficiently than CTLs generated with the native epitope. Similar results supporting our findings have been reported by the Scheinberg group (Pinilla-Ibarz et al., 2006). They showed that the WT1A1 analogue (the same as 126Y analogue) generated more potent CD8 CTL responses and was able to recognize and lyse $WT1^+$ CML cell lines. In a subsequent study, the same group identified an HLA-DR-restricted epitope (WT1-122A1, SGQA**Y**MFPNAPYLPSCLES) spanning the WT1 amino acids 122-140 and showed that inclusion of the heteroclitic peptide WT1A1/126Y within the sequence of the helper peptide induces a robust CD4 and CD8 T cell response (May et al., 2007). In addition, the modified WT1-122A1 epitope was more potent in inducing cytotoxic $CD8^+$ CTL responses than the native sequence, WT1-122A (May et al., 2007). These results along with results presented in this thesis demonstrate that the analogue 126Y epitope is able to cross-react with its native sequence and it is more efficient than the native epitope in inducing anti-WT1 responses. All together these data should open the door for a potential immunotherapeutic approach utilizing WT1-126Y peptide analogue to target malignant tumour cells in HLA-matched leukaemia and possibly in other WT1-expressing tumours.

The ability of the 126Y peptide analogue to induce reliable immune responses in vivo has been tested by the Scheinberg group via a recently conducted pilot study using multivalent-WT1 peptides to vaccinate patients with AML, mesothelioma and non-small cell lung cancer (Maslak et al., 2010, Krug et al., 2010). The vaccine consisted of the analogue peptide WT1A1/126Y and three class II peptides including the modified WT1-122A1. A total of ten AML patients in complete remission (CR) following chemotherapy who had measurable WT1 transcript were enrolled. Patients received the four WT1 peptides plus the immune adjuvant montanide and GM-CSF. In this trial, robust CD4 and CD8 T-cell immune responses were observed to the

WT1 peptides in high proportion of patients with minimal toxicity. Importantly, the WT1-122A1 heteroclitic peptide was more efficient in inducing anti-126N immune responses than its native sequences (Maslak et al., 2010). Data from this trial suggested that it is possible to induce effector cells capable of recognizing WT1-expressing malignant cells in vitro. However, further clinical trials that evaluate the clinical effectiveness of this vaccine as a post-remission therapy for leukaemia patients are needed.

Despite the promise of using heteroclitic peptides as a strategy to break tolerance to the WT1 antigen, the mono-therapy vaccine alone will not be able to achieve robust and persistent anti-tumour responses that will prevent relapse. For the best clinical benefit it will probably be necessary to combine multiple therapeutic interventions which include blockade of host-negative immunoregulatory mechanisms (checkpoints) such as CTLA-4, PD-1 and most importantly regulatory T cells (Tregs). Tregs have been shown to play an important role in modulating both natural and adoptive immune responses in cancer patients. It has been demonstrated that Tregs directly suppress the anti-tumour immune responses in cancer patients (Beyer and Schultze, 2006, Ghebeh et al., 2008), and depletion of this T-cell population resulted in an enhancement of vaccine-mediated anti-tumour immunity in cancer patients (Dannull et al., 2005, Mahnke et al., 2007). Furthermore, Tregs have been shown to be inversely related to the outcome of several human malignancies (Salama et al., 2009, Heimberger et al., 2008). Importantly, fully functional Tregs specific for LAGE1 and ARTC1 were demonstrated in melanoma patients (Wang et al., 2004, Wang et al., 2005a). Greiner et al (2006) investigated the influence of the expression levels of several LAAs on the clinical outcome of patients with AML. High expression of three LAAs was found to be associated with favourable clinical outcome, inducing strong $CD8^+$ CTL responses. However, there was no correlation with the clinical outcome nor induction of natural anti-WT1 $CD8^+$ CTL response in these patients (Greiner et al., 2006). In line with this, Nishikawa et al (2005) has shown the existence of tumour-specific Tregs, which actively suppress antigen-specific anti-tumour immunity in cancer patients. Another study by Nadal and colleagues suggested that Tregs exert an inhibitory effect on graft versus leukaemia and this was associated with relapse after allogeneic stem cell transplantation (Nadal et al., 2007). Importantly, it has been shown that anti-WT1 responses can be largely

affected by the presence of $CD4^+CD25^+$ Tregs in which depletion of this T-cell population was necessary for the generation of an effective WT1-specific CTL responses (Asemissen et al., 2006). Moreover, our group has identified a human HLA-DRB1*0402–restricted $CD4^+CD25^+FOXP3^+$ Tregs population specific for the WT1 antigen and showed that this antigen is a novel target for leukaemia-specific $CD4^+$ Tregs (Lehe et al., 2008). In this study, we have generated Tregs cell lines and clones using a pool of 110 15-mer overlapping peptides across the entire WT1 protein. These T-cell lines and clones specifically recognized a $WT1_{333-347}$-derived peptide (RYFKLSHLQMHSRKH) designated WT1-84 in an HLA-DRB1*0402–restricted manner. The Treg clones were able to recognize HLA-DRB1*04–matched fresh leukemic cells expressing the WT1 antigen and were able to significantly inhibit the proliferative activity of allogeneic T cells. We also detected anti–WT1-84 Tregs in HLA-$DR4^+$ AML patients. Furthermore, we have shown that the presence of Tregs strongly inhibited the induction of anti–WT1-126 $CD8^+$ CTL responses (Lehe et al., 2008). Therefore, the existence of such tumour-specific Tregs can actively suppress the WT1-specific anti-tumour immunity in cancer patients as shown in other systems (Nishikawa et al., 2005). In addition to our previous findings, a novel MHC class II epitope derived from WT1 antigen and presented by DRB1*04 ($WT1\text{-}60_{237-251}$, TMNQMNLGATLKGVA) was identified in this presented study. This peptide was recognized by $CD4^+$ T cells obtained from a healthy HLA-DRB1*0406,0701 donor. The anti-WT1-60- peptide T-cell line was generated using the same methodology and conditions used to generate the anti-WT1-84 Tregs. Moreover, the cytokines secreted by these cells was of a Th2 cytokine profile similar to that of generated Tregs. WT1-60 may thus represent another target for Tregs. However, these T cells should be further examined for the presence of Treg markers, to confirm the Treg-phenotype, and for their suppressor effect, to confirm Treg function.

Current interests in cancer immunotherapy should be focused on determining the therapeutic implications of Treg cells ‘‘regulating the regulators.’’ Interestingly, elimination of Tregs by IL-2 conjugated to diphtheria toxin (ONTAK) enhanced vaccine-mediated anti-tumour immunity in cancer patients (Dannull et al., 2005, Mahnke et al., 2007). However, targeting the CD25 molecule may eliminate Tregs, leading to an increase in the susceptibility to autoimmunity, and it can also deplete

activated $CD25^+$ effector cells, which may be important for clearance of cancer and infection (Curtin et al., 2008). Therefore, studies should be tailored toward developing selective depletion strategies directed against Treg-related markers. Alternatively, triggering of TLR8 or OX40, and potentially blocking adenosine, might improve the chances of neutralizing Tregs immunosuppression in cancer patients (Colombo and Piconese, 2007).

Although the WT1 antigen represents an ultimate target for tumour immunotherapy, targeting a single tumour antigen may result in tumour immune escape by losing or down-regulating the expression of the parent protein. Therefore, immunotherapeutic approaches targeting more than one antigen may be more effective in inducing anti-tumour immune responses. In this respect, Rezvani et al (2008) showed that a combined peptide vaccine consisting of PR-1 (VLQELNVTV) and WT1-126 (RMFPNAPYL) peptides was safe and could elicit immunologic responses associated with a reduction in WT1 mRNA expression in patients with myeloid leukaemia. In line with this strategy, the work presented in chapter 5 focused on the identification of novel potential multi-antigenic epitopes, derived from two potential antigens (MPP11 and PR-3), that are presented in the context of HLA-A*0201 molecule and recognized by T cells. The data showed that CTLs directed against the MPP-4 (STLCQVEPV) peptide derived from the MPP11 antigen could be generated form peripheral blood mononuclear cells of an HLA-A*0201 normal donor. These findings strongly suggest that MPP-4-specific T cells should be further investigated as they may be used for the development of an efficient T-cell based leukaemia immunotherapy. MPP-4 specific T cells could be expanded ex vivo for adoptive transfer to leukaemia patients or MPP-4 peptide may be utilized in vaccination trials as a single agent or in combination with epitopes derived from other antigens such as WT1.

Collectively, data presented in this thesis expanded our knowledge of improving T-cell based immunotherapeutic approaches against leukaemia by demonstrating the concept of introducing a modified epitope within a self tumour antigen and by identifying a novel epitope derived from the MPP11 antigen. Indeed the commentary by Justin Kline on our study of the WT1-126 peptide modification "altering tumour antigens may be the next logical step to take on the road to

improving cancer immunotherapy" supports such strategy (Kline, 2009). Epitope-modification represents a promising approach for improving current strategies in immunotherapy for cancer patients. However, tumour immunotherapy should focus on the development of strategies that will further increase the immunogenicity of self-epitopes and decrease tolerance. In addition, such approaches should be incorporated into strategies that specifically deplete or inhibit Tregs in cancer patients as the existence of this T cell population may contribute to the impairment of anti-tumour responses and to the limited success of cancer immunotherapy in humans.

In order to develop immunotherapeutic strategies for a broad range of cancer bearing patients, future studies will investigate whether WT1, MPP11 and PR-3 antigens containing potential epitopes presented by common HLA alleles other than HLA-A*0201. Modifications in the sequence of PR-169 and/or MPP-4 epitopes using the same approach used to promote WT1 peptides and generation of T cell responses using the wild type PR-169 and MPP-4 peptides and their analogues should be also investigated.

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8. APPENDIX

Contents

Papers

Paper I: Enhancement of lytic activity of leukemic cells by $CD8^+$ cytotoxic T lymphocytes generated against a WT1 peptide analogue.

Paper II: The Wilms' tumor antigen is a novel target for human $CD4^+$regulatory T cells: implications for immunotherapy.

Paper III: Identification of a novel peptide derived from the M-phase phosphoprotein 11 (MPP11) leukemic antigen recognized by human $CD8^+$ cytotoxic T lymphocytes.

Posters

Poster I: Investigation of M-Phase Phosphoprotein (MPP11) as a Novel Target for Leukemia T Cell Immunotherapy. Presented at the *British Society of Immunology Congress, Harrogate, UK, 6-9 December 2005.* Published in the *Immunology, 2005, 116, Suppl 1, p 89.*

Poster II: CD8+ cytotoxic T lymphocytes generated against a WT1 peptide analog enhance the lytic activity of leukemic cells. Presented at the *20th Meeting of the European Association for cancer research Conference Lyon, France, 5-8 June 2008.* Published in *European Journal of Cancer Supplements, 2008, 6, P 168.*

Output of HLA Peptide-Binding Prediction Programs

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ORIGINAL ARTICLE: RESEARCH

Enhancement of lytic activity of leukemic cells by CD8$^+$ cytotoxic T lymphocytes generated against a WT1 peptide analogue

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Abstract
The Wilms tumor antigen 1 (WT1) antigen is over-expressed in human leukemias, making it an attractive target for immunotherapy. Most WT1-specific Cytotoxic T Lymphocytes (CTLs) described so far displayed low avidity, limiting its function. To improve the immunogenicity of CTL epitopes, we replaced the first-amino-acid of two known immunogenic WT1-peptides (126 and 187) with a tyrosine. This modification enhances 126Y analogue-binding ability, triggers significant number of IFN-γ-producing T cells ($P = 0.0003$), induces CTL that cross-react with the wild-type peptide, exerts a significant lytic activity against peptide-loaded-targets ($P = 0.0006$) and HLA-A0201-matched-leukemic cells ($P = 0.0014$). These data support peptide modification as a feasible approach for the development of a leukemia-vaccine.

Keywords: *WT1 Db126 analogue, CD8+ CTL, AML, peptide modification, dendritic cells, immunotherapy*

Introduction

Cytotoxic T Lymphocytes (CTLs) recognise tumor antigens in the form of short peptides (CTL epitopes) presented by MHC class I molecules on the cell surface of an antigen presenting cell [1]. In the last few years, a large number of tumor antigens and their corresponding CTL epitopes have been identified in several hematological malignancies and solid tumors [1,2]. The Wilms tumor antigen 1 (WT1), a gene that has been shown to be responsible for the childhood renal cancer [3], performs an oncogenic function in various types of leukemia [4] and solid tumors [5]. This antigen has been shown to serve as marker for disease burden [6–9]. It is overexpressed in acute myelocytic and lymphocytic leukemia, chronic myelocytic leukemia and also in various types of solid tumors including lung and breast cancer [10–12]. For this reason, it has been considered as an attractive target for immunotherapy. Because WT1 has been shown to be expressed at low levels in some normal cells such as CD34$^+$ stem cells [13,14], many of the potential CTL epitopes against this antigen may be absent or sub-optimal. This is mainly due to clonal deletion of high-avidity CTLs during maturation of the immune system [15]. To this end, different groups introduced modifications in the sequence of these 'sub-optimal' peptides to improve their binding to HLA class I molecules and to increase their immunogenicity [16–19]. For example, substitution of amino acid residues pointing to the T cell

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receptor has been shown to enhance epitope immunogenicity [20,21]. Some of these modified peptides have been effectively used to vaccinate cancer patients [22], to improve the detection of anti-tumor immunity [19], or to reverse the irresponsiveness of T lymphocytes to wild-type tumor antigens [21]. Indeed, vaccination of cancer patients with WT1-modified peptides induced WT1-specific CTLs and resulted in tumor regression [23].

It has been demonstrated that WT1 specific CTLs isolated from leukemia patients exert a low-avidity response [24,25]. In the present study, we explored the feasibility of using an approach that enhances the immunogenicity of these low-avidity restricted-peptides without altering their antigenic specificity [26]. This approach consists of replacing the first amino acid of the epitope by a tyrosine (P1Y). The advantage of this approach is that the P1Y variants are able to efficiently trigger *in vivo* wild-type peptide-specific CTLs which also recognised the naturally occurring epitope [26]. We have carried out the Y amino acid substitution in the sequence of the 126 and 187 WT1-derived peptides. We have selected these two peptides as they were reported to be presented by the most common HLA allele (HLA-A0201 present in 50% of the population) and exert specific lysis against WT1-expressing leukemia cells [27].

Our study demonstrated that the replacement of the first amino acid of WT1–126 with tyrosine (126Y) enhanced its binding to HLA-A0201 molecule and increased the CTL lytic activity not only to the 126Y epitope but also to the wild-type 126N counterpart. This modification also enhanced the frequency of the IFN-γ producing T cells and increased the CTL lytic activity against HLA-A0201-matched leukemic cells. Interestingly, we found that the primed T cells generated against this modified peptide can cross-react with the wild-type peptide.

Materials and methods

Cell lines

The T2 and K562 cell lines were purchased from ATCC (Manassas, VA). B-lymphoblastoid cell Lines (LCL) were established by transformation of B cells using Epstein–Barr virus using standard techniques [28]. The T2, LCL and K562 cells were maintained in complete medium (CM) consisting of RPMI 1640 medium (Sigma, MO) supplemented with 10% FCS (Cambrex Bio Science, MD), 2 mM L-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin (Sigma).

Peptides

A **Y** amino acid substitution in the sequence of the following WT1 peptides was carried out: Peptide 126 (wild type 126N = **R**MFPNAPYL, analogue 126Y = **Y**MFPNAPYL), and peptide 187 (wild type 187N = **S**LGEQQYSV, analogue 187Y = **Y**LGE QQYSV). The peptides were synthesised and HPLC-purified to $\geq$90% purity by Alta Bioscience, Birmingham, UK.

*Prediction of peptide-binding to the HLA-A*0201 molecule and in vitro binding ability of the peptides using the T2 binding assay*

We first compared the predictive binding ability of the wild-type (126N and 187N) peptides with their corresponding analogues (126Y and 187Y) to the HLA-A*0201 molecule using a computer-based epitope prediction program (http://bimas.dcrt.nih.gov/molbio/hla_bind). Peptide binding capacity to the HLA-A*0201 molecule was then measured using the T2 binding assay described previously [29]. T2 cells are a hybrid human cell line which lacks most of the MHC class I region including the known transporter proteins for antigenic peptide (TAP) and proteasome genes. They contain the gene HLA-A*0201, but express very low levels of cell-surface HLA-A*0201 and are unable to present endogenous antigens [30]. Stable HLA-A2.1 expression in T2 cells is observed only when peptides that are capable of binding to and stabilising the cell surface expression of the HLA-A2.1 molecule are added exogenously. T2 cells were washed three times in serum-free CM and suspended at 10^6 cells/mL. Cells (10^6 cells/well) were incubated for 18 h in serum-free CM containing different concentrations of peptides and 1 μg/mL β_2-microglobulin (Sigma). The cells were washed twice with cold PBS containing 2% FCS and incubated for 30 min at 4°C with anti-HLA-A2.1 BB7.2 monoclonal antibody conjugated to FITC ((AbD Serotec, NC). The cells were washed three times and fixed with 4% paraformaldehyde. The level of HLA-A2 expression was analysed using FACS Scan (Becton & Dickinson, Immunocytometry Systems, CA). HLA-A2 expression was quantified as fluorescence index according to the following formula: fluorescence index = (mean fluorescence intensity with peptide (mean fluorescence intensity without peptide)/mean fluorescence intensity without peptide.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from patients with leukemia and healthy donors were

isolated from blood samples by density gradient centrifugation over Ficoll–Paque PLUS (Amersham Biosciences AB, Uppsala, Sweden). Written informed consent was obtained from patients and donors prior to sample collection. The cells were aliquoted and cryopreserved in 90% FCS + 10% DMSO according to pre-established procedures. The HLA typing of these samples were carried out at the Immunopathology Laboratory (King Faisal Specialist Hospital and Research Centre) and HLA-A*0201$^+$ subjects were selected. The patients and donor information are presented in Table I.

Generation of autologous monocytes derived dendritic cells

Monocytes (CD14$^+$ cells) were isolated from PBMCs of a healthy donor, designated as BC-32, using MACS human Monocytes Isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The CD14+ cells were adhered and cultured in 24-well plates in X-vivo 15 medium (BioWhittaker, MD) supplemented with 2 mM L-glutamine (DC-medium) in the presence of 50 ng/mL rhIL-4 and 100 ng/mL rhGM-CSF (R&D Systems, MN). 20 ng/mL rhTNF-α, (R&D Systems), 10 ng/mL rhIL-1β (eBioscience, CA) and 25 μg/mL Poly I:C (Sigma) were added on day 6 and mature dendritic cells (DCs) were harvested on day 8.

Generation of anti-WT1 126N and 126Y peptide specific CTLs

CD8+ T lymphocytes were separated from PBMCs obtained from healthy HLA-A2-positive donors using MACS Cell Isolation Kits according to manufacturer's protocols. CD8+ T lymphocytes were stimulated *in vitro* with the 126N or 126Y peptides loaded on DCs. Briefly, the DCs growing in X-vivo 15 medium were washed once in the same medium and incubated with the peptides at concentration of 50 μM/mL and 5 μg/mL β_2-microglobulin for 2 h at 37°C. The peptide loaded DCs were then X irradiated (2500 rads), washed once and added to the CD8+ T lymphocytes at 5:1 T lymphocytes: DCs ratio. IL-7 (20 ng/mL) and IL-12 (100 pg/mL) were added to each culture. The cells were incubated in 2 mL DC-medium + 10% heat inactivated human AB serum (Sigma) in 24-well plates. After 7 days of co-culture, a similar second stimulation was performed, and on the following day, IL-2 (20 IU/mL) was added (the cultured cells were maintained by weekly stimulation with peptide-loaded DCs and feeding with IL-2 the next day). Two T cell lines against the two different peptides were generated. Responder T lymphocytes were tested for cytotoxic activity (Chromium Release Assay) and IFN-γ production (ELISPOT Assay) against different targets. Cross-reactivity between the peptides was also tested to determine if the analogue peptide 126Y is able to induce immune response against the native 126N peptide.

Quantitative real time RT-PCR for WT1 expression in patients' leukemic cells

Total RNA was isolated from PBMC-32, AML-27 and AML-28 samples (see Table I) and treated with DNase1 (Invitrogen, CA). For cDNA synthesis, 1 μg RNA using oligo-dT-primers and M-MLV RT (Invitrogen) were used in a total volume of 25 μL. The WT1 mRNA expression was quantified using Light Cycler FastStart DNA master SYBR Green 1 kit (Roche, Mannheim, Germany) in a Light Cycler (Roche). PBGD was used as house-keeping gene. 2 μL cDNA from the K562 cells was used to generate standard curves [31] in 5-log steps for WT1 and PBGD. Amplification was conducted in a total volume of 20 μL for 40 cycles/10 sec at 95°C, 4 sec/64°C and 35 sec/72°C. Samples were run in triplicates and their relative expression was determined by normalising the expression of each target to PBGD and then comparing this to the normalised expression in a reference sample (K562) to calculate a fold-change-value. Forward primer: TTCATC AAACAGGAGCCGAGC and reverse primer:

Table I. Information for patients and donors used in the study.

Donor/patient samples	HLA-A type	% Blasts*	QRT-PCR WT 1 mRNA†	Status‡	FAB-type§
PBMCC-32	A0201, 29	NA	60	NA	NA
AML-27	25, 29	81	7950	New	M4
AML-28	A0201, 26	61	1800	New	M4

*% blasts were determined routinely by the hematopathology laboratory.
†Quantative real-time PCR data for the expression level of WTl which normalised from K562 set on 1000.
‡New indicates that samples were taken at first diagnosis.
§French–American–British classification.
Na, not applicable.

GGTGCGAGGGCGTGTGA were used for WT1. For PBGD, forward primer: CATGTCTGG TAACGGCAATG and reverse primer: TCTTCT CCAGGGCATGTTCAA were used.

Chromium release assay

Target cells were removed from culture, washed in RPMI1640 serum-free medium, re-suspended in a minimal volume of RPMI1640 and incubated with ^{51}Cr (100 μCi) per target for 90 min. Target cells labelled with peptide were concurrently incubated with the appropriate peptide at 50 μM/mL. The target cells were then washed and placed in 96 V-bottomed wells at 10^3 cells/100 μL/well. The effector T cells were washed, and added in triplicate at varying quantities to the target cells, to give varying effector to target ratios (E:T ratios) in a final volume of 200 μL/well. The plates were spun for 5 min (300*g*) and incubated for 4 h at 37°C before 100 μL supernatants were removed and transferred to 1450 Microbeta Plus Wallac plates (Wallac, Turku, Finland). 150 μL scintillation fluid, Optiphase HiSafe 2 (Wallac) was added to each well and the plates were heat-sealed using a Microsealer system (Wallac). Chromium release was assessed by a liquid scintillation counter (Wallac). Target cells were also incubated with 0.2% Tween 20 or medium alone to assess the maximum and minimal (spontaneous) release of the chromium respectively. Spontaneous release was never exceeding 20% of the maximum release. Percent specific lysis was calculated as:

$$\%\ \text{Specific lysis} = (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release}) \times 100.$$

In some experiments blocking antibody (10 μg/mL) anti-class I ABC (AbD Serotec, NC) was added to target cells before adding T cells.

ELISPOT assay

An ELISPOT assay for IFN-γ production by the generated T cell lines was performed using an IFN-γ kit (Mabtech, OH) according to the manufacturer's protocol. 20 000 T cells/well and 2000 cells/well of different stimulators were seeded in Multiscreen 96-well plates (Millipore, MA) pre-coated overnight (4°C) with catching-antibody (1-D1K). After 40 h incubation (37°C, 5% CO_2), cells were removed and after washing, Biotinylated-IFN-γ antibody was added for 3 h at room temperature (RT). After washing, Streptavidin ALP was added to each well and incubated at RT for 2 h. After washing, 100 μL/well of BCIP/NBT substrate (Sigma) was added to each well and incubated at RT until colour developed according to the manufacturer's instructions. Spots were counted using an automated ELISPOT reader (AID, Strasberg, Germany). Antigen-specific T cell frequencies were considered to be increased when they were at least two-fold higher than in the control wells.

Statistical analysis

Comparative analysis was performed by the Student *t* test. A *P* value less than or equal to 0.05 was deemed to be significant in all experiments.

Results

Identification of peptides predicted to highly bind to the HLA-A0201 molecule

HLA-A0201 is one of the most common allele in the whole population. Thus, we aimed to identify peptides with enhanced binding to HLA-A0201 that can be recognised by the T cell receptors. This approach may afford peptides that would induce CTL recognising modified peptides with stronger affinity compared with CTL induced with unmodified peptides. To this end, we modified the two WT1 peptides, which are known to be HLA-A0201 restricted peptides [27], by substituting the amino acid at position 1 with tyrosine (Y). The binding of the wild-type peptides (126N and 187N) was compared with their corresponding analogues (126Y and 187Y) to the HLA-A0201 molecule using a computer-based epitope prediction program (http://bimas.dcrt.nih.gov/molbio/hla_bind/). Our data (Figure 1) demonstrated that this tyrosine

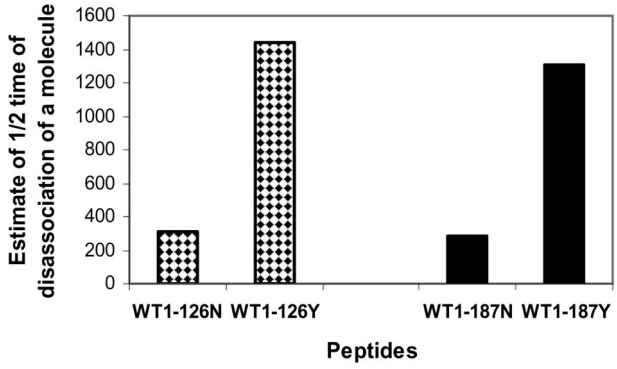

Figure 1. Prediction of the wild-type (WT1-126N, WT1-187N) and the modified (WT1-126Y, WT1-187Y) peptides and their binding affinities to the HLA-A02 01 molecule as predicted by the computer-based epitope prediction program (http://bimas.dcrt.nih.gov/molbio/hla_bind/).

modification resulted in 4.6-fold increase in the estimated half-time of disassociation of the modified peptides to HLA-A0201 compared with their native counterparts.

The computer-based epitope prediction program has only 60–80% predictive accuracy. Therefore, we directly measured the strength of the interaction between these peptides and the HLA-A0201 molecules using the T2 binding assay described above. We observed enhanced binding of the 126Y analogue peptide compared to the wild-type 126N peptide at all doses tested (Figure 2A). Although 50 μM of the 126N peptide is needed to achieve a 2.5 fluorescence index, only 15 μM of the 126Y peptide was able to reach the 2.5 fluorescence index. However, modification of the 187 peptide (187Y) did not enhance its binding ability and stabilisation of the HLA-A0201 molecules (Figure 2B). These data show a selective enhancement of peptide binding to HLA-A0201 after modification, thus give us a good model to test the effect of this process on the function of T cell generated.

Enhanced cytotoxicity of CTL generated against WT1-126Y

Because only the 126Y analogue peptide but not the 187Y peptide showed a high binding affinity to the HLA-A0201 molecule compared to their peptides counterparts, we tested the effect of tyrosine modification of the WT-126 peptide on its ability to induce specific CTL compared with its wild-type peptide. We used an optimised T-cell activation protocol, with $CD14^+$ monocytes-derived DC as antigen presenting cells, and purified $CD8^+$ T cells to investigate whether the new synthetic WT1 (126Y) analogue could stimulate peptide-specific CTLs. After three to five rounds of stimulations, the generated T cell lines were tested against the T2 cells in the presence or absence of autologous peptide using a Chromium Release Assay and an IFN-γ ELISPOT assay. Cross-reactivity between the two peptides was also tested to determine if the analogue peptide 126Y is able to induce immune response to the native peptide (126N).

The lysis activity of the T cell lines generated against 126N and 126Y are presented in Figure 3(A) and (B), respectively. Specific lysis was recorded against the T2 cells for both T cell lines raised against the 126N peptide and its 126Y analogue, because there was no T cell response against the T2 cells in the absence of the peptides. However, a significantly ($P = 0.0006$) higher lytic activity was recorded when the T cell line raised against the 126Y modified peptide was used (Figure 3A *versus* Figure 3B). Interestingly, we found cross reactivity of each T cell line with the opposite peptide. In this, the T cell line generated against the wild-type 126N peptide was able to equally lyse the T2 cells loaded with the 126N or the 126Y. Similar results were obtained for the T cell line generated against the analogue 126N peptide. To confirm that the T cells response generated against both the wild-type 126N and the 126Y peptides is restricted to the HLA-A0201 molecule, an HLA blocking assay was performed. Blocking of the HLA-ABC resulted in a strong inhibition of the cytotoxic activity of both T cell lines against peptide-loaded T2 cells [Figure 3(C)]. However, the isotype control did not inhibit the cytotoxic activity of this T cell lines. These data show that the tyrosine modification of the WT1-126 peptide, which enhanced their binding to HLA-A0201, can increase its lytic activity not only to the 126Y epitope, but also to the wild-type 126N counterpart.

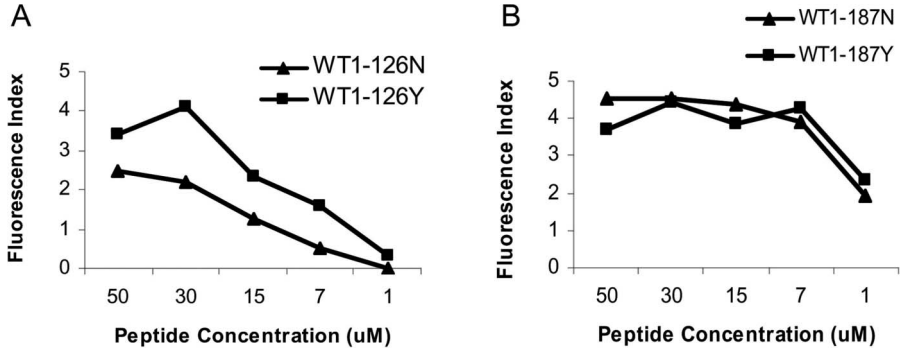

Figure 2. HLA-A0201 epitope enhancement of the WT1-126N wild-type peptide by an amino acid substitution (Y) at position 1 (126Y) analogue peptide (A) and no enhancement in the case of peptide 187Y (B) as measured by the T2 stabilisation. The assay was conducted as described in Materials and methods section. The HLA-A0201 expression by the T2 cells was quantified as fluorescence index according to the following formula: fluorescence index = (mean fluorescence intensity with peptide – mean fluorescence intensity without peptide)/mean fluorescence intensity without peptide.

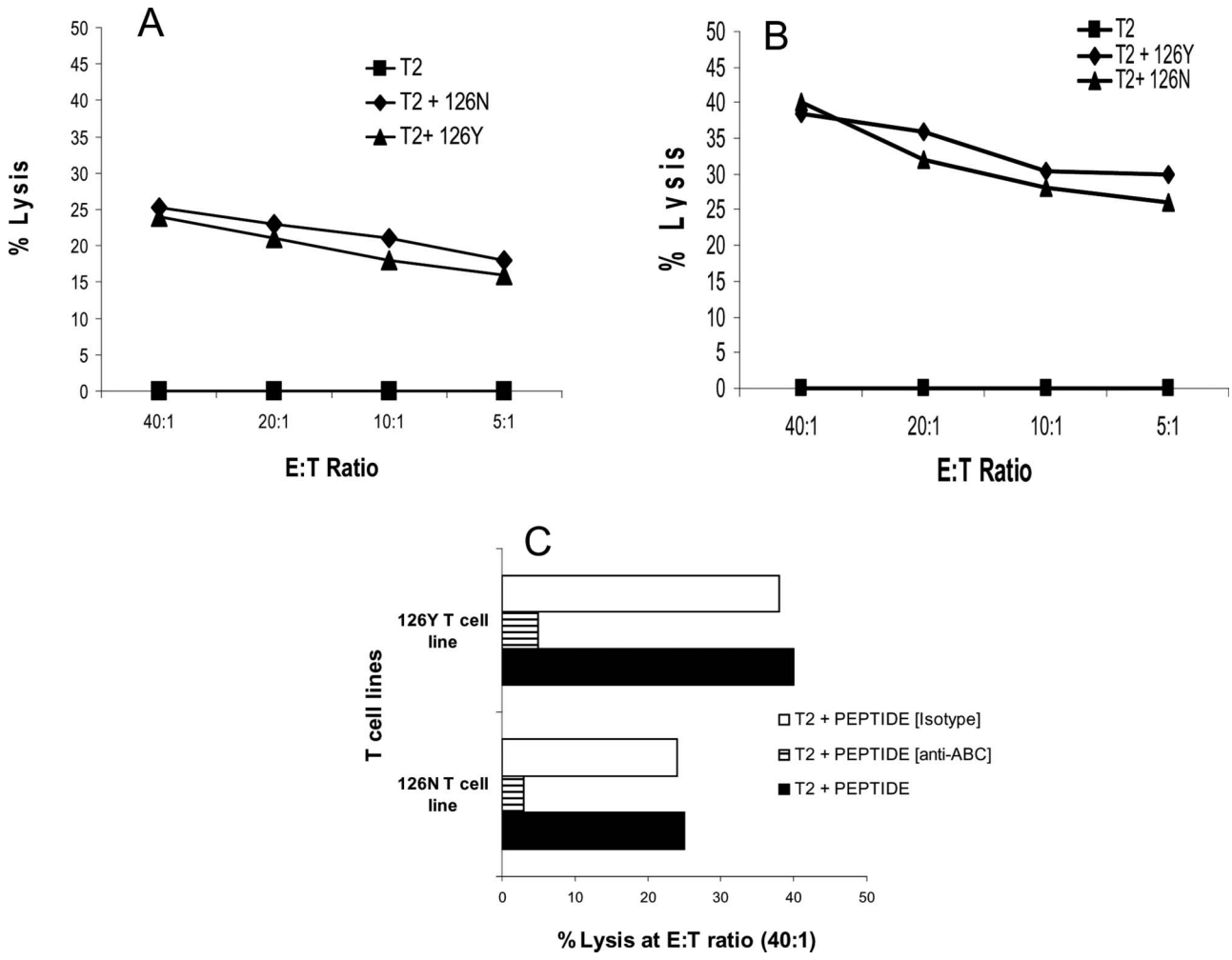

Figure 3. Cytotoxicity assay with T cells from a healthy HLA-A0201 donor after multiple stimulations *in vitro*. T cells were stimulated *in vitro* following the protocol described in Material and methods section with the peptides. (A) Specific lysis of the 126N T cell line and its cross-reactivity with the analogue 126Y peptide. (B) Specific lysis of the 126Y T cell line and its cross-reactivity with the wild-type 126N peptide. (C) Inhibition of lytic activity after blocking with anti-ABC antibody showing the restriction response to the HLA-A0201 molecule. Ab was added to the wells containing T2 cells 20 min before the addition of T cells. *P* value between A and B = 0.0006.

WT1-126Y peptide increased the frequency of IFN-γ producing T cells

The 126N and the 126Y T cell lines were tested for the frequency of IFN-γ producing T cells after three rounds of *in vitro* stimulation using an ELISPOT assay. Both T cell lines produced specific IFN-γ in response to stimulation with autologous LCL loaded with their corresponding peptide (Figure 4). However, the T cell line generated with the analogue 126Y peptide exerted a significantly higher number ($P = 0.0003$) of IFN-γ producing T cells compared with that generated against the 126N wild-type peptide (Figure 4). These data demonstrate that modification of the WT1-126 peptide can increase the frequency of IFN-γ producing T cells and thus making them better effector cells.

Evaluation of WT1 expression and lytic activity of anti-WT1 T cell lines against HLA-A0201-matched leukemic cells

Given the above, we tested the feasibility of using this modification approach of the WT1-126 peptide to generate CTLs that effectively kill HLA-A0201-matched leukemic cells. We first examined the expression of the WT1 mRNA in fresh AML cells (AML-27 and AML-28) using Q-RT-PCR. K562 cells, known to express high levels of WT1 [31] were used as a positive-control. PBMC-32 from a normal donor served as a negative control. Higher levels of WT1 expression were observed in AML samples (Table I). The lytic activity of the two T cell lines generated against the 126N and 126Y peptides were then measured against PBMCs from HLA-A0201-matched (AML-28) and

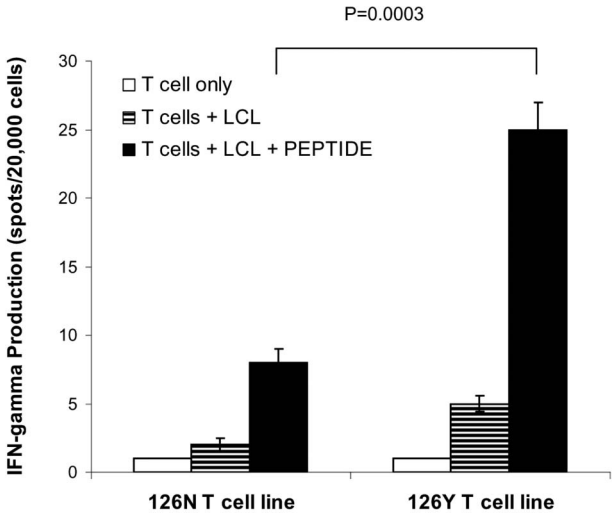

Figure 4. Specific production of IFN-γ by the two T cell lines generated against the wild type 126N and its analogue 126Y peptides as measured by an ELISPOT assay. T cells (2×10^4 cell/well) were stimulated with 2×10^3 cell/well autologous LCL $\pm$ peptide for 40 h in anti-IFN-γ pre-coated wells before development of the spots. T cells incubated without stimulators (T cell only) served as a baseline.

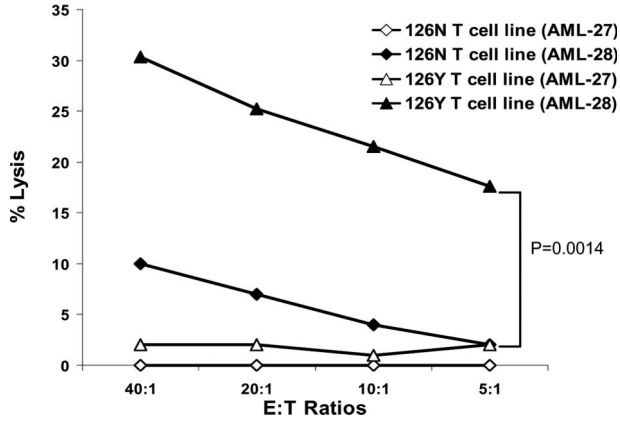

Figure 5. Cytotoxicity assay showing the lytic activity of the 126N and 126Y T cell lines against HLA-A0201-matched (AML-28) and non-matched AML-27 cells. T cells were used after three rounds of stimulations.

non-HLA-A0201 matched (AML-27) AML patients. Specific lytic activity was recorded for both T cell lines against the AML-28 cells expressing the HLA-A0201 molecule (Figure 5). However, both T cell lines failed to recognise non-HLA-A0201 AML-27 although they express high levels of WT1 (Figure 5). Interestingly, the T cell line generated with the analogue 126Y peptide exerted a significantly higher lytic activity ($P = 0.0014$) compared with that generated against the 126N wild-type peptide (Figure 5). Altogether, the modified WT1-126Y enhanced its binding to HLA-A0201, increased the frequency of IFN-γ producing T cells, and boosted the lytic activity against HLA-matched leukemia cells. Thus, modification of the WT1-126 peptide provides a potential approach for the development of leukemia vaccine.

Discussion

Most tumor associated antigens which have been identified so far are derived from self-antigens. Immunological tolerance can prevent the development of immune responses against such self-antigens. Therefore, most self/tumor antigens used in clinical trials so far showed poor immunogenicity. To circumvent the problem of tolerance, Stauss *et al.* [32,33] developed an allo-restricted CTL against leukemia. In this approach, T cells from HLA-A0201+ donors were stimulated with peptide-loaded HLA-A0201-matched APCs. This allows the generation of high-avidity allo-restricted CTLs against an HLAA0201 WT1-derived epitope. Interestingly, the generated CTLs specifically lysed $WT1^+$, HLA-A0201-matched leukemia cells. More importantly, only the high-avidity CTLs were able to eliminate the progenitors of CFU-GM and BFU-GM from CD34+ CML samples, whereas normal CD34+ progenitors/stem cells were spared [32,33]. However, as allogeneic APCs usually provoke dominant CTL responses against allogeneic epitopes, the isolation of HLA-A0201-WT1 peptide-specific allogeneic CTLs was unsuccessful in many cases. Another drawback of this strategy is the difficultly in applying such an approach in the clinical settings. A more promising approach has been recently developed by the same group, which rely on introducing the WT1-specific TCR into autologous T cells for possible future clinical applications [34].

Because the WT1 is a self-protein expressed at low levels in some normal cells [35,36], many of the potential CTL epitopes from this antigen may be absent or 'sub-optimal'. This is due to clonal deletion of high-avidity CTLs during maturation of the immune system. Therefore, the induction of anti-tumor immune responses frequently needs to be addressed against those 'sub-optimal' CTL epitopes to enhance their immunogenicity, frequency and hence their therapeutic potential. In addition, naturally arising CD25+CD4+ regulatory T cells (T_{regs}), play an important role in the maintenance of immunologic self-tolerance [37]. Although this T cell population plays a key role in regulating the immune response to self antigen, it has been shown recently that T_{regs} can directly suppress the anti-tumor immune responses in cancer patients [38–41]. Indeed, another group demonstrated that the depletion of this T cell population resulted in an enhancement of vaccine-mediated anti-tumor

immunity in cancer patients [42]. Furthermore, a recent study showed that such response can be largely affected by the presence of CD4+CD25+ T_{regs} and depletion of this T cell population was necessary for the generation of an effective WT1-specific cytotoxic response [43].

It is known that the immunogenicity of antigenic peptides is dependent on their binding affinity to MHC class I molecules; stability of the peptide-MHC complexes; and the avidity of the TCR binding to the peptide complex [44]. Indeed, it has been found that the poor immunogenicity of some self/tumor antigens is due to the instability of the peptide-MHC complex rather than low affinity of T cell receptors to peptide-MHC complexes [44]. Therefore, many studies have put emphasis on introduced a peptide modification to improve the binding of self antigenic peptides to their corresponding MHC class I molecules and consequently enhance the immunogenicity of these peptides [45–51]. In the present study, we have applied a strategy used by others and shown to enhance the immunogenicity of low-avidity HLA-A0201-restricted peptides without altering their antigenic specificity [26]. This approach consists of substituting the first amino acid of the epitope with a tyrosine (Y). The importance of this approach is that the resulting peptide analogues are able to efficiently trigger *in vivo* wild-type peptide-specific CTLs which also recognised the naturally occurring epitope [26]. We have carried out the Y amino acid substitution in the sequence of the two known HLA-A0201-restricted Db126 and WH187 WT1-derived peptides [27]. Using a computer-based epitope prediction program, the 126Y and 187Y analogue peptides were predicted to bind 4.6-fold more to the HLA-A0201 molecule compared to their native 126N and 187N counterparts. However, a significant enhancement of the actual binding ability was only recorded for the 126Y peptide analogue when we used the T2 binding assay. Therefore, we generated CTLs lines against the wild-type 126N and its 126Y peptide analogue. Although both peptides were able to induce specific cytotoxic immune response against autologous peptide-loaded T2 cells, the CTL line generated with the 126Y peptide showed a higher and significant lytic activity compared with the CTL line generated with the 126N wild-type peptide. Interestingly, CTL generated against the 126Y analogue peptide was able to lyse the T2 cells target when loaded with the wild-type peptide. Therefore, modification of the 126 peptide resulted in enhancement of its immunogenicity without altering its antigenic specificity. Another important finding is the significant high lytic activity observed for the 126Y CTL line against HLA-A0201-matched leukemic cells expressing the WT1 antigen. These data confirm that T cells generated against the 126Y analogue peptide cross-react also with the naturally processed 126N native peptide. Our results suggest that stimulation with the 126Y peptide analogue induced CTLs with a high TCR avidity. Finally, the high lytic activity provoked by the 126Y CTL may be also attributed to the significant high number of anti-126 T cells in this T cell line as demonstrated by γ-IFN production.

During the course of our study, Pinilla-Ibarz *et al.* [52] reported on a similar study that uses the same strategy to modify several HLA-A0201 WT1-derived peptides including the 126N, 187N and their analogues described in the present work. Although, this group did not find any difference in the binding affinity between the 2 126N and 126Y peptides, they reported that stimulation with the 126Y peptide analogue was able to generate an earlier and stronger specific CTL than that generated with the 126N wild-type peptide. Consistent with our findings the 126Y CTL line was able to recognise the 126N wild-type peptide on the target cells and specifically lyse HLA-A0201-matched leukemia cells expressing the WT1 antigen. Another group has introduced a tyrosine modification in position two of a 9-mer WT1-derived peptide which is known to bind to the HLA-A2402 molecule and showed a better binding affinity and elicitation of higher lytic activity [53]. Moreover, the modified peptide has been used to vaccinate cancer patients where a clear correlation was observed between an increase in the frequencies of WT1-specific CTL after vaccination and clinical responses [23].

In conclusion, our study and others' [23,52,53] shed light on the importance of peptide modification as a new approach in elucidating a better immune response, needed to combat cancer. On the basis of these findings, leukemia patients could be clinically tried for vaccination with the current WT1-126Y peptide analogue or adoptively treated with *ex vivo* anti-WT1-126Y T cells to specifically enhance the graft-*versus*-leukemia effect and anti-WT1 CTL frequency, which are known to be very low in these patients.

Acknowledgements

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The Wilms' Tumor Antigen Is a Novel Target for Human CD4+ Regulatory T Cells: Implications for Immunotherapy

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Abstract

Compelling evidences indicate a key role for regulatory T cells (T_{reg}) on the host response to cancer. The Wilms' tumor antigen (WT1) is overexpressed in several human leukemias and thus considered as promising target for development of leukemia vaccine. However, recent studies indicated that the generation of effective WT1-specific cytotoxic T cells can be largely affected by the presence of T_{regs}. We have generated T-cell lines and clones that specifically recognized a WT1-84 (RYFKLSHLQMHSRKH) peptide in an HLA-DRB1*0402–restricted manner. Importantly, they recognized HLA-DRB1*04–matched fresh leukemic cells expressing the WT1 antigen. These clones exerted a T helper 2 cytokine profile, had a $CD4^+CD25^+Foxp3^+GITR^+CD127^-$ T_{reg} phenotype, and significantly inhibited the proliferative activity of allogeneic T cells independently of cell contact. Priming of alloreactive T cells in the presence of T_{regs} strongly inhibited the expansion of natural killer (NK), NK T, and $CD8^+$ T cells and had an inhibitory effect on NK/NK T cytotoxic activity but not on $CD8^+$ T cells. Furthermore, priming of T cells with the WT1-126 HLA-A0201–restricted peptide in the presence of T_{regs} strongly inhibited the induction of anti–WT1-126 $CD8^+$ CTL responses as evidenced by both very low cytotoxic activity and IFN-γ production. Moreover, these T_{reg} clones specifically produced granzyme B and selectively induced apoptosis in WT1-84–pulsed autologous antigen-presenting cells but not in apoptotic-resistant DR4-matched leukemic cells. Importantly, we have also detected anti–WT1-84 interleukin-5^+/granzyme B^+/$Foxp3^+$ $CD4^+$ T_{regs} in five of eight HLA-$DR4^+$ acute myeloid leukemia patients. Collectively, our *in vitro* and *in vivo* findings strongly suggest important implications for the clinical manipulation of T_{regs} in cancer patients. [Cancer Res 2008;68(15):6350–9]

Introduction

The Wilms' tumor (*WT1*) gene exerts an oncogenic function in various types of leukemias (1). It is also overexpressed in several solid tumors (2), and therefore, it has been considered as an attractive target for cancer immunotherapy. Specific anti-WT1 immune responses have been described in which $CD8^+$ cytotoxic T cells (3, 4) have been generated *in vitro*. However, a recent study showed that such response can be largely affected by the presence of $CD4^+CD25^+$ regulatory T cells (T_{reg}) in which depletion of this T-cell population was necessary for the generation of an effective WT1-specific cytotoxic response (3).

It has been shown recently that T_{regs} directly suppress the antitumor immune responses in cancer patients (5, 6), and depletion of this T-cell population resulted in an enhancement of vaccine-mediated antitumor immunity in cancer patients (7). This highlights the role of T_{regs} in modulating both natural and adoptive immune responses in cancer patients. T_{regs} may directly modulate the $CD8^+$ T-cell response (8) or alternatively promote tolerization of $CD8^+$ T cells by preventing the licensing of antigen-presenting cells (APC) by $CD4^+$ T helper cells (9). It has became clear that $CD8^+$ T cells generated in the absence of $CD4^+$ T cells help may have a normal primary response; however, their cytotoxic memory response is severely weakened (10). A recent study by Greiner and colleagues (11) investigated the influence of the expression levels of several leukemia-associated antigens (LAA) on the clinical outcome of patients with acute myeloid leukemia (AML). High expression of three LAAs, which was found to be associated with favorable clinical outcome, induced strong $CD8^+$ T-cell responses. However, there was no correlation with the clinical outcome nor induction of natural detectable anti-WT1 $CD8^+$ T-cell response in these patients (11). In line with this, a recent study has shown the existence of tumor-specific T_{regs}, which actively suppress antigen-specific antitumor immunity in cancer patients (12). Furthermore, fully functional T_{regs} specific for LAGE1 (13) and ARTC1 (14) were shown in melanoma patients. Another study by Nadal and colleagues (15) suggested that T_{regs} exert an inhibitory effect on graft versus leukemia and this was associated with relapse after allogeneic stem cell transplantation.

To this end, we asked whether an anti-WT1 T_{reg} population exist in leukemia patients, which may contribute to the impairment of anti-WT1 responses. We have identified a human HLA-DRB1*0402–restricted $CD4^+$ T_{reg} population and showed that the WT1 is a novel target for leukemia-specific $CD4^+$ T_{regs}.

Materials and Methods

Patients and donors. Peripheral blood mononuclear cells (PBMC) from leukemia patients and healthy donors were isolated by density gradient centrifugation. This study was conducted in accordance with Helsinki Declaration and all patients and donors signed a consent form approved by the Research Ethics Committee of King Faisal Specialist Hospital and Research Center (KFSH&RC). The study was approved by the KFSH&RC Research Advisory Council (RAC#2030006). Patient and donor information is listed in Supplementary Data 1.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Peptides. A pool of 110 peptides derived from the WT1 protein designated as WT1-Pepmix and a microscale WT1 peptide set containing each peptide in a single well were obtained from JPT Peptide Technologies (Jerini AG, GmbH; Supplementary Data 2). The $WT1_{333-347}$ (RYFKLSHLQMHSRKH), WT1-84, $HPV33_{73-87}$ (ASDLRTIQQLLMGTV) HLA-DR*0402–restricted peptide (16), and the WT1-db126 (RMFPNAPYL) HLA-A0201–restricted peptide (4) were synthesized and high-performance liquid chromatography purified to ≥90% purity by Alta Bioscience.

Cell lines. B-lymphoblastoid cell lines (LCL) were established by transformation of B cells using EBV using standard techniques (17). K562 and T2 cell lines were purchased from the American Type Culture Collection. All cell lines were maintained in RPMI 1640 (Sigma) supplemented with 10% FCS (Cambrex Bio Science), 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin (Sigma).

Generation of autologous monocyte-derived dendritic cells. $CD14^+$ cells were isolated from PBMCs of three healthy donors, designated as BC-29, BC-52, and BC-62, using MACS Monocytes Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. $CD14^+$ cells were adhered and cultured in 24-well plates in X-VIVO 15 medium supplemented with 2 mmol/L L-glutamine [dendritic cell (DC) medium] in the presence of 50 ng/mL recombinant human interleukin (rhIL)-4 and 100 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems). Recombinant human tumor necrosis factor-α (20 ng/mL; R&D Systems), 10 ng/mL rhIL-1β (eBioscience), and 25 μg/mL polyinosinic acid:poly-CMP (Sigma) were added on day 6 and mature DCs were harvested on day 8.

Generation of anti–WT1-specific T_{regs} and clones. Irradiated DCs (3,000 rads) were pulsed with 10 μg/mL WT1-Pepmix in DC medium and incubated for 4 h at 37°C, 5% CO_2. DCs were cocultured with autologous PBMCs at a 10:1 T-cell to DC ratio. DC medium containing 10 ng/mL rhIL-7, 20 pg/mL rhIL-12 (R&D Systems), and 10% heat-inactivated human serum (Sigma) was used. T cells were restimulated at weekly intervals and 260 IU/mL rhIL-2 was added 2 d after each restimulation. Three of three anti–WT1-Pepmix-specific T-cell lines were obtained after the fourth restimulation. Anti–WT1-Pepmix T-cell clones were generated as described before (18). T cells were screened for their peptide specificity against the microscale WT1 peptide set.

Proliferation and ^{51}Cr cytotoxicity assay. T-cell proliferation was assessed by $[^3H]$thymidine incorporation as described before (18). Briefly, T cells were cocultured with X-irradiated PBMCs, LCLs (±antigens), or leukemic cells at different ratios in DC medium containing 10% human serum for 3 d. $[^3H]$thymidine (1 μCi/well; Amersham) was added for the last 18 h. $[^3H]$thymidine uptake was measured using a 1450 Micro Beta PLUS liquid scintillation counter (Wallac). In some experiments, the following blocking antibodies (50–100 μg/mL) were used: anti-ABC (AbD Serotec), anti–HLA-DR (BD Biosciences), anti–HLA-DP, and anti–HLA-DQ (Leinco Technologies). Mouse isotypes were used as controls. Cytotoxic activity was measured by a standard ^{51}Cr release assay as described before (18).

ELISA. Supernatants from 48-h T-cell cultures were harvested, and ELISA for the human IFN-γ, IL-4, IL-5, IL-10, and GM-CSF ELISA kits (Mabtech) and transforming growth factor (TGF)-β1 and TGF-β2 matched antibody pairs (R&D Systems) was performed according to the manufacturer's instructions.

Flow cytometry. T cells were phenotypically analyzed using the human T_{reg} staining kit (eBioscience) according to the manufacturer's instructions and also stained with CTLA-4-PE (Dako Corp.), CD127-RPE, and GITR-APC (eBioscience). The T-cell receptor (TCR) Vβ profile was determined using the IOTest Beta Mark, TCR Vβ Repertoire kit (Beckman Coulter). Cells were analyzed using FACScan (Becton Dickinson).

Immunostaining. Immunostaining on cytospins was carried out as described previously (19). Anti-Foxp3 antibody (eBioscience), anti-CD3 antibody (Dako), and anti-WT1 antibody (Dako) were used. Isotype-matched controls for all antibodies were used. The staining was evaluated by two independent scientists.

Reverse transcription-PCR for Foxp3 expression. Total RNA was isolated using RNeasy Micro kit (Qiagen). The forward primer CAGCTGCCCACACTGCCCCTAG and the reverse primer CAGTGCCATTTTCCCAGCCAG were used for Foxp3. The PCR conditions were 95°C/3 min, 35 cycles of 95°C/1 min, 67°C/40 s, and 72°C/1 min. The β-actin forward primer ATCTGGCACCACACCTTCTACAATGAGCTGCG and the reverse primer CGTCATACTCCTGCTTGCTGATCCACATCTGC were used as a control. PCR products were separated by electrophoresis on a 1% agarose gel (Sigma).

Quantitative real-time reverse transcription-PCR for WT1 expression. Total RNA was isolated as above and treated with DNase1 (Invitrogen). For cDNA synthesis, 1 μg RNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen) was used in a total volume of 25 μL. The WT1 mRNA expression was quantified using LightCycler FastStart DNA Master SYBR Green 1 kit (Roche) in a LightCycler (Roche). *Porphobilinogen deaminase* (*PBGD*) was used as housekeeping gene. cDNA (2 μL) from K562 was used to generate standard curves (20). Amplification was conducted in a total volume of 20 μL for 40 cycles/10 s at 95°C, 4 s/64°C, and 35 s/72°C. The forward primer TTCATCAAACAGGAGCCGAGC and the reverse primer GTGCGAGGGCGTGTGA were used for WT1. For PBGD, the forward primer CATGTCTGGTAACGGCAATG and the reverse primer TCTTCTCCAGGGCATGTTCAA were used.

T_{reg} suppression assays and Transwell experiments. To examine the suppressive effect of the T_{reg} clones on the induction of T helper alloresponses, a standard mixed lymphocyte reaction (MLR) was carried out as described before (21). Briefly, fresh BC-29 PBMCs (10^5 per well, responders) were cultured in DC medium containing 10% human serum for 5 d with XR, 3,000 rads allogeneic PBMCs (10^5 per well, stimulators) with different concentrations of T_{regs} + autologous LCL ± WT1-84 peptide. The proliferative activity was determined as before. For some experiments, neutralizing antibodies against TGF-β1/β2, IL-4, IL-5 (R&D Systems), IL-10 (Biosource), and GM-CSF (Biolegend) were added in the assay at an optimum final concentration of 5 μg/mL. The effect of T_{reg} inhibition on nonirradiated autologous LCL was also examined. Transwell experiments were performed in 24-well plates with pore size of 0.4 μm (Corning Costar). BC-29 PBMCs (0.75×10^6 per well, responders) were cultured in the outer wells of 24-well plates in DC medium containing 10% human serum and XR, 3,000 rads allogeneic (BC-8) PBMCs (1.5×10^6 per well, stimulators). T_{reg} clones (0.15×10^6 cells per well) were added into the inner wells of autologous LCL ± WT1-84 peptide. After 4 d in culture, the cells in the outer wells were harvested and transferred to 96-well plates and the proliferative activity was determined as before.

The suppressive effect of T_{regs} on the induction of alloreactive and anti–WT1-specific cytotoxic responses was examined. For alloreactive CTLs, BC-29 PBMCs (2×10^6 per well, responders) were cultured in DC medium containing 10% human serum for 7 d with XR, 7,500 rads allogeneic LCL (0.5×10^6 cells per well, stimulators) in 24-well plates ± 0.5×10^6 cells per well TCC 29.B.42 T_{reg} clone + WT1-84 peptide. This procedure was used for the generation of anti-WT1 CTLs, except that autologous DC pulsed with the WT1-126 (RMFPNAPYL) HLA-A0201–restricted peptide (4) was used. IL-2 (25 units/mL) was added on day 4 and a similar stimulation was repeated after 7 d. Several stimulations were carried out without addition of the T_{regs} every 7 d. Flow cytometry analysis was used to determine the CD8, CD56, and CD8/CD56 cell population in the culture. CD8 T cells were purified using MACS human CD8-negative selection kit (Miltenyi Biotec). Cytotoxic and IFN-γ production activity were determined by ^{51}Cr release and enzyme-linked immunospot (ELISPOT) assays, respectively.

ELISPOT assay. ELISPOT assay was performed using IFN-γ, granzyme B, and perforin kits (Mabtech). Autologous LCLs or AML cells were used as stimulators. T_{regs} (10^3 cells per well) and stimulators (2×10^4 cells per well) were seeded in Multiscreen 96-well plates (Millipore) precoated with catching antibody. After 40 h of incubation, cells were removed and plates were processed according to the manufacturer's instructions. Spots were counted using an automated ELISPOT reader (AID). Antigen-specific T-cell frequencies were considered to be increased when they were at least 2-fold higher than in the control wells.

Apoptosis assay. An apoptosis assay was carried out using Vybrant Apoptosis Assay Kit #2 (Invitrogen). T_{regs} (2×10^4) and target cells (2×10^4) were incubated in DC medium + 10% human serum for 24 h. Targets were

gated after staining with monoclonal antibodies (mAb) to CD19-PE, CD40-PE, and CD33-PE (BD Biosciences) for LCL, DCs, and AML, respectively. Apoptosis was measured by flow cytometry assessment of phosphatidylserine externalization cells stained with Alexa Fluor 488 Annexin V in combination with propidium iodide.

Intracellular cytokine staining and determination of anti–WT1-84/Foxp3$^+$ T_{reg} frequencies in AML patients. PBMCs (2×10^6/mL) were cultured in DC medium + 10% human serum + 125 units IL-2 for 48 h ± WT1-84 peptide. Brefeldin A (eBioscience) was added at 5 μg/mL in the last 12 h of culture. Cells were surface stained with anti-CD4 antibody (BD Biosciences) followed by fixation/permeabilization using eBioscience buffer. Cells were intracellularly stained with anti–granzyme B, anti–IL-5 antibodies (BD Biosciences), and anti-Foxp3 antibody (eBioscience) and analyzed using FACScan as above.

Results

Generation of T-cell lines and clones against the WT1-Pepmix and analysis of their HLA restriction. PBMCs from three healthy individuals, BC-29, BC-52, and BC-62 (Supplementary Data 1), sharing the HLA-DRB1*0402 molecule, were stimulated repeatedly with autologous DCs pulsed with WT1-Pepmix. Corresponding T-cell lines designated TCL 29, TCL 52, and TCL 62 with specific proliferative activity against autologous WT1-Pepmix–pulsed LCLs were generated (Fig. 1*A*). The TCL 29 line, which gave high proliferative activity, was cloned by limiting dilution, and 48 clones were obtained and screened by proliferation assay against autologous LCL ± WT1-Pepmix. Eight clones showed specific proliferative activity against the WT1-pepmix. Four clones (TCC 29.B.9, TCC 29.B.16, TCC 29.B.19, and TCC 29.B.42), which showed the highest and most stable proliferative activity (Fig. 1*A*), were selected and expanded for further analysis. TCL 29 was then screened by proliferation assay against the individual 110 WT1-Pepmix peptides. A peptide designated WT1-84 with the $WT1_{333-347}$ RYFKLSHLQMHSRKH sequence induced the strongest specific proliferative activity of TCL 29 (Fig. 1*B*). Interestingly, both TCL 52 and TCL 62 lines also showed specific proliferative activity against the WT1-84 peptide (data not shown). The rest of the peptides did not induce significant proliferative activity, indicating the

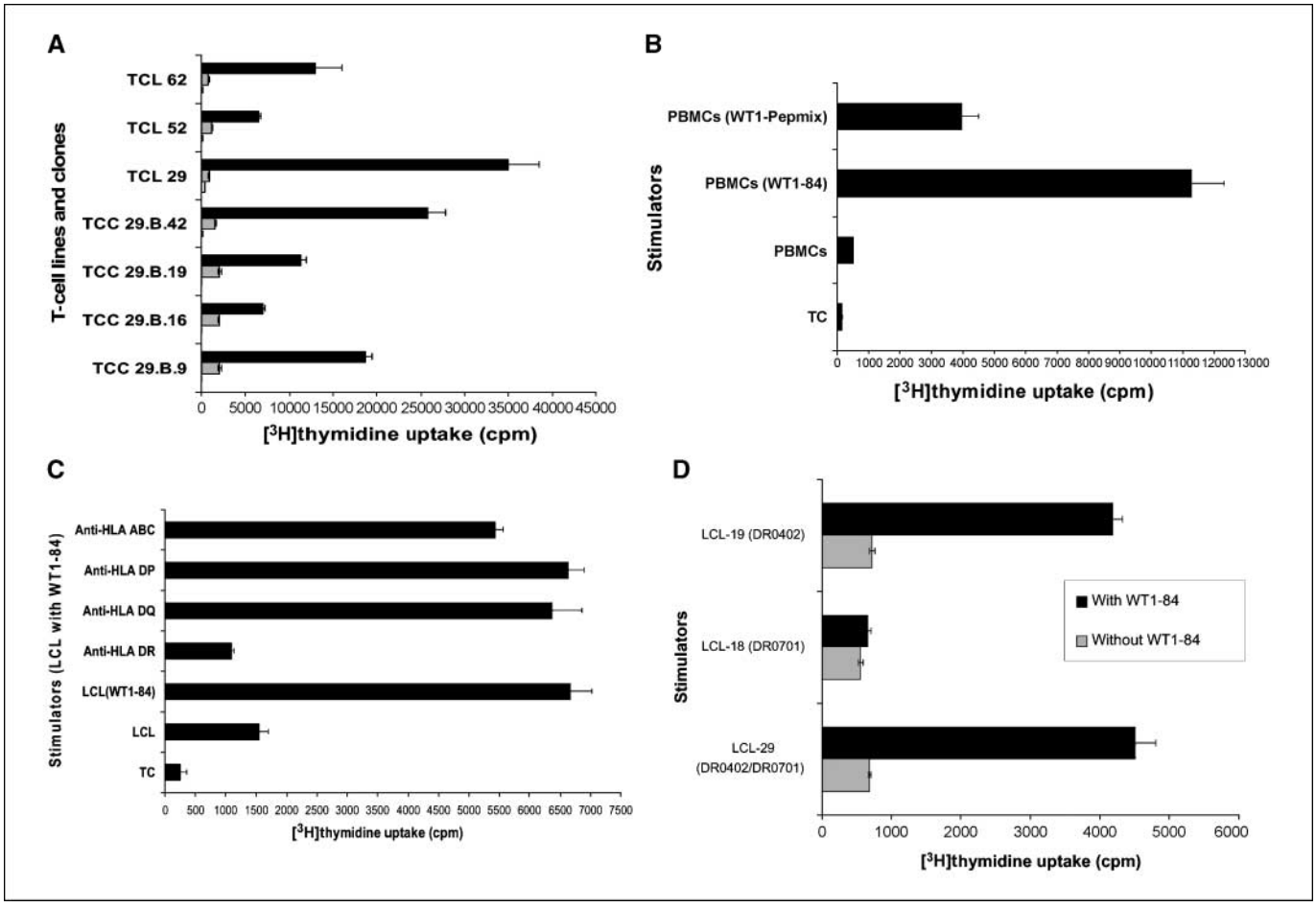

Figure 1. Proliferative activity of T-cell lines and clones generated against the WT1-Pepmix. *A,* T cells (5×10^4 per well) from three T-cell lines (TCL 29, TCL 52, TCL 62) and four expanded T-cell clones (TCC 29.B.9, TCC 29.B.16, TCC 29.B.19, and TCC 29.B.42) were challenged for 72 h with irradiated (8,000 rads, XR) autologous LCL (10^5 per well) in the absence (*gray columns*) or presence (*black columns*) of WT1-Pepmix. Cells were pulsed with [^{3}H]thymidine for additional 18 h and then harvested. T cells only (*TC*; *white columns*) were used as negative control. *B,* the TCL 29 cells (2.5×10^4 per well) were mixed with irradiated autologous PBMCs (5×10^4 per well) pulsed with the different 110 single WT1 peptides. The culture conditions were the same as described in *A. C,* representative T-cell clone (TCC 29.B.42) showing the restriction response to the HLA-DR molecule. Experimental conditions were the same as described in *A*. mAbs were added to the wells containing LCLs 20 min before the addition of T cells. *D,* proliferative activity of a representative T-cell clone (TCC 29.B.42) against the HLA-DR0402–matched LCL-19 and the HLA-DR0701–matched LCL-18 in the absence (*gray columns*) or presence (*black columns*) of the WT1-84 peptide. Experimental conditions for *D* were the same as described in *A* with the exception that 10^4 T cells per well were challenged for 72 h with 2×10^4 per well irradiated LCLs.

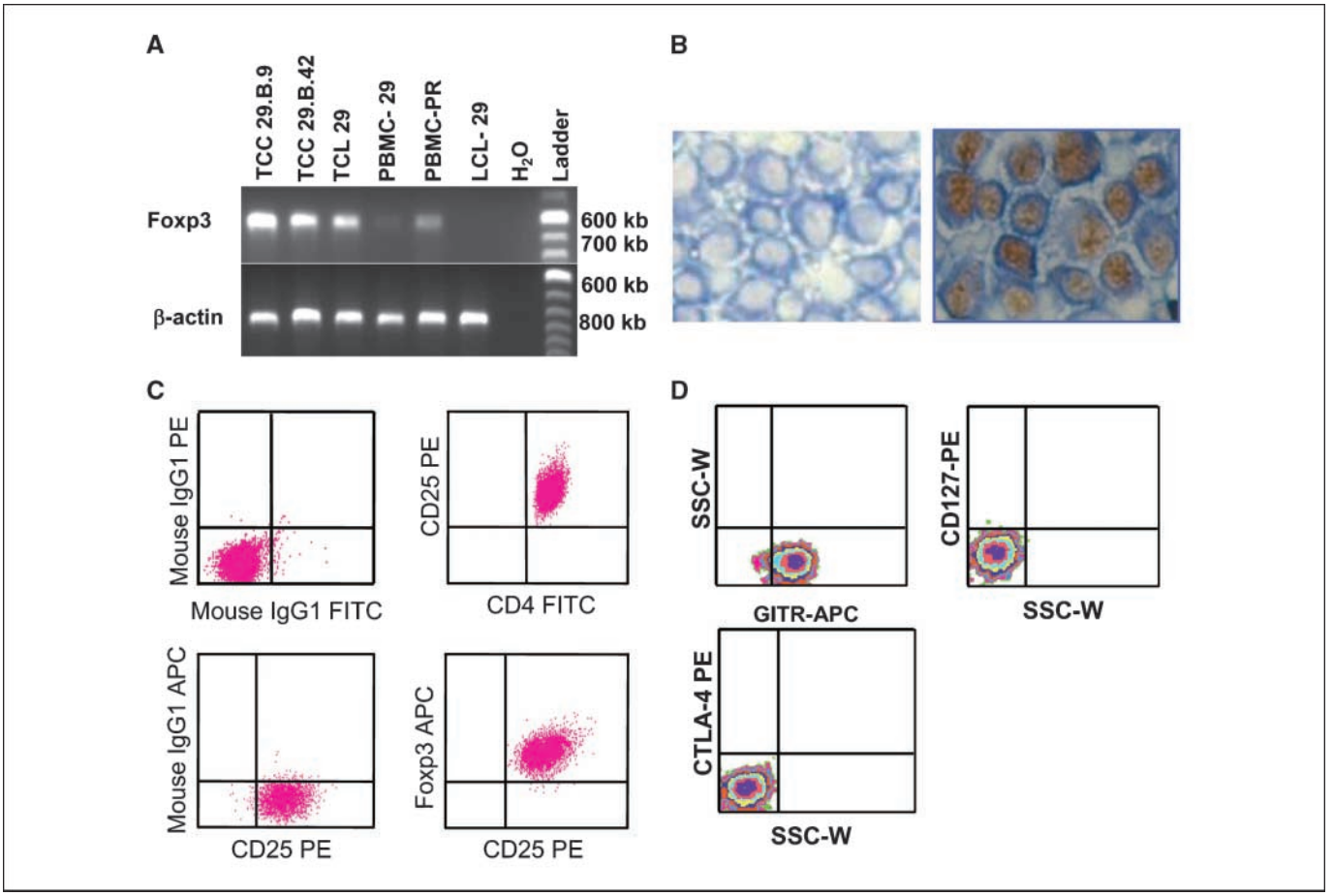

Figure 2. Phenotypic analysis of the TCL 29 T-cell line and the expanded T-cell clones generated against the WT1-Pepmix. *A,* RNA expression of Foxp3 in the T-cell line (TCL 29) and clones (TCC 29.B.9 and TCC 29.B.42). PBMCs from a 7-mo pregnant women (PBMC-PR) and LCL-29 were used as a positive and a negative control. PBMC-29 from which the TCL 29 was generated was used as a baseline. *β-Actin* acts as a housekeeping gene. Total RNA was isolated from different individual cell types and analyzed by RT-PCR. *B,* cytospin immunocytochemical double staining of a representative T-cell clone (TCC 29.B.9) after more than 1 mo in culture showing (*right*) the nuclear expression of the Foxp3 protein (*brown*) and the membranous expression of the CD3 T-cell marker (*blue*). *Left,* anti–CD3-stained cells with an isotype Foxp3-matched negative control. Magnification, ×520. *C* and *D,* FACS analysis of a representative T-cell clone (TCC 29.B.9) showing a T_{reg} phenotype after more than 1 mo in culture.

immunodominance of this epitope. All clones had specific proliferative activity to the WT1-84 peptide and a dose-dependent response was shown in all clones with the optimal concentration at 40 μmol/L (data not shown). The HLA restriction of the clones was determined in which anti–HLA-DR mAb was found to significantly inhibit their proliferative activity, whereas their corresponding isotypes had no inhibition effect (Fig. 1*C*). To define more precisely the HLA-DR restriction, two HLA-DR–matched allogeneic LCLs sharing the DRB1*0402 (LCL-19) or DRB1*0701 (LCL-18) with the TCC 29.B.42 clone were used. LCL-18 failed to present the WT1-84 peptide, ruling out the involvement of the DRB1*0701 in this presentation. However, LCL-19 sharing the DRB1*0402 with the clone induced a specific proliferative activity against the WT1-84 peptide, showing the requirement of DRB1*0402 restriction in this process (Fig. 1*D*). No proliferative response was recorded when LCL pulsed with a negative control $HPV33_{73-87}$ HLA-DR*0402–restricted peptide was used (data not shown). All clones were $CD4^+$ (data not shown). Because $CD4^+$ T cells can exert a cytotoxic effect (22), we used ^{51}Cr release assay to examine cytotoxic effect against autologous LCL ± WT1-84. No lytic activity was detected (data not shown), ruling out this possibility.

Evaluation of the cytokine profile of the T-cell clones. Cytokines generated during an immune response dictate the outcome of this response. Hence, we evaluated the cytokine profile generated by the anti–WT1-84 clones. Various cytokines released were determined from cell culture supernatants of T cells cultured for 48 h with irradiated autologous PBMCs or LCL ± WT1-84 or ±WT1-Pepmix. All clones secreted very high amounts of IL-5 and GM-CSF, and high IL-4, specifically to WT1-84 and WT1-Pepmix, but little or no IL-10, TGF-β1/β2, or IFN-γ (Supplementary Data 3). This clearly shows a T helper 2 (Th2)-polarized immune response.

Characterization of the phenotypic profile and TCR usage of the T-cell clones. The cytokine profile of the clones suggested that they represent a Th2 phenotype. It has been recently shown (23) that human Th2 cells exert a T_{reg} phenotype in which a generated Th2 clone also expressed Foxp3, a specific marker for T_{reg} lineages (24, 25). Foxp3 is also expressed transiently in activated non-T_{regs} (26). Therefore, we tested whether TCL 29 and clones express Foxp3 after long-term culture. Figure 2*A* shows the presence of Foxp3 mRNA in TCL 29 and two other clones. Because T_{regs} are elevated during pregnancy (27), we used PBMCs from a pregnant

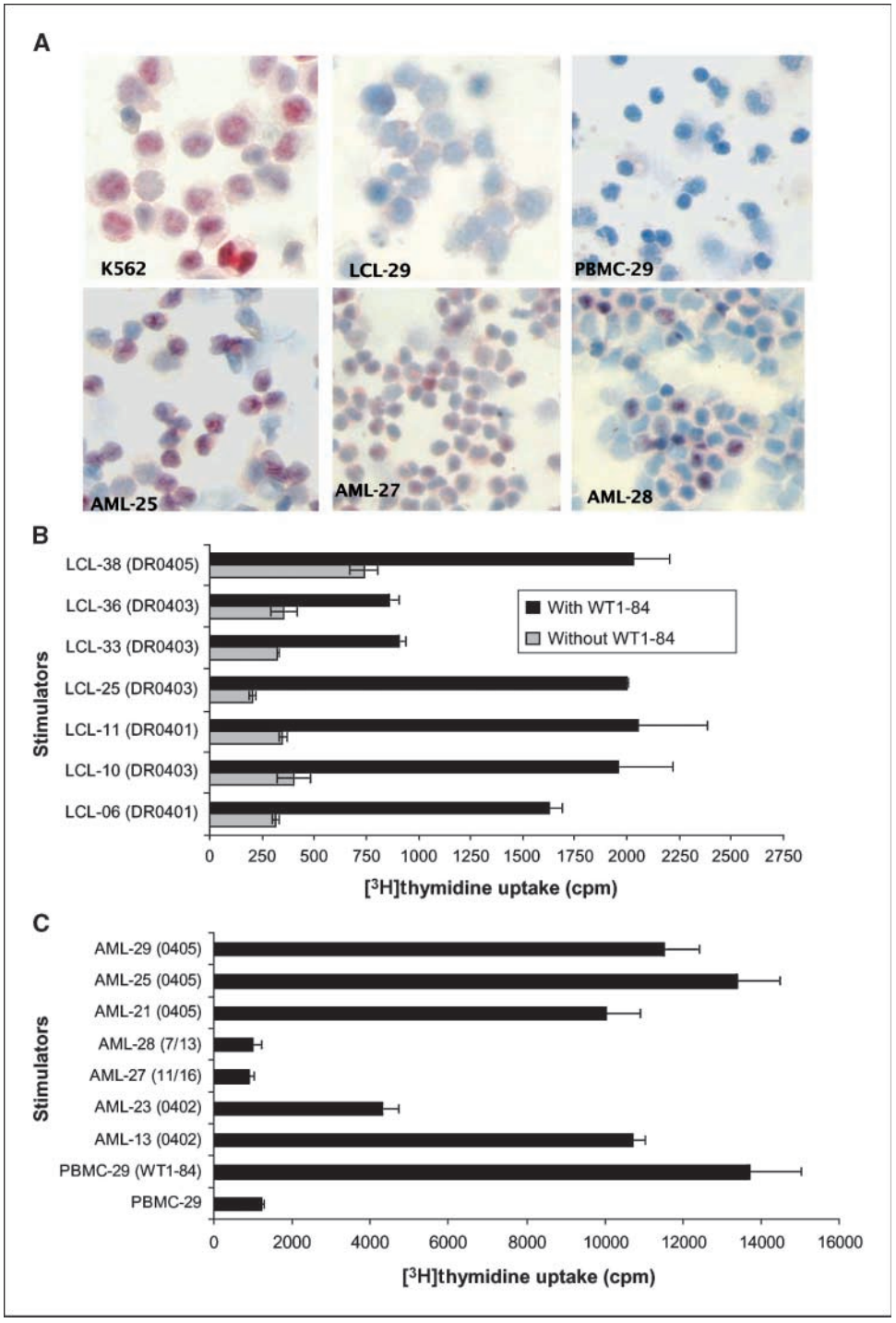

Figure 3. Expression of the WT1 antigen by leukemic cells and proliferative activity of the anti-WT1 T_{regs} to different HLA-DR4–matched leukemias. *A,* cytospin immunocytochemical staining of three representative PBMCs from AML patients (AML-25, AML-27, and AML-28) showing the nuclear expression of the WT1 protein in the leukemic blasts (*red*). The K562 cell line was used as a positive control, and LCL-29 and PBMC-29, from which the TCL 29 and clones were generated, were used as negative controls. Cells were counterstained in instant hematoxylin. Magnification, ×520. *B,* proliferative activity of a representative T-cell clone (TCC 29.B.42) against the HLA-DR04 suballelic positive LCLs (LCL-06, LCL-11, LCL-10, LCL-25, and LCL-38) in the absence (*gray columns*) or presence (*black columns*) of the WT1-84 peptide. *C,* proliferative activity of the TCC 29.B.42 T_{reg} clone against PBMCs from DR0402-matched (AML-13 and AML-23), nonmatched (AML-27 and AML-28), and HLA-DR04 suballelic positive AML (AML-21, AML-25, and AML-29) patients. T cells (5×10^4 per well) were challenged for 72 h with irradiated (3,000 rads, XR) PBMCs (10^5 per well) from AML patients, incubated for additional 18 h with [^{3}H]thymidine, and harvested. T cells challenged with irradiated autologous PBMC-29 in the absence or presence of the WT1-84 peptide served as negative and positive control, respectively.

woman as a positive control. Foxp3 expression was further confirmed at the protein level (Fig. 2*B*). We further confirmed the protein expression in these clones by fluorescence-activated cell sorting (FACS) analysis and found to exert a $CD4^+CD25^+Foxp3^+$ T_{reg} phenotype (Fig. 2*C*). Finally, we examined the clones for the expression of other T_{reg}-related markers. GITR (28, 29) and CTLA-4 (30) molecules are constitutively expressed at high levels in T_{regs}. All clones expressed GITR but were negative for CTLA-4 and CD127, which has been recently used to discriminate between human regulatory $CD127^-$ and activated T cells (Fig. 2*D*; ref. 31). Altogether, these data indicate that the current anti-WT1 clones are phenotypically T_{regs}.

The TCR profile of the generated T-cell line and clones was determined using a TCR Vβ kit, which is used to detect the 24 most common human TCR Vβ molecules. All clones shared the same TCR Vβ8 chain (Supplementary Data 4A). We also determined the frequency of TCR Vβ8 in TCL 29 and PBMC-29 from the same donor. The TCR Vβ profile of PBMC-29 scattered between 1% and 8% in which 5% of T cells were TCR Vβ8 (Supplementary Data 4B). The frequency of other two T-cell populations with unknown

specificities was increased (Vβ2, 8–21%; Vβ3, 7–12%) after seven rounds of stimulation (Supplementary Data 4C). Interestingly, the TCR Vβ8$^+$ T-cell frequency was enriched to 40% in TCL 29 after seven rounds of stimulation, indicating the immunodominance of anti–WT1-84 TCR Vβ8$^+$ T-cell population in TCL 29.

Evaluation of WT1 expression and proliferative activity of anti-WT1 T_{regs} against DR4-matched healthy and leukemic cells. We examined the expression of the WT1 mRNA in fresh AML cells using quantitative reverse transcription-PCR (RT-PCR). WT1 protein expression was also evaluated by immunostaining and scored by two independent scientists. K562 cells, known to express high levels of WT1 (20), were used as a positive control. PBMCs and LCLs from normal donors served as negative controls. Higher levels of WT1 expression were recorded in AML samples (Supplementary Data 5). Figure 3*A* shows immunostaining of PBMCs from three AML patients indicating different levels of WT1 expression.

Because HLA-DR was the restriction element for the clones (Fig. 1*C*), it was important to evaluate its expression in the AML samples. All AML samples expressed HLA-DR, although at different levels (Supplementary Data 5). The proliferative activity of a selective clone was then measured against PBMCs from two DR0402-matched (AML-13 and AML-23) and two non-DR0402 (AML-27 and AML-28) AML patients. Specific proliferative activity was recorded for the clone against AML-13 and AML-23 cells expressing the DR0402 molecule (Fig. 3*B*). However, this clone failed to recognize non-DR0402 AML-27 and AML-28 cells, although they express high levels of WT1 (Fig. 3*C*). Percentages of leukemic blasts used in this study are shown in Supplementary Data 4, column 2. T cells challenged with PBMC-29 ± WT1-84

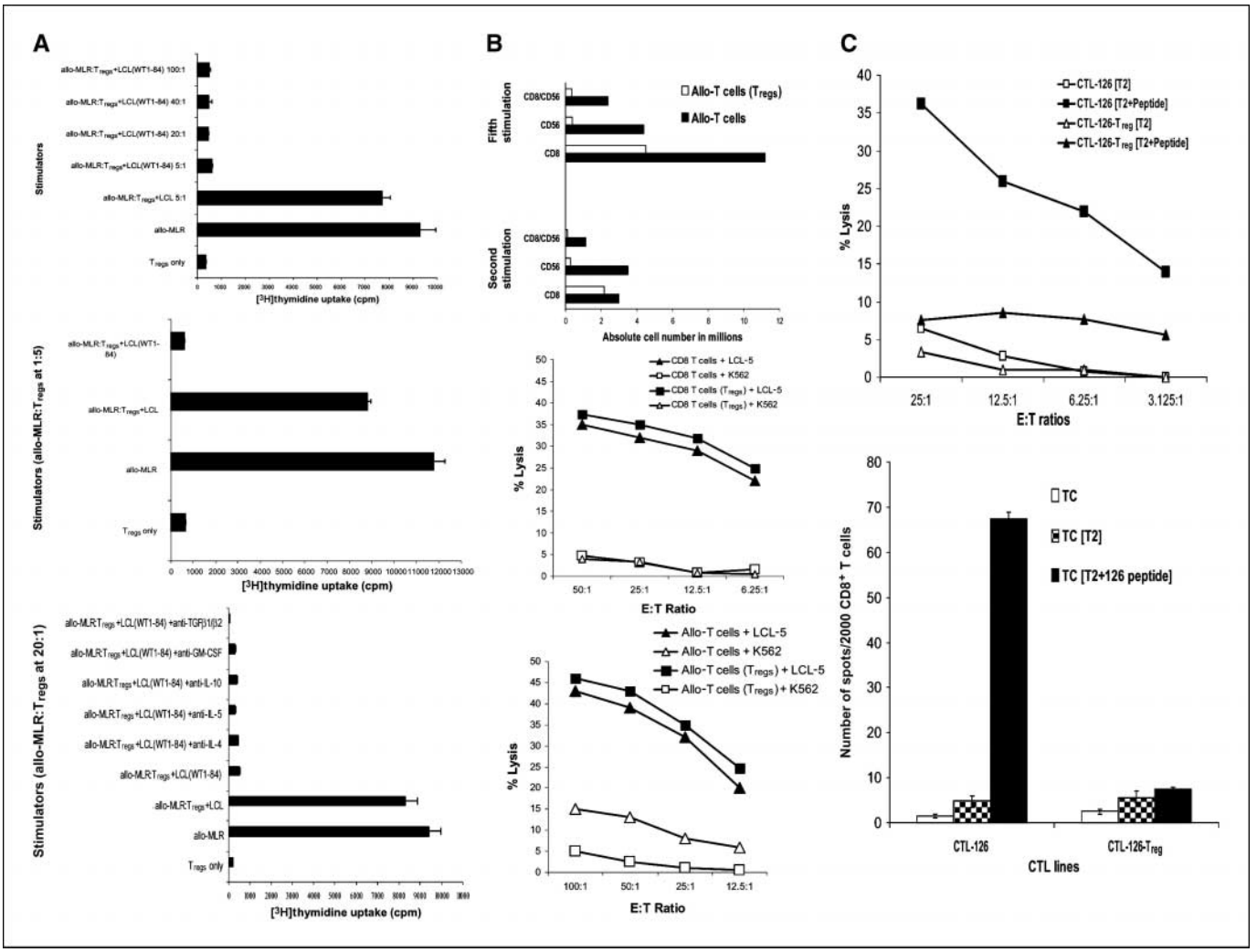

Figure 4. Suppressive function of the TCC 29.B.42 T_{reg} clone. *A, top,* the proliferative activity of allo-MLR was strongly suppressed by a representative clone TCC 29.B.42 at different clone to allo-PBMCs ratios in which specific activation of the clones with WT1-84 peptide was required for such suppressive activity; *middle,* Transwell experiments showing that T_{regs} cultured in inner wells were able to significantly suppress the proliferative activity of the allo-MLR cells cultured in the outer wells (ratio was at 1:5); *bottom,* blocking antibodies to TGF-β1/β2, IL-4, IL-5, IL-10, or GM-CSF did not restore the allo-MLR (ratio was at 1:20). *B, top,* the suppressive effect of the TCC 29.B.42 T_{reg} clone on the expansion of alloreactive cytotoxic lymphocytes (inhibition effect of the T_{regs} on the expansion of NK (CD56), NK T (CD8/CD56), and CD8 cells after second and fifth stimulation; *middle,* the cytotoxic activity of purified CD8$^+$ alloreactive T lymphocytes generated in the presence or absence of T_{regs} and tested against allogeneic LCL-5 and K562 cells; *bottom,* the cytotoxic activity of mixed alloreactive T lymphocytes generated in the presence or absence of T_{regs} and tested against allogeneic LCL-5 and K562 cells. *C, top,* the suppressive effect of the TCC 29.B.42 T_{reg} clone on the induction of anti–WT1-126 CTLs; *bottom,* the suppressive effect of the TCC 29.B.42 T_{reg} clone on the induction of anti–WT1-126 IFN-γ–producing T lymphocytes. CTLs were challenged with irradiated T2 cells in the absence (*TC[T2]*) or presence (*TC[T2+126 peptide]*) of peptide. T cells only (*TC*) were used as negative control.

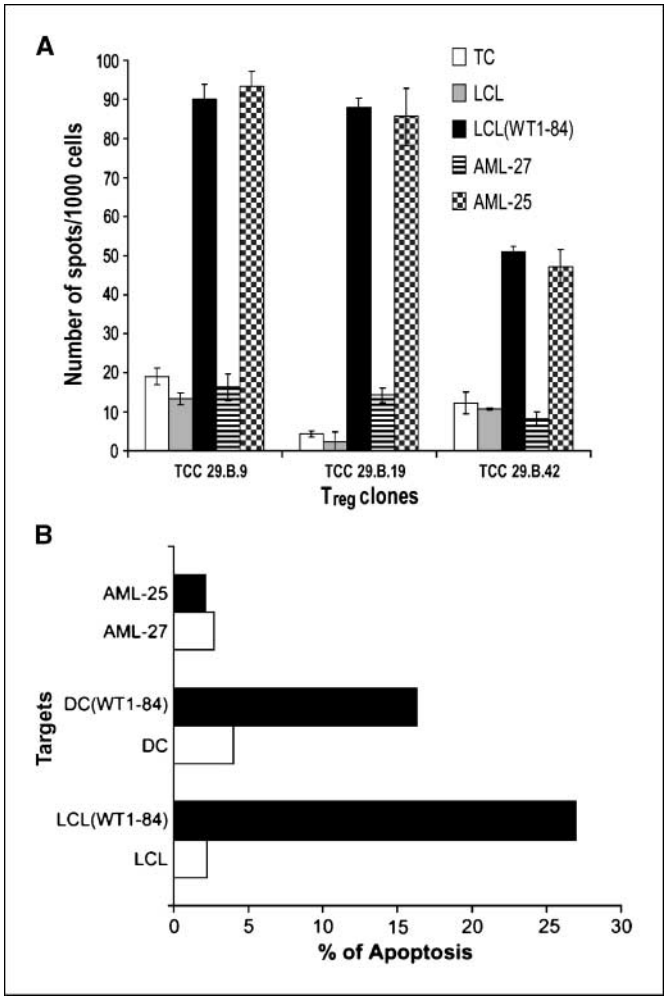

Figure 5. Specific production of granzyme B by anti-WT1 T_{reg} clones and induction of apoptosis in target cells. *A,* ELISPOT assay for granzyme B production by three T_{reg} clones. T_{regs} (10^3 per well) were stimulated with autologous LCL ± WT1-84, HLA-matched AML-25, or nonmatched AML-27 cells (2×10^4 per well) for 40 h in anti–granzyme B precoated wells before development of the spots. T cells incubated without stimulators (*TC*) served as a baseline. *B,* proportion of apoptosis induction by TCC 29.B.42 T_{reg} clone after 24 h of incubation with target cells. Apoptosis was determined by FACS using Annexin V in combination with propidium iodide on CD19 (LCL), CD40 (DCs), and CD33 (AML) gated cells.

served as negative and positive controls. We have also showed that the WT1-84 peptide was able to induce a specific proliferative cross-reactivity in the T-cell clones after challenging with LCLs sharing different HLA-DR04 suballeles: DR-0401 (LCL-06 and LCL-11), DR-0403 (LCL-10, LCL-25, LCL-33, and LCL-36), and DR-0405 (LCL-38; Fig. 3*B*). Therefore, we tested the proliferative activity of the clones against WT1-expressing PBMCs from different HLA-DR04 suballelic positive AML patients (AML-21, AML-25, and AML-29). The clones recognized all AML cells used (Fig. 3*C*). We conclude that leukemic cells express relatively high levels of the WT1 antigen and able to process it to the WT1-84 peptide, which then presented to T_{regs} through the HLA-DR04 molecule.

Evaluation of the inhibition effect of the T_{reg} clones on alloreactive T helper/cytotoxic lymphocytes and induction of anti-WT1 CTL responses. To evaluate whether the T_{reg} clones exert a suppressor function on alloreactive T helper lymphocytes, we used an allo-MLR (32) as a functional readout. All clones significantly suppressed the proliferative activity of the allo-MLR and showed a strong inhibition effect even at 1:100 T-cell to allo-PBMC ratio (Fig. 4*A, top*). Specific activation of the clones with WT1-84 peptide was required for such suppressive activity, as stimulation with LCL alone did not have a significant effect. To test whether cell-cell contact was required for such inhibition, we carried out experiments in Transwell plates. All clones cultured in inner wells were able to significantly suppress the proliferative activity of the allo-MLR cells cultured in the outer wells (Fig. 4*A, middle*), showing that cell-cell contact was not required for such inhibition. This inhibition effect was not mediated with TGF-β1/β2, IL-4, IL-5, IL-10, or GM-CSF, as mAbs to these cytokines did not restore the allo-MLR (Fig. 4*A, bottom*). The clones inhibited also the proliferation of autologous LCL (data not shown), suggesting the involvement of other soluble factors in such inhibition.

We next examined the suppressive effect of TCC 29.B.42 T_{reg} clone on the expansion and function of alloreactive cytotoxic lymphocytes. The presence of T_{regs} had a strong inhibition effect on the expansion of both natural killer (NK; CD56) and NK T (CD8/CD56) cells in early cultures, whereas a strong inhibition effect on the expansion of CD8 was recorded in late cultures (Fig. 4*B, top*). However, the cytotoxic activity of alloreactive $CD8^+$ CTLs generated in the presence of T_{regs} was comparable with that generated in the absence of T_{regs} when allogeneic LCL-5 cells were used as a target (Fig. 4*B, middle*). Interestingly, NK activity against K562 was recorded only in mixed alloreactive cells generated in the absence of T_{regs} (Fig. 4*B, bottom*).

Finally, we examined the suppressive effect of TCC 29.B.42 T_{reg} clone on the induction of anti-WT1 CTL responses. The presence of T_{regs} had a strong inhibition effect on the induction of anti–WT1-126 $CD8^+$ CTL responses as evidenced by both very low cytotoxic activity (Fig. 4*C, top*) and IFN-γ production (Fig. 4*C, bottom*) recorded for the CTL-126 T_{regs} after challenge with the $HLA\text{-}A0201^+$ T2 cell line as a target.

Production of granzyme B and induction of apoptosis by the T_{reg} clones. We next sought to determine what other soluble factors are involved in such inhibition. Granzyme B is produced by T_{regs} and induces apoptosis in target cells in a perforin-independent (33) and perforin-dependent (34) manners. Therefore, we examined our T_{reg} clones for granzyme B and perforin production using ELISPOT and intracellular staining assays. Granzyme B was specifically produced in response to autologous LCL in the presence of the WT1-84 peptide and also to stimulation with DR4-matched AML-25 cells but not to non–DR4-matched AML-27 cells (Fig. 5*A*). Perforin was not detected by either assays (data not shown). We further examined the apoptotic effect of these clones. A specific apoptosis was induced in autologous DCs and LCL pulsed with WT1-84 peptide but no apoptotic effect was detected for either DR4-matched AML-25 or non–DR4-matched AML-27 cells (Fig. 5*B*). These data show the capability of T_{regs} to selectively induce apoptosis in APCs and not in AML cells.

Determination of anti–WT1-84 T_{reg} frequencies in AML patients. Our generated T_{reg} clones exerted a $CD4^+Foxp3^+$ phenotype and specifically produced IL-5 and granzyme B in response to the WT1-84 peptide. Therefore, we have used intracellular cytokine staining to test for specific granzyme B and IL-5 production and Foxp3 expression by $CD4^+$ T cells in AML patients. Of eight $HLA\text{-}DR\text{-}4^+$ patients (Supplementary Data 1)

tested, five patients showed significant increase in granzyme B/Foxp3 expression and IL-5 production in the presence of the WT1-84 peptide (Fig. 6). Non–HLA-DR-4 (AML-27 and AML-28) and HLA-DR-4$^+$ donors (BC-29 and BC-62; Supplementary Data 1) were negative (data not shown). These findings support our *in vitro* data and show the amplification of tumor-specific anti–WT1-84 T_{regs} in AML patients in HLA-DR4–restricted manner.

Discussion

In this study, we identified novel human anti-WT1 T_{reg} and clones using a pool of 110 15-mer overlapping peptides across the entire WT1 protein. These T-cell lines and clones specifically recognized a $WT1_{333-347}$ (RYFKLSHLQMHSRKH) peptide designated WT1-84 in an HLA-DRB1*0402–restricted manner. The T-cell clones produced GM-CSF, IL-4, and IL-5 in response to the WT1-84 peptide exerting a Th2 cytokine profile. During the course of our study, Fujiki and colleagues (35) described an anti-WT1 T helper 1 (Th1) T-cell clone restricted to HLA-DRB1*0405 and specific to a 16-mer $WT1_{332-347}$ peptide that has an additional amino acid compared with the 15-mer $WT1_{333-347}$ epitope described in our study. It has been shown that the affinity of the antigen for TCR and the antigen dose can influence the differentiation of Th1 and Th2 cells (36). Th2 cells are generated only in the presence of a low dose of an antigen, and a weak TCR signal is needed for their generation (37). Fujiki and colleagues used high dose (50 μg/mL) of the $WT1_{332-347}$ as a single peptide to prime T cells, whereas only 10 μg/mL of the 110 Pepmix including the $WT1_{333-347}$ peptide were used in the current study. Therefore, the antigen dose and peptide competition in the case of the WT1-Pepmix may contribute to the generation of two different T helper cell types. Finally, we used high concentration of IL-2 (260 IU/mL) for the T-cell generation, whereas Fujiki and colleagues used only 20 IU/mL in their study. High dose of IL-2 has been shown to increase the frequency of T_{regs} in cancer patients (38) and selectively up-regulate the *in vitro* expression of Foxp3 in T_{regs}. It is possible that the presence of high dose of IL-2 in our T-cell cultures played a role in the polarization of T cells to Th2 phenotype as it has been described in other systems (39) and up-regulated the expression of Foxp3 in this T-cell population.

A recent study by Durinovic-Bello and colleagues (23) showed that human Th2 cells that exert a down-regulatory T_{reg} phenotype also express Foxp3. However, Foxp3 has also been shown to be expressed transiently in activated non-T_{regs}, whereas

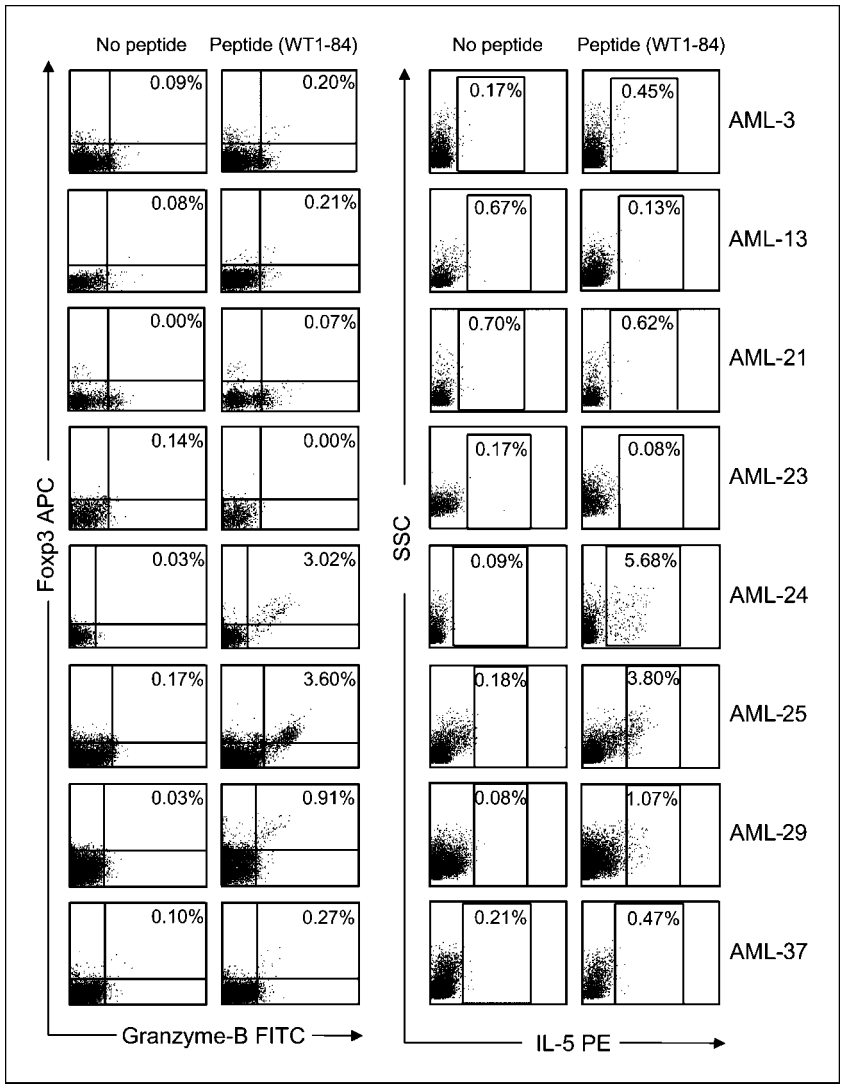

Figure 6. Intracellular staining and determination of anti–WT1-84 Foxp3$^+$ T_{reg} frequencies in AML patients. PBMCs (2×10^6/mL) were cultured in DC medium as described in Materials and Methods + 125 units of IL-2 for 48 h ± WT1-84 peptide. Brefeldin A was added at 5 μg/mL in the last 12 h of culture. Granzyme B-FITC/Foxp3-APC double-positive cells were analyzed on gated CD4-PE$^+$ cells. IL-5-PE$^+$ cells were analyzed on gated CD4$^-$APC$^+$ cells. AML-3, AML-24, AML-25, AML-29, and AML-37 showed significant increase in granzyme B/Foxp3 expression and IL-5 production in response to the WT1-84 peptide. Inserted numbers indicate the percentage of positive cells within the quadrant or the gate.

it is stably expressed in T_{regs} (26). We have shown that the generated T-cell clones were stably expressing Foxp3 and exerted a $CD4^+CD25^+Foxp3^+$ T_{reg} phenotype. Furthermore, the T-cell clones were examined for the expression of other T_{reg}-related markers (GITR, CTLA-4, and CD127) and confirmed to be $CD4^+CD25^+Foxp3^+GITR^+CD127^-$ antigen-specific T_{regs}. The lack of CD127 expression rules out the activation-induced Foxp3 expression. These clones recognize the WT1-84 epitope through their TCR Vβ8 chain. The immunodominance of the anti–WT1-84/TCR $V\beta8^+$ T-cell population in the generated T-cell line may have had a down-regulatory effect on the generation of Th1 T cells against other epitopes in the WT1-Pepmix. Importantly, these T_{reg} clones recognized HLA-DR4–matched leukemic cells expressing the WT1 antigen, showing the natural processing and presentation of the WT1-84 epitope. The WT1-84 peptide described in this study seems to be a promiscuous HLA class II–restricted T-cell epitope as it has been reported to be recognized by both HLA-DP5–restricted cytotoxic (22) and HLA-DR0405–restricted T helper (35) $CD4^+$ T cells.

Demonstration of functional T_{regs} will reside eventually on their ability to inhibit immune responses in functional assays (40). Several molecular and cellular events have been attributed to the suppressive effect of T_{regs} (41). The T_{reg} clones described in the present study were able to significantly inhibit the proliferative activity of allogeneic T cells independently of cell contact. Moreover, priming in the presence of anti–WT1-84 T_{regs} had a strong inhibition effect on the expansion and cytotoxic activity of NK and NK T-cell populations. This agrees with the recent study showing a direct inhibitory effect of T_{regs} on the generation and function of NK cells (42). However, the presence of anti–WT1-84 T_{regs} had a strong inhibition effect on the expansion of alloreactive CD8 T cells but no effect on their cytotoxic activity. The animal model study by Edinger and colleagues (43) showing that T_{regs} suppress alloantigen-driven expansion of CD8 T cells without inhibition of their cytotoxic effect supports our observation. More importantly, we have shown that the presence of T_{regs} strongly inhibited the induction of anti–WT1-126 $CD8^+$ CTL responses. The existence of such tumor-specific T_{regs} can actively suppress the WT1-specific antitumor immunity in cancer patients as it has been shown in other systems (12).

Furthermore, the present T_{regs} inhibited the proliferation of LCL, which does not depend on cytokines in their proliferation, suggesting the involvement of other soluble factors. It has been shown that production of granzyme B by T_{regs} results in the induction of apoptosis in T and B cells in a perforin-independent fashion (33). The current T_{regs} specifically produced granzyme B, but not perforin, in response to the WT1-84 peptide and induced apoptosis in LCL and DCs, suggesting a direct effect of granzyme B on the suppressive activity of these T_{regs}. However, they failed to induce apoptosis in HLA-matched AML cells, although granzyme B was produced in response to these cells. It is known that leukemic cells use several antiapoptotic signals to escape killing (44). Apoptosis in response to granzyme B involves activation of caspase-dependent cell death pathways (45). Blockade in caspase activation pathways is a common feature in leukemia (46), and this may explain the current resistance of AML cells to apoptosis. Our findings raise the possibility that leukemic cells may function as APC to preferentially induce anti–WT1-specific T_{regs} and hence down-regulate the patients' immune response through induction of apoptosis in APCs, such as DCs and B cells (34), and/or inhibition of specific and nonspecific anti-leukemia immune responses. Another important finding is the detection of anti–WT1-84 IL-5^+/granzyme B^+/$Foxp3^+$ $CD4^+$ T_{regs} in five of eight $HLA\text{-}DR4^+$ AML patients. The recent demonstration by Zhou and colleagues (47) that tumor-specific T_{regs} are amplified *in vivo* following cancer vaccination supports our data.

Current interests in cancer immunotherapy should be focused on determining the therapeutic implications of T_{regs} "regulating the regulators." Interestingly, elimination of T_{regs} by IL-2 conjugated to diphtheria toxin (ONTAK) enhanced vaccine-mediated antitumor immunity in cancer patients (7). However, targeting the CD25 molecule may eliminate T_{regs}, leading to an increase in the susceptibility to autoimmunity, and it can also deplete activated $CD25^+$ effector cells, which may be important for clearance of cancer and infection (48). Therefore, studies should be tailored toward developing selective depletion strategies directed against T_{reg}-related markers. Alternatively, triggering of TLR8 or OX40, and potentially blocking adenosine, might improve the chances of neutralizing T_{reg} immunosuppression in cancer patients (49). In conclusion, our findings should open opportunity for the clinical manipulation of anti-WT1 T_{regs} for the treatment of leukemia patients.

Disclosure of Potential Conflicts of Interest

All authors read and approved the final manuscript. The authors declare that they have no financial competing interests.

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original research report

Identification of a novel peptide derived from the M-phase phosphoprotein 11 (MPP11) leukemic antigen recognized by human CD8+ cytotoxic T lymphocytes

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BACKGROUND AND OBJECTIVES: There is an urgent need for the development of leukemia-targeted immunotherapeutic approaches using defined leukemia-associated antigens that are preferentially expressed by most leukemia subtypes and absent or minimally expressed in vital tissues. M-phase phosphoprotein 11 protein (MPP11) is extensively overexpressed in leukemic cells and therefore is considered an attractive target for leukemia T cell therapy. We sought to identify potential CD8+ cytotoxic T lymphocytes that specifically recognised peptides derived from the MPP11 antigen.

MATERIALS AND METHODS: A computer-based epitope prediction program SYFPEITHI, was used to predict peptides from the MPP11 protein that bind to the most common HLA- A*0201 molecule. Peptide binding capacity to the HLA-A*0201 molecule was measured using the T2 TAP-deficient, HLA-A*0201-positive cell line. Dendritic cells were pulsed with peptides and then used to generate CD8+ cytotoxic T lymphocytes (CTL). The CML leukemic cell line K562-A2.1 naturally expressing the MPP11 antigen and engineered to express the HLA-A*0201 molecule was used as the target cell.

RESULTS: We have identified a potential HLA-A*0201 binding epitope (STLCQVEPV) named MPP-4 derived from the MPP11 protein which was used to generate a CTL line. Interestingly, this CTL line specifically recognized peptide-loaded target cells in both ELISPOT and cytotoxic assays. Importantly, this CTL line exerted a cytotoxic effect towards the CML leukemic cell line K562-A2.1.

CONCLUSION: This is the first study to describe a novel epitope derived from the MPP11 antigen that has been recognized by human CD8+ CTL.

Despite major advances in molecular biology and targeted therapy of leukemia, current treatment strategies induce adverse side effects and fail to achieve and maintain remission in many patients. In acute myeloid leukemia (AML), the most deadly form of leukemia,[1,2] the majority of adult patients younger than 60 years achieve a complete remission following consolidation therapy. However, only approximately 30% to 40% maintain durable remission.[3] Furthermore, the complete remission rate and duration attained by older AML patients is less, with an overall survival rate of 20% to 40%.[3] Therefore, there is an urgent need for the development of leukemia-targeted immunotherapy designed to eliminate residual leukemic cells thus enhancing the graft-versus-leukemia (GVL) effect observed after allogeneic hematopoietic stem cell transplantation (HSCT) and/or prolonging a complete remission achieved by chemotherapy.[4,5]

Leukemia cells express unique antigens or overexpress normal cellular antigens.[6,7] The overexpressed

normal antigens constitute attractive targets for the development of leukemia immunotherapy.[6] Among this group of antigens are the M-phase phosphoprotein 11 (MPP11) proteins. MPP11 was discovered by Matsumoto-Taniura et al[8,9] using an MPM2 monoclonal antibody, which recognizes several important mitosis phosphoproteins found to be present during mitosis.[8-10] Recently, it has been reported that MPP11 functions as a ribosome-associated molecular chaperone.[11,12] MPP11 maps to the region of chromosome 7q22-31.1, which is a critical common position in human cancer.[10,13] MPP11 has been detected by serological analysis of cDNA expression libraries (SEREX) in solid tumours and hematological malignancies including melanoma, breast, renal cell carcinoma, small cell lung cancer and leukemia.[14,15] The humoral immune responses to MPP11 have been detected in patients with acute (AML) and chronic myeloid leukemia (CML), but not in healthy donors or patients with autoimmune diseases.[15] Moreover, this protein has been found to be expressed extensively in AML and CML patients as compared to normal controls.[15-18] Immunohistochemical staining of primary tumor sections and Western blot analysis of head and neck squamous cell cancer (HNSCC) cell lines revealed tumor-specific overexpression of MPP11 protein.[13,15] Additionally, fluorescence in situ hybridization analysis carried out on HNSCC cell lines showed an increase in the copy number of MPP11 along with chromosome 7 suggesting an oncogenic role for MPP11.[13] The specific overexpression of MPP11 by tumor tissues (with the exception of very low expression in testis, kidney, and lung) makes it an attractive target for cancer immunotherapies.[15,17]

We sought to identify potential CD8+ cytotoxic T lymphocyte (CTL) epitopes in the leukemia-associated MPP11 antigen that bind to the most common HLA-A*0201 molecule and provoke specific CTL responses. Although various antigenic epitopes derived from leukemia antigens have been identified, progress in targeting specific immunotherapeutic approaches depends on the recognition of a wider range of leukemia antigens. This would allow the use of these peptides in leukemia immunotherapy by either expanding ex vivo tumor-reactive specific CTLs for adoptive T cell therapy or actively inducing leukemia antigen-specific T-cell immunity in vivo by vaccinating patients with well-defined leukemia-associated antigens.

MATERIALS AND METHODS

Leukocyte-rich buffy coats not older than eight hours were obtained from healthy volunteers from the blood bank. All samples were obtained with informed consent at King Faisal Specialist Hospital and Research Centre (KFSHRC). Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from heparinized blood by density gradient centrifugation over Ficoll-Paque PLUS (Amersham Biosciences) according to the manufacturers' instructions. PBMCs were suspended in 90% Fetal Calf Serum (FCS) + 10% dimethyl sulfoxide (DMSO), aliquoted and cryopreserved in liquid nitrogen until further processing. The HLA typing of these samples was carried out at the routine immunopathology laboratory.

T lymphocyte epitope prediction and peptide synthesis

The SYFPEITHI prediction algorithm is a database comprising more than 4500 peptide sequences known to bind class I and class II MHC molecules. The scoring system of the SYFPEITHI program is based on the presence of certain amino acids in certain positions along the MHC-binding groove.[19] Peptides qualified as positive if the score ranked ≥22. The amino acid sequences of MPP11 were entered into the specified program and candidate epitopes were selected based on their predicted ability to bind to the HLA- A*0201 molecule. Four 9-mer peptides derived from MPP11 were selected as potential epitopes for the generation of CTL responses. The four peptides were: MPP-1N=MMP11-421-429 (QLLIKAVNL), MPP-2=MP11-419-427 (DLQLLIKAV), MPP-3=MPP11-313-321 (AIMLLLPSA), and MPP-4=MPP11-437-445 (STLCQVEPV). In addition to the predicted peptides, we modified the sequence of the first predicted peptide derived from MPP11 by introducing a Y at its N-terminus (wild type MPP-1N= QLLIKAVNL, analog MPP-1Y=MPP11-1Y(YLLIKAVNL) according to our recent study.[20] This approach enhanced both the binding and the immunogenicity of the modified peptide. The amino acid sequences of the nonameric peptides with a purity of ≥90% were synthesized and purified by Alta Bioscience, Birmingham, UK.

T2 binding assay

Peptide binding capacity to the HLA-A*0201 molecule was measured using the TAP-deficient, HLA-A*0201-positive cells as described previously.21 Briefly, T2 cells were washed three times, suspended at 10^6 cells/mL and incubated for 18 hours at 37°C in serum-free medium containing 30 μM of each peptide and 1 mg/mL β2-microglobulin (Sigma). Cells were then washed twice with cold FACS buffer (phos-

phate buffered saline containing 2% FCS). Purified rabbit IgG (Sigma) was added to the cell suspension and incubated for 15 minutes on ice in order to block the FC receptors. FITC-conjugated mouse anti-HLA-A2 antibody was added to the cell suspension (1 μg/10^6 cells), with a control sample labelled with isotype-matched monoclonal antibody and incubated for 30 min at 4°C in the dark. The cells were washed twice with cold FACS buffer, and fixed with 300 μL of PBS/4% paraformaldehyde. The level of HLA-A2 expression was analyzed using fluorescence-activated cell sorter (FACS) scan (Becton & Dickinson, Immunocytometry Systems, CA, USA). HLA-A2 expression was quantified as fluorescence index (FI) according to the following formula: fluorescence index = (mean fluorescence intensity with peptide-mean fluorescence intensity without peptide)/mean fluorescence intensity without peptide. All T2 binding assays were carried out in duplicate.

Cell lines

The mutant TAP-deficient cell line T2 (ATCC number CRL-1992) and the HLA-class I and II negative human CML cell line K-562 (ATCC number CCL-243) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). K-562 cells transfected with the HLA-A*0201 gene22 were kindly provided by Dr. Wolfgang Herr (University of Mainz, Germany). The T2 and K562 cells were maintained in complete medium (CM) consisting of RPMI 1640 medium (Sigma, MO, USA) supplemented with 10% FCS (Cambrex Bio Science, MD, USA), 2 mM L-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin (Sigma). K562/ A*0201 were cultured in CM containing 0.5 mg/mL geneticin (G418, Sigma). B-lymphoblastoid cell lines (LCLs) were established from normal donors by transformation of B cells using Epstein-Barr virus (EBV) according to a standard technique.23 Briefly, PBMCs (10^7 cells) were incubated with the virus (EBV virus stock, B95.8) for an hour at 37°C. The cells were cultured in CM containing 1μg/mL PHA (PHA-P, Sigma) and 0.1 ug/mL cyclosporin A, and established LCLs lines were used after 3-4 weeks of culture.

Generation of DC2d dendritic cells (Fast-DC)

Monocytes (CD14+ cells) were isolated from PBMCs of healthy HLA-A*0201 positive donors by plastic adherence. PBMCs were cultured at 5×10^6 cells/mL/well of a 6-well plate (Sigma) in X-vivo 15 medium (DC-medium), and allowed to adhere in a 5% CO_2 incubator at 37°C for 90-min. Non-adherent cells and media were removed and fresh DC-medium was added to the cells. After a second incubation period, non-adherent cells and media were removed and the adherent cells were washed carefully with pre-warmed medium. The DC2d were generated according to previously published protocols[24,25] with minor modifications. Briefly, DC-medium supplemented with 100 ng/mL rhGM-CSF and 50 ng/mL rhIL-4 (R&D Systems) was added to monocytes cultured in 6-well plates. Maturation cytokines, 10 ng/mL rhTNFa, 10 ng/mL rhIL-1b, 10 ng/mL rhIL-6 (R&D Systems) and 1 mM PGE2 (Sigma), were added to the cultures on the next day. Cultures were incubated for an additional day before mature DC2d were harvested.

Generation of anti-peptide specific CD8+ T lymphocytes

CD8+ T lymphocytes were negatively separated from PBMCS obtained from healthy HLA-A*0201 positive donors using MACS CD8+ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's protocols. Some of the CD8+ T lymphocytes lines were generated using a protocol adapted from previous studies.21,26 Briefly, T2 cells were washed three times in serum-free medium and incubated with each peptide at concentrations of 50 μM/mL and 1 μg/mL β2-microglobulin at 37 °C, 5% CO2 for 2 hours. The peptide-pulsed T2 cells were then X-irradiated (7500 rads), washed and added to freshly purified CD8+ T lymphocytes (T lymphocytes:T2 ratio of 5:1). After 7-10 days of stimulation, the second stimulation was performed and on the following day, 20 IU/mL of rhIL-2 (R&D Systems) was added. The cultured T lymphocytes were maintained by re-stimulations with peptide-pulsed T2 cells. After the third in vitro stimulation, the generated T lymphocytes were tested for their specificity and cytotoxic activity using enzyme-linked immunospot assay (ELISPOT) and chromium release assays. DC2d-loaded peptides were also used to in vitro prime some of the purified CD8+ T lymphocytes. Briefly, mature DC2d cells were harvested, washed in serum-free medium and incubated with the peptides at concentration of 50 μM/mL and 5 μg/mL β2-microglobulin for 2 hours at 37°C, 5% CO_2. The peptide loaded DCs were then X-irradiated (2500 rads), washed once and added to the CD8+ T lymphocytes at 20:1 to 5:1 T lymphocytes: DCs ratio. rhIL-7 (20 ng/mL) and rhIL-12 (100 pg/mL) (R&D Systems) were added to each culture. The cells were incubated in 2 mL T lymphocyte culture medium in 24-well plates. After 7-10 days, T lymphocytes were harvested, washed and re-stimulated with peptide

loaded DC2d as previously described and rhIL-2 (20 IU/mL) was added the following day. The cultured T lymphocytes were maintained by re-stimulation with autologous peptide-loaded adherent monocytes and feeding with rhIL-2 the next day. T lymphocytes lines against the different peptides were generated. Responder T lymphocytes were tested for their peptide specificity and cytotoxic activity using enzyme-linked immunospot assay (ELISPOT) and chromium release assay against different targets.

Generation of T lymphocyte clones

T lymphocytes lines were cloned by limiting dilution as described before25,27 with minor modifications. T lymphocytes were seeded at 1, 2 and 5 cells/well/200 µL T lymphocytes culture medium in 96-well U-bottomed plates. Allogeneic PBMCs were X-irradiated (2500 rads) and used as a feeder layer (25×10^3 cells/well). T2 cells were incubated separately in X-Vivo 15 medium (5×10^6 cells/mL) with 5 µg/mL β2-microglobulin and 50 µM/mL of MPP11 peptides for 2 hours at 37°C. T2 cells were then irradiated (7500 rads), washed once and used for T lymphocytes stimulation (50×10^3 cells/well) in the presence of 130 IU/mL of rhIL-2.5ng/mL rhIL15 (R&D Systems), and 30ng/mL OKT3 (R&D Systems), were also added to the wells. The plates were incubated for 2-4 weeks. Cultures were re-fed with fresh medium containing rhIL-2 (130 IU/mL) every 3-4 days. Wells with positive signs of growth were selected for expansion. Growing clones were transferred to 96-well flat-bottomed plates, and each clone was stimulated with X-irradiated peptide-pulsed T2 cells. The activation cycle was repeated after 7-10 days of culture at T lymphocyte: T2 ratios of 20:1 to 5:1. Clones were screened for their lytic activity against T2-pulsed with or without peptide using chromium release assay.

Cytotoxicity assay (chromium release assay)

Cytotoxicity assays were performed 5 days post in vitro stimulation. Target cells were removed from culture, washed in RPMI1640 serum-free medium, re-suspended in a minimal volume (± 50 µL) of RPMI1640 and incubated with 51Cr (100 µCi) per target for 90 min. Target cells labelled with peptide were concurrently incubated with the appropriate peptide at 50 µM/mL. The cells were then washed and placed in 96 V-bottomed wells at 10^3 cells/100 µL/well. The effector T cells were washed, and added in triplicate at varying quantities to the target cells, to give varying effector to target ratios (E:T ratios) in a final volume of 200 µL/well. The plates were spun for 5 min (300 g) and incubated for 4 hours at 37°C and 5% CO_2 before 100 µL supernatants were removed and transferred to 1450 Microbeta Plus Wallac plates (Wallac, Turku, Finland). 150 µL scintillation fluids, Optiphase HiSafe 2 (Wallac), were added to each well and the plates were heat-sealed using a Microsealer system (Wallac).

Chromium release was assessed by a liquid scintillation counter (Wallac). Target cells were also incubated with 0.2% Tween 20 or medium alone to assess the maximum and minimum (spontaneous) release of the chromium respectively. Spontaneous release was never exceeded 20% of the maximum release. The percentage of specific lysis was calculated as: % specific lysis = (experimental release−spontaneous release)/(maximal release−spontaneous release)×100. In some of the experiments 10 µg/mL anti-class I ABC blocking antibody (clone: W6/32, AbD Serotec) was added to target cells before T lymphocytes addition.

Enzyme-linked immunospot assay (ELISPOT)

ELISPOT assay was performed using IFN-γ, granzyme B, and perforin kits (Mabtech, Mariemont, OH) according to the manufacturer's protocols. 5×10^3 to 5×10^4 T lymphocytes/well and 2×10^4 to 10^5 cells/well of different stimulators were seeded in Multiscreen 96-well plates (Millipore, MA) pre-coated overnight (4°C) with catching-antibody. The plates were then blocked with T lymphocytes culture medium. After 40 hours incubation (37°C, 5% CO_2), cells were removed and after washing, biotinylated monoclonal antibodies specific for IFN-γ, granzyme B, or perforin were added and incubated for 3 hours at room temperature (RT). After washing, Streptavidin-alkaline phosphatase[28] or Streptavidin-horseradish peroxidase (HRP) were added to each well and incubated at RT for 2 hours. After washing, the appropriate substrate (BCIP/NBT in case of ALP and AEC in case of HRP) was added to each well and incubated at RT until color developed according to the manufacturer's instructions. Spots were counted using an automated ELISPOT reader (AID, Strasberg, Germany). Antigen-specific T lymphocytes frequencies were considered to be significantly higher if they were at least two-fold higher than in the control wells.

RESULTS

Selection of potential CTL epitopes derived from the MPP11 protein

The amino acid sequences of MPP11 were entered into the computer program and potential candidate epitopes were selected, synthesized and tested for their capacity to bind to the HLA-A*0201 molecule and/or generate cytotoxic T lymphocytes responses. We selected four

native peptides derived from MPP11 protein using the Syfpeithi server available at www.syfpeithi.de. These peptides showed high theoretical binding affinity to the HLA-A*0201 molecule with binding scores ranging from 22 to 25. In addition to the predicted peptides, we have carried out tyrosine amino acid substitution in the P1 position of the first predicted epitope MPP-1Y (QLLIKAVNL). The binding score of the modified epitope, MPP-1Y (YLLIKAVNL), was enhanced from 25 to 27 as assessed by the Syfpeithi program. The sequences of the peptides as well as their binding scores are presented in Table 1.

We used the standard T2 binding assay to evaluate the ability of MPP11 candidate peptides to bind and stabilize the HLA-A*0201 molecule using the well-characterized A2-binding peptides WT1-187 as a positive control (WT1 derived peptide known to effectively bind to the HLA-A*0201 molecule.[20,29] The human processing-defective T2 cell line express empty HLA class I molecules. The binding assay is based on the stabilization of HLA class I molecules on the cell surface by the addition of peptides exogenously. The T2 cells are incubated with peptides overnight and if the peptide binds to the HLA-A*0201, the surface expression will be stabilized and there will be an upregulation of this molecule. Changes in its expression were assessed by staining HLA-A*0201 and quantifying the fluorescence intensity by flow cytometry analysis. The peptides are assumed to bind if their fluorescence ratio is greater than 1. All T2 binding assays were done in duplicate. As shown in Figure 1, the MPP-3 and MPP-4 peptides (Fluorescence Index [FI] =1.1 and 2.5 respectively) stably bind to the HLA*A-0201 molecule. The affinity of the MPP-4 peptide to the molecule was comparable with that observed for the positive control WT1-187 epitope (FI=2.6). Because of their high binding affinities, we selected MPP-3 and MPP-4 peptides for the generation of peptide-specific T lymphocytes lines. On the other hand, the peptides MPP-1N, MPP1-Y, and MPP-2 were excluded from testing for immunogenicity since they did not show any binding or only very weak binding to the HLA-A*0201 molecule.

Table 1. Peptide Sequences derived from the MPP11 protein and their binding prediction to the HLA- A*0201 molecule.

Given name	Start position	Sequence	SYFPEITHI Score
MPP-1N	421	QLLIKAVNL	25
MPP-1Y	Y-422	YLLIKAVNL	27
MPP-2	419	DLQLLIKAV	24
MPP-3	13	AIMLLLPSA	23
MPP-4	37	STLCQVEPV	22

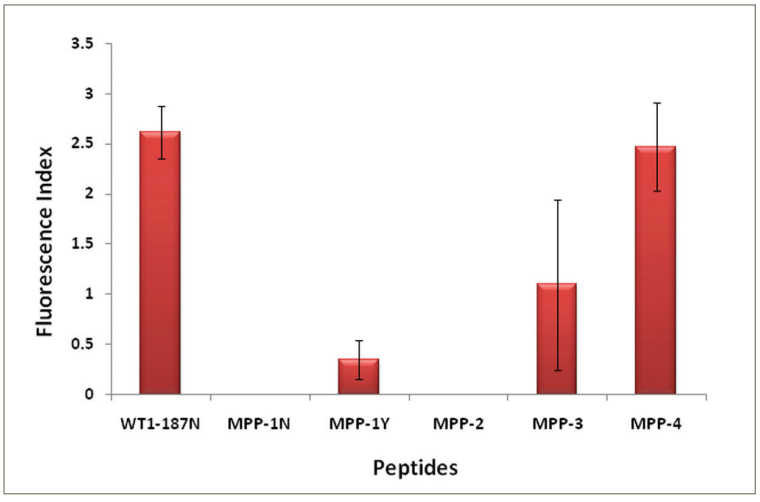

Figure 1. The binding ability of MPP11-derived peptides to the HLA-A*0201 molecule as measured by the T2 peptide-binding assay. The WT1-187N peptide served as a positive control. MPP-3 and MPP-4 peptides stably bind to the HLA-A*0201. The peptides MPP-1N, MPP-2 and the modified peptide MPP-1Y showed no binding to HLA-A*0201 and thereby were not used to generate CTLs. Each value represents the average of duplicate assays and error bars indicate the SD.

Generation of MPP11-specific T lymphocytes lines

We tested the ability of the MPP11-derived peptides to induce specific cytotoxic T lymphocytes (CTLs). We used PBMCs obtained from four different HLA-A*0201 positive healthy volunteers to generate CTLs specific to the selected peptides. The four donors were designated as BC-21, BC-32, BC-37 and BC-41. PBMCs from each donor were used as the source of the responder cells and antigen presenting cells. The responder cells were CD8+ T cells purified by MACS negative selection and stimulated in vitro with antigen presenting cells loaded with peptide for 7-10 days. Initially, we used T2 cells pulsed with peptides to prime and activate T lymphocytes raised against MPP11-derived peptides. However, we did not observe any specific CD8+ T lymphocyte responses to any of the peptides tested including the WT1-126N positive control peptide. Therefore, we used another protocol for the generation of CD8+ T lymphocyte lines where peptide-pulsed DC2d were used to prime the CD8+ T lymphocytes and peptide-pulsed monocytes were used for subsequent T lymphocyte stimulations. To investigate whether the MPP11 synthetic peptides could stimulate peptide-specific CTLs, the generated T cell lines were tested against different HLA-A*0201+ targets in the presence or absence of autologous peptide using a chro-

mium release assay and an ELISPOT assay after 3 to 8 rounds of stimulations.

Cytotoxic T cell lines generated against MPP-4 (STLCQVEPV) peptide produced specific IFN-γ

After the third round of stimulation, the specificity of in vitro primed CTLs was first tested for specific IFN-γ release by stimulation with the corresponding peptides using an ELISPOT assay. The CTLs generated against the MPP-4 (STLCQVEPV) peptide produced specific IFN-γ spots when stimulated with T2-loaded peptide whereas non-specific production was seen in case of CTLs generated against the MPP-3 (AIMLLLPSA) peptide as shown in Figure 2A and B.

Because CD8+ T lymphocytes are known to use cytotoxic factors such as granzyme B and/or perforin to induce killing of their target cells, so we tested the MPP-4- CTL for the release of granzyme B and/or perforin after 5 rounds of stimulation. MPP-4 pulsed T2 or autologous LCLs were used as stimulators. There was no specific production of granzyme B and/or perforin to the peptide MPP-4 due to very high background responses seen when T lymphocytes were used alone in the assay (Figures 2c and 2d).

T lymphocytes generated against the MPP-4 peptide specifically lyse MPP-4-pulsed target cells and leukemic cells

The expanded MPP11 T lymphocyte lines generated against the MPP-4 and MPP-3 peptides were tested for their specific cytotoxicity against T2 cells and autologous LCLs using a chromium release assay. Specific lysis of 17.8% against the T2 cells loaded with the MPP-4 peptide compared to 0% in the absence of peptide was recorded for TCL21 MPP-4 at effector to target ratio (E:T) of 50:1 (Figure 3A). In addition, 35% specific lysis for the same T lymphocytes line was recorded against autologous LCL loaded with the MPP-4 peptide compared to 6% against autologous LCL in the absence of the peptide at E: T ratio of 50:1 (Figure 3b).

The cytotoxic activity of the MPP-4 T lymphocytes line against the leukemic K562-A2.1 cell line was also measured (K562-A2.1 cells which naturally express the MPP11 antigen was transfected with the HLA-A*0201 molecule). Interestingly, the MPP-4 CTLs exerted a lytic activity of 14% against this leukemia cell line at an E:T ratio of 50:1, whereas only 7% lysis towards the wild type K-562 (lacking the

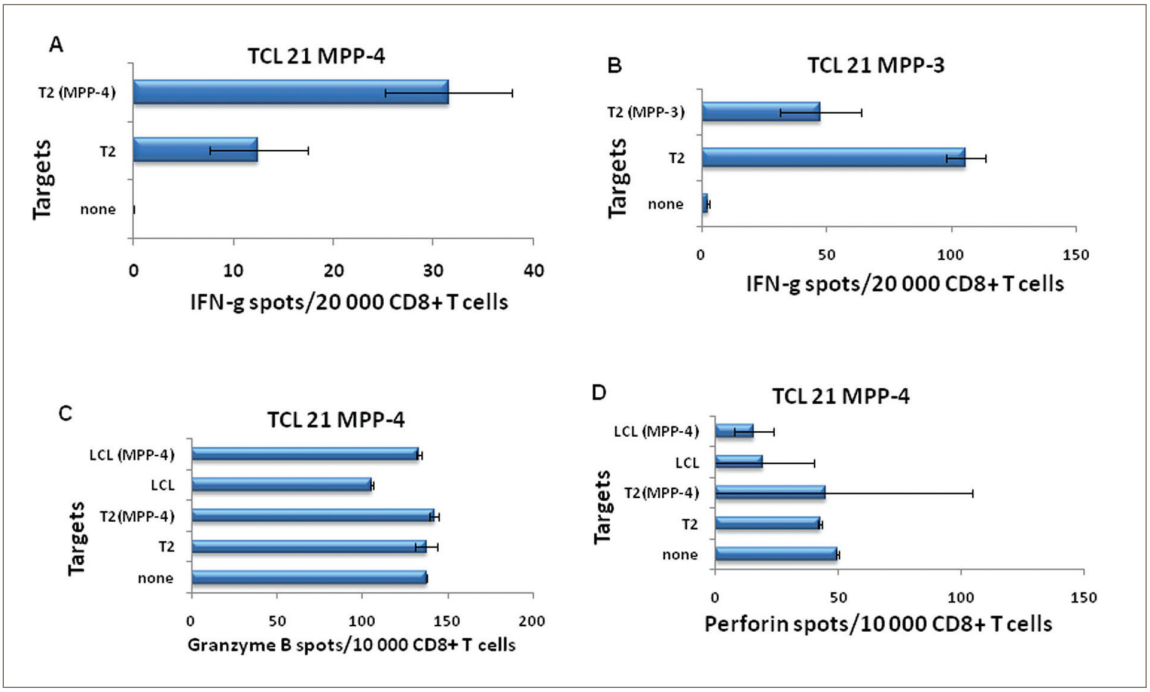

Figure 2. Specificity of CTLs generated against the MPP-3 and MPP-4 peptides using an ELISPOT assay. The CTL lines were established from BC-21, an HLA-A0201+ donor, following three to five rounds of in vitro T lymphocyte stimulations. MPP-4-CTLs releases specific IFN-g upon re-stimulation with T2 cells pulsed with MPP-4 peptide (A). CTLs generated against the MPP-3 peptide did not release specific IFN-g (B). High and non-specific granzyme B (C) and perforin secretion by CTL-MPP-4 (D). All assays were performed in triplicate and error bars indicate the SD.

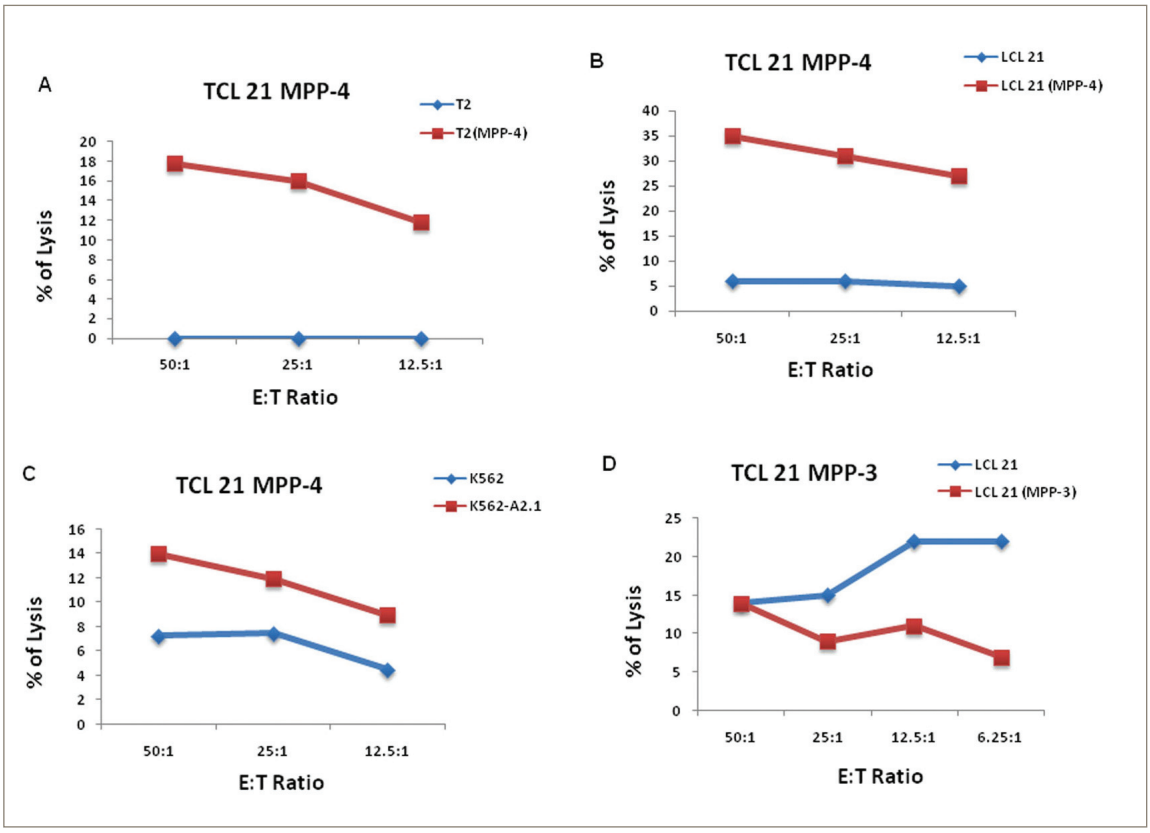

Figure 3. Cytotoxic activity of MPP11 CTL lines generated from the BC-21 donor as tested using a standard chromium release assay. MPP11-CTL lines were challenged with different HLA-A0201+ target cells at different E: T ratios. T2 and autologous LCLs were pulsed with each peptide before challenge. MPP-4 specific cytotoxic T cells specifically lysed peptide loaded T2 cells (A) and autologous LCL pulsed peptide (B). The same CTL lines lysed the leukemic cell line K-562-A2.1 and K-562 by TCL21 MPP-4 (C). Non-specific lysis of CTL line generated against MPP-3 (D).

HLA-A2 expression) was recorded at the same E:T ratio (Figure 3c). Similar to the ELISPOT results, the T lymphocytes line generated against the MPP-3 peptide did not show any specific cytotoxic activity (Figure 3d).

DISCUSSION

CD8+ CTLs recognize endogenously processed peptides3,30 consisting of 8-11 amino acids, most commonly 9 amino acids long, presented on the cell surface of target cells in the context of major MHC class I molecules. These cells are believed to play an important role in identifying and eliminating tumor cells.31 To date, several CD8+ CTL epitopes derived from leukemia antigens have been identified, and some of them have been investigated in clinical trials.[32-34] Importantly, the graft-versus-leukemia (GVL) effect often seen after allogeneic hematopoietic stem cell transplantation (AHSCT) has been found to be mediated mostly by donor-derived CD8+ T lymphocytes recognizing peptides derived from leukemia antigens and/or minor histocompatibilty antigens (mHAs) presented on recipient cells.[35-37] Overexpressed antigens such as WT1, PR-3 and MUC1 represent possible targets of specific GVL reactions.[38-40]

Previous studies have reported the existence of a humoral response against the MPP11 protein as well as the overexpression of this protein in patients with myeloid leukemias.[15-17] However, the identification of CD8+ CTL epitopes derived from this protein is a crucial contribution to the field of leukemia immunotherapy. In this study, we investigated potential CD8+ CTL epitopes derived from the MPP11 antigen using a reverse immunology approach.[41] The reverse immunology approach permits the identification of class I as well as class II candidate epitopes derived from known tumor antigens on the basis of HLA-binding motifs.[42,43] Using this approach, numerous epitopes were identified in leukemia[21,29,44,45] as well as in solid tumours.[46-51] We first examined the amino acid

sequences derived from the MPP11 protein for the presence of peptides containing binding motifs for one of the most frequent A*0201 class I alleles,[52] using a computer-based epitope prediction program. We identified four MPP11-derived peptides predicted to bind with high affinity to the HLA-A*0201 molecule. The peptides were named MPP-1N, MPP-2, MPP-3 and MPP-4. Using the T2 binding assay, we tested the actual binding capacity of the selected peptides. The MPP11-derived peptides MPP-3 and MPP-4 recorded binding scores lower than MPP-1N, and MPP2 scores as determined by the SYFPEITHI software. However, in the actual T2 binding assay, MPP-3 and MPP-4 were found to have higher binding affinities compared to the two other peptides. This is probably due to a very poor dissolution of the MPP-1N and MPP-2 peptides since more hydrophobic amino acids are contained within their sequence. In addition to the four predicted peptides, we modified the amino acid sequence of the highest scoring peptide i.e., MPP-1N (QLLIKAVNL). We[20] and others[53,54] have shown that introducing a tyrosine amino acid residue at the first position (P1Y) of a peptide enhances epitope immunogenicity. Therefore, a tyrosine amino acid residue was introduced in P1 of the MPP-1N peptide to boost its binding affinity and/or immunogenicity. The modification of MPP-1N peptide enhanced the binding affinity of the modified MPP-1Y peptide using the algorithm prediction but in the actual binding assay, the analogue peptide could not stabilize the HLA-A*0201 molecule which is again may be due to its poor dissolution.

Consistent with other groups[21,26,29] who have succeeded in identifying CD8+ CTL epitopes using a simple method that employs T2 cells as antigen-presenting cells, we could not generate specific immune responses using this method to both MPP11-derived peptides as well as WT1-126N peptide which was included as a positive control. Since MPP11 and WT1 are self proteins, the majority of high avidity CD8+ T lymphocytes may have been deleted during thymic maturation.[55] Thus it is more difficult to generate CTL responses from the remaining low avidity-naïve precursors because their activation requires more stringent co-stimulatory requirements.[56,57] Therefore, we used DCs as antigen-presenting cells for the generation of anti-MPP11 CTL responses. DCs are known as the professional antigen presenting cells because of their unique ability to stimulate naïve T lymphocyte responses. The conventional protocol for generating monocyte-derived DCs included 7-9 days of culture, however, Dauer et al,[24] described a rapid protocol for the generation of mature DCs from monocytes within only two days of in vitro culture. Such cells, termed fast DC or DC2d, were found to be capable of inducing tumor-specific CD8+ CTL responses as effectively as DCs generated according to the conventional 7-day protocol.[25,58] Moreover, CTL lines primed with the DC2d cells expanded more effectively and showed greater lytic activity than lines stimulated with the DC7d cells.[25,58] Using the DC2d protocol, we were able to detect antigen-specific responses by the CTL-21 line generated against the MPP-4 peptide. MPP-4-CTLs selectively recognized peptide-loaded T2 cells and specifically responded to the targets by releasing IFN-g in an ELISPOT assay. Importantly, these T lymphocytes specifically killed T2 cells and autologous LCLs when loaded with the MPP-4 peptide. Moreover, they were capable of recognizing the leukemia cell line K562-A2.1 expressing the MPP11 protein. Even though T2 cells were not efficient at priming and generating specific-CD8+ CTL immunity, these cells were very useful as targets when conducting functional assays such as cytotoxicity and ELISPOT assays.

We have attempted to generate MPP-4-specific CTLs from four healthy HLA-A*0201 positive individuals; however, specific responses could only be observed in one CTL line established from one donor (BC-21). The failure to obtain specific CTL responses to tumor antigens in all donors tested was observed in a previous study was thought to be due to the low frequency of peptide-specific CD8+ T lymphocyte precursors in healthy individuals.[59] We aimed to clone the MPP-4 CTL line before testing its capability of recognizing fresh A*0201-positive leukemic cells that overexpress the MPP11 protein. Unfortunately, regardless of our attempts to generate MPP-4-specific clones, we could not generate any specific clones. Standard techniques for cloning T cells employ stimulation with antigen loaded-APCs in the presence of IL-2 and allogeneic feeder cells in limiting dilution cultures followed by repeated re-stimulations to maintain growth and specificity. In the first attempt, the MPP-4 CTL line was cloned by stimulation with peptide pulsed-T2 cells in the presence of allogeneic feeder cells in the presence of IL-2, IL-15 and OKT3. IL-2 is the cytokine frequently used to promote the survival and expansion of cultured T cells.60 IL-2 and IL-15 share the β-chain and the common γ-chain receptor (IL-2/IL-15 Rβγc), but each cytokine has its unique α-chain receptor.[61,62] IL-15 was initially identified by its capability to promote the development of NK cells and the survival of CD8+ memory T lymphocytes in vivo.[63-65] However, it has been reported that antitumor-CTLs expanded and survived for relatively long time periods in vitro in the

presence of IL-15 whereas CTLs maintained in IL-2 died at earlier stages.[66] Furthermore, IL-15-maintained CTLs retained their effector function as CTLs and did not switch into memory cells.[66] For TCR triggering, we used OKT3 as reported previously.25 Because all of the generated clones were not specific, we conducted another attempt to clone the line but without the addition of either IL-15 or OKT3 antibody. The generated T lymphocytes clones were very few and died at early stage perhaps as a result of an old T lymphocyte culture.

In summary, our data suggests that CD8+ CTL reactive to the MPP-4 peptide could be generated from the T lymphocytes repertoire of HLA-A*0201+ healthy individuals. More importantly, these CTLs were able to specifically lyse the K562-A2.1 leukemic cells naturally expressing the MPP11 antigen.

Acknowledgments

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Investigation of M-phase phosphoprotein (MPP11) as a novel target for leukemia T cell immunotherapy

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MPP11 is an important protein during the mitotic phase of the cell cycle. It recognizes several important mitosis phosphoproteins and maps to the critical region of chromosome 7q22–31.1, which is a common place in human cancer. The high expression of MPP11 by tumors makes it an attractive tumour-restricted antigen. MPP11 has been detected in gastric, breast, small cell lung cancer and in leukaemias especially in AML, and CML patients, but not in healthy people. This makes the MPP11 as a unique target for cancer immunotherapy. The present PCR analysis showed high expression of the MPP11 gene in PBMCs from leukaemia patients (13/20 ALL, 16/20 AML) but only in 6/28 normal subjects. We observed that screening of the MPP11 protein sequence against the SYFPEITHI computer algorithm, resulted in 5 peptides predicted to bind to the HLA-class I A2.1 molecule. Further, the ability of these peptides to bind to the HLA-A2.1 molecule was evaluated using the T2-binding assay. We have found that peptides 3 & 4 have a high binding activity. T cells are being sensitized with T2-pulsed with these peptides to be tested against different targets mainly HLA-A2.1-matched patients' leukemic cells.

CD8+ cytotoxic T lymphocytes generated against a WT1 peptide analog enhance the lytic activity of leukemic cells

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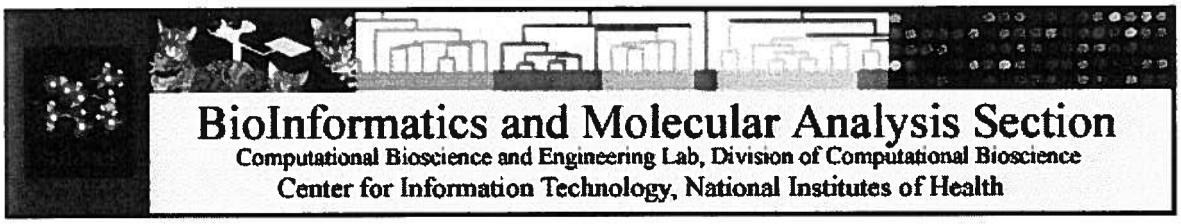
Background: The WT1 antigen performs an oncogenic function in various types of cancer. It is overexpressed in human leukemias and therefore it has been considered as an attractive target for immunotherapy. Most WT1-specific CTLs described displayed a low avidity and exerted minimal lytic activity against cancer cells.

Materials & Methods: We used an approach to improve the immunogenicity of CTL epitopes consisting of substituting the first-amino-acid, of 2 known HLA-A0201-restricted WT1-derived peptides (Db126 and WH187), with tyrosine (Y).

Results: This modification resulted in the enhancement of the binding ability of the 126Y analog and CTL generated against this peptide exerted a significantly lytic activity against the 126Y peptide-loaded target cells and importantly cross-reacted with the 126N native peptide. Another interesting finding is the significant high lytic activity recorded for the 126Y CTL against freshly isolated HLA-A0201-matched leukemic cells expressing the WT1 antigen. This data confirms that T cells generated against the 126Y analog peptide cross-react also with the naturally processed 126N native peptide. Moreover, it seems that stimulation with the peptide analog induced CTLs with a high TCR avidity. Finally, the high lytic activity provoked by the 126Y CTL may be also attributed to the significant high number of anti-126 T cell frequencies in this T cell line as demonstrated by IFN-gamma production in the ELISPOT assay.

Conclusions: This study provide evidence that peptide modification results in a better immune response against cancer and further support the use of this strategy as a potential approach for the development of a leukemia-vaccine.



HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A_0201
A_0205
A24
A3
A68.1

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(1/2)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

RMFPNAPYL

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

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HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	20
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	9
number of subsequence scores calculated	1
number of top-scoring subsequences reported back in scoring output table	1

Scoring Results			
Rank	**Start Position**	**Subsequence Residue Listing**	**Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)**
1	1	RMFPNAPYL	313.968

Echoed User Peptide Sequence (length = 9 residues)

1 RMFPNAPYL

Return to HLA peptide motif search page

query date: Tue Jul 27 2010, 05:19 EDT

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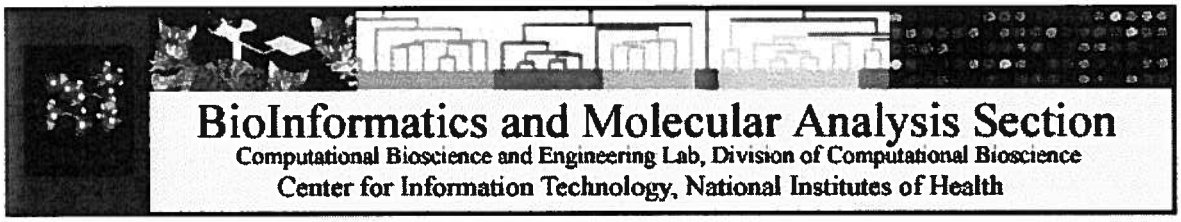
www-bimas@bimas.cit.nih.gov Ronald Taylor of BIMAS / CBEL / CIT / NIH



HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A1
A_0201
A_0205
A24
A3

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(1/2)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

YMFPNAPYL

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

Return to BIMAS home page

HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	20
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	9
number of subsequence scores calculated	1
number of top-scoring subsequences reported back in scoring output table	1

Scoring Results			
Rank	**Start Position**	**Subsequence Residue Listing**	**Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)**
1	1	YMFPNAPYL	1444.253

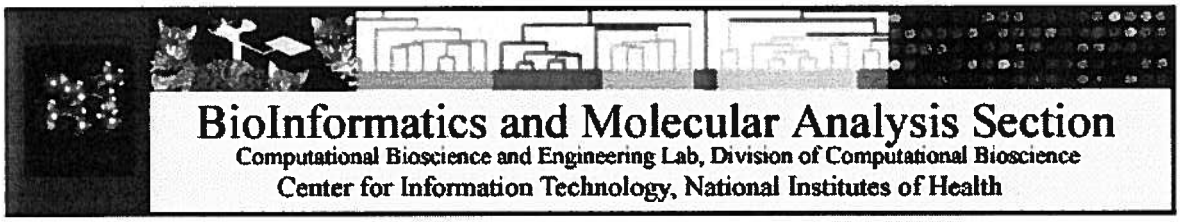
Echoed User Peptide Sequence (length = 9 residues)

1 YMFPNAPYL

Return to HLA peptide motif search page

query date: Tue Jul 27 2010, 05:20 EDT

www-bimas@bimas.cit.nih.gov Ronald Taylor of BIMAS / CBEL / CIT / NIH



HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A1
A_0201
A_0205
A24
A3

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(1/2)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

SLGEQQYSV

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

Return to BIMAS home page

HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	20
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	9
number of subsequence scores calculated	1
number of top-scoring subsequences reported back in scoring output table	1

Scoring Results			
Rank	**Start Position**	**Subsequence Residue Listing**	**Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)**
1	1	SLGEQQYSV	285.163

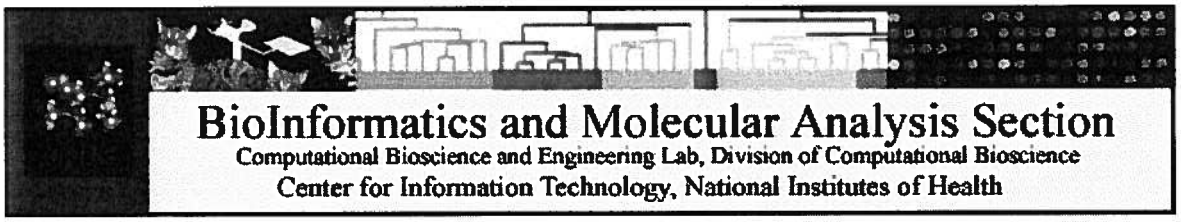
Echoed User Peptide Sequence (length = 9 residues)

```
1       SLGEQQYSV
```

Return to HLA peptide motif search page

query date: Tue Jul 27 2010, 05:21 EDT

www-bimas@bimas.cit.nih.gov Ronald Taylor of BIMAS / CBEL / CIT / NIH



HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A1
A_0201
A_0205
A24
A3

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(1/2)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

YLGEQQYSV

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

Return to BIMAS home page

HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	20
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	9
number of subsequence scores calculated	1
number of top-scoring subsequences reported back in scoring output table	1

Scoring Results			
Rank	**Start Position**	**Subsequence Residue Listing**	**Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)**
1	1	YLGEQQYSV	1311.751

Echoed User Peptide Sequence (length = 9 residues)

1 YLGEQQYSV

Return to HLA peptide motif search page

query date: Tue Jul 27 2010, 05:30 EDT

www-bimas@bimas.cit.nih.gov Ronald Taylor of BIMAS / CBEL / CIT / NIH

Epitope prediction

This page allows you to find out the ligation strength to a defined HLA type for a sequence of aminoacids. The algorithmus used are based on the book "MHC Ligands and Peptide Motifs" by H.G.Rammensee, J.Bachmann and S.Stevanovic. The probability of being processed and presented is given in order to predict T-cell epitopes.

1. Select MHC type

If you chose "all", max. sequence length is 100 aminoacids (letters)!

- all
- H2-Ad
- H2-Ak
- H2-Db
- H2-Ed

Hold down ctrl key when clicking to select multiple items

2. Choose a mer

- octamers (8 aa)
- nonamers (9 aa)
- decamers (10 aa)
- endecamers (11 aa)
- 15 - mers (15 aa) for MHC Type II only
- all mers

3. Paste your sequence here:

Max. input 2048 aminoacids (letters)!
Letters only, no numbers or non-ASCII-symbols please.
You may use 'SYFPEITHI' with H2-Kd to see an example.

```
DVSRCAHRARPGAIMLLLPSAADGRGTAITHALTSASTLCQVEPVGRWFEAF
VKRRNRNASASFQELEDKKELSEESEDEELQLEEFPMLKTLDPKDWKNQDHY
AVLGLGHVRYKATQRQIKAAHKAMVLKHHPDKRKAAGEPIKEGDNDYFTCIT
KAYEMLSDPVKRRAFNSVDPTFDNSVPSKSEAKDNFFEVFTPVFERNSRWSN
KKNVPKLGDMNSSFEDVDIFYSFWYNFDSWREFSYLDEEEKEKAECRDERRW
IEKQNGATRAQRKKEEMNRIRTLVDNAYSCDPRIKKFKEEEKAKKEAEKKAK
```

4. Choose Run to start analysis

Run Reset Home

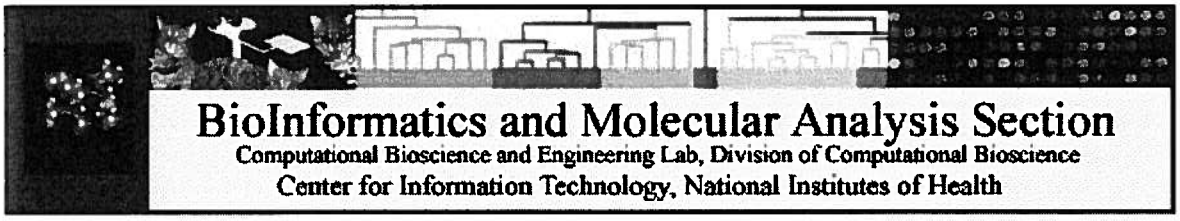
Search Report

Return to search conditions
HLA-A*0201 nonamers

HLA-A*0201 nonamers

go to top

Pos	1 2 3 4 5 6 7 8 9	score
421	Q **L** L I K A V N **L**	25
419	D **L** Q L L I K A **V**	24
13	A **I** M L L L P S **A**	23
37	S **T** L C Q V E P **V**	22
104	Y **A** V L G L G H **V**	22
350	L **L** A K K E K D **I**	22
521	K **L** L E Q A L K **T**	22
362	A **I** K K E R Q K **L**	21
28	A **I** T H A L T S **A**	19
422	L **L** I K A V N L **F**	19
459	D **V** I G K A K S **L**	19
462	G **K** A K S L Q K **L**	19
540	K **I** A E A V P G **R**	19
8	R **A** R P G A I M **L**	18
9	A **R** P G A I M L **L**	18
14	I **M** L L L P S A **A**	18
16	L **L** L P S A A D **G**	18
31	H **A** L T S A S T **L**	18
108	G **L** G H V R Y K **A**	18
122	K **A** A H K A M V **L**	18
207	S **N** K K N V P K **L**	18
217	D **M** N S S F E D **V**	18
375	K **I** E E I N E Q **I**	18
526	A **L** K T Y P V N **T**	18
553	C **M** K R Y K E L **V**	18
20	S **A** A D G R G T **A**	17
21	A **A** D G R G T A **I**	17
82	L **Q** L E E F P M **L**	17
154	C **I** T K A Y E M **L**	17
279	R **I** R T L V D N **A**	17
349	A **L** L A K K E K **D**	17
569	A **A** Q E Q V L N **A**	17
15	M **L** L L P S A A **D**	16



HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A1
A_0201
A_0205
A24
A3

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(½)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

VLQELNVTV

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

Return to BIMAS home page

HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	20
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	9
number of subsequence scores calculated	1
number of top-scoring subsequences reported back in scoring output table	1

Scoring Results			
Rank	**Start Position**	**Subsequence Residue Listing**	**Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)**
1	1	VLQELNVTV	484.777

Echoed User Peptide Sequence (length = 9 residues)

1 VLQELNVTV

Return to HLA peptide motif search page

query date: Tue Jul 27 2010, 07:40 EDT

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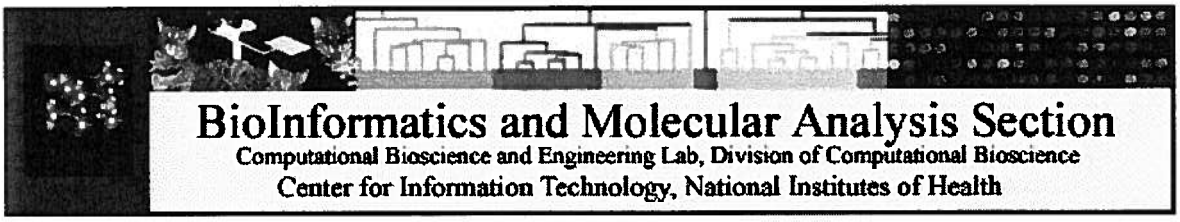
www-bimas@bimas.cit.nih.gov Ronald Taylor of BIMAS / CBEL / CIT / NIH



HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A1
A_0201
A_0205
A24
A3

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(1/2)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

NLSASVATV

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

Return to BIMAS home page

HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	20
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	9
number of subsequence scores calculated	1
number of top-scoring subsequences reported back in scoring output table	1

Scoring Results			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	1	NLSASVATV	159.970

Echoed User Peptide Sequence (length = 9 residues)

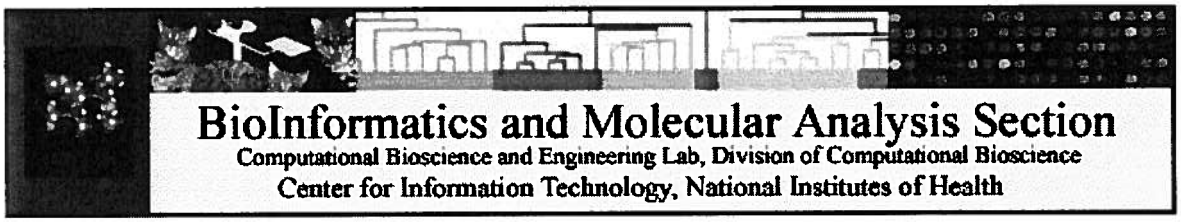
```
1       NLSASVATV
```

 Return to HLA peptide motif search page

query date: Tue Jul 27 2010, 07:40 EDT

---

www-bimas@bimas.cit.nih.gov Ronald Taylor of BIMAS / CBEL / CIT / NIH



HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A1
A_0201
A_0205
A24
A3

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(1/2)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

ALASVLLAL

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

Return to BIMAS home page

HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	20
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	9
number of subsequence scores calculated	1
number of top-scoring subsequences reported back in scoring output table	1

Scoring Results			
Rank	**Start Position**	**Subsequence Residue Listing**	**Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)**
1	1	ALASVLLAL	49.134

Echoed User Peptide Sequence (length = 9 residues)

```
1       ALASVLLAL
```

Return to HLA peptide motif search page

query date: Tue Jul 27 2010, 07:41 EDT

---

www-bimas@bimas.cit.nih.gov Ronald Taylor of BIMAS / CBEL / CIT / NIH

Epitope prediction

This page allows you to find out the ligation strength to a defined HLA type for a sequence of aminoacids. The algorithmus used are based on the book "MHC Ligands and Peptide Motifs" by H.G.Rammensee, J.Bachmann and S.Stevanovic. The probability of being processed and presented is given in order to predict T-cell epitopes.

1. Select MHC type

If you chose "all", max. sequence length is 100 aminoacids (letters)!

- all
- H2-Ad
- H2-Ak
- H2-Db
- H2-Ed

Hold down ctrl key when clicking to select multiple items

2. Choose a mer

- octamers (8 aa)
- nonamers (9 aa)
- decamers (10 aa)
- endecamers (11 aa)
- 15 - mers (15 aa) for MHC Type II only
- all mers

3. Paste your sequence here:

Max. input 2048 aminoacids (letters)!
Letters only, no numbers or non-ASCII-symbols please.
You may use 'SYFPEITHI' with H2-Kd to see an example.

VLQELNVTV

4. Choose Run to start analysis

Run Reset Home

Search Report

Return to search conditions
HLA-A*0201 nonamers

HLA-A*0201 nonamers

go to top

Pos	1 2 3 4 5 6 7 8 9	score
1	V **L** Q E L N V T **V**	28

Epitope prediction

This page allows you to find out the ligation strength to a defined HLA type for a sequence of aminoacids. The algorithmus used are based on the book "MHC Ligands and Peptide Motifs" by H.G.Rammensee, J.Bachmann and S.Stevanovic. The probability of being processed and presented is given in order to predict T-cell epitopes.

1. Select MHC type

If you chose "all", max. sequence length is 100 aminoacids (letters)!

- all
- H2-Ad
- H2-Ak
- H2-Db
- H2-Ed

Hold down ctrl key when clicking to select multiple items

2. Choose a mer

- octamers (8 aa)
- nonamers (9 aa)
- decamers (10 aa)
- endecamers (11 aa)
- 15 - mers (15 aa) for MHC Type II only
- all mers

3. Paste your sequence here:

Max. input 2048 aminoacids (letters)!
Letters only, no numbers or non-ASCII-symbols please.
You may use 'SYFPEITHI' with H2-Kd to see an example.

NLSASVATV

4. Choose Run to start analysis

Run Reset Home

Search Report

Return to search conditions
HLA-A*0201 nonamers

HLA-A*0201 nonamers

go to top

Pos	1	2	3	4	5	6	7	8	9	score
1	N	**L**	S	A	S	V	A	T	**V**	28

Epitope prediction

This page allows you to find out the ligation strength to a defined HLA type for a sequence of aminoacids. The algorithmus used are based on the book "MHC Ligands and Peptide Motifs" by H.G.Rammensee, J.Bachmann and S.Stevanovic. The probability of being processed and presented is given in order to predict T-cell epitopes.

1. Select MHC type

If you chose "all", max. sequence length is 100 aminoacids (letters)!

- all
- H2-Ad
- H2-Ak
- H2-Db
- H2-Ed

Hold down ctrl key when clicking to select multiple items

2. Choose a mer

- octamers (8 aa)
- nonamers (9 aa)
- decamers (10 aa)
- endecamers (11 aa)
- 15 - mers (15 aa) for MHC Type II only
- all mers

3. Paste your sequence here:

Max. input 2048 aminoacids (letters)!
Letters only, no numbers or non-ASCII-symbols please.
You may use 'SYFPEITHI' with H2-Kd to see an example.

ALASVLLAL

4. Choose Run to start analysis

Run Reset Home

Search Report

Return to search conditions
HLA-A*0201 nonamers

HLA-A*0201 nonamers

go to top

Pos	1 2 3 4 5 6 7 8 9	score
1	A **L** A S V L L A **L**	30

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AND RESEARCH CENTRE

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مستشفى الملك فيصل التخصصي
ومركز الأبحاث

RESEARCH CENTRE
OFFICE OF RESEARCH AFFAIRS

MBC 03: Fax: 27894 Ext: 32969

INTERNAL MEMORANDUM

TO: Said Dermime, PhD
Tumor Immunology Unit
Department of Biological & Medical Research

DATE: 16 Dhu Al Qada 1425
28 December 2004

FROM: Mohamed M. Al Turki, CCRP
Co-Director
Office of Research Affairs

REF: ORA/2059/25

SUBJECT: Proposal # 2040 038
Enhancing the Immunogenicity of Low-Affinity HLA-A2 Wilms Tumor-Restricted CTL Epitopes by Selective Amino Acid Replacements: Implication in the Generation of Effective cancer Vaccines and Adoptive T Lymphocyte Therapy in Leukaemias and Breast Cancer in the Saudi Population.

Further to our memo, ORA/1928/25 dated 24 Shawwal 1425/07 December 2004, your reply (received on 20 December 2004) to the concern raised by the Research Ethics Committee (REC) in regard to the above-referenced proposal was reviewed by REC on 15 Dhu Al Qada 1425/27 December 2004.

It is my pleasure to inform you that the REC accept the reply and recommended the proposal for approval.

The proposal can start as soon as approval by King Abdulaziz City for Science and Technology (KACST) is obtained. Please forward to the Office of Research Affairs (ORA) a copy of the correspondence indicating such approval.

cc: Chairman, Research Ethics Committee
Chairman, Department of Biological & Medical Research

KINGDOM OF SAUDI ARABIA
KING FAISAL SPECIALIST HOSPITAL
AND RESEARCH CENTRE

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مستشفى الملك فيصل التخصصي
ومركز الأبحاث

RESEARCH CENTRE
OFFICE OF RESEARCH AFFAIRS
MBC 03: Fax 27894 Ext: 32969
INTERNAL MEMORANDUM

TO: **Said Dermime, BSc.,MSc., PhD**
Scientist, Tumor Immunology Unit
Department of Biological and Medical Research

DATE: 02 Jumada I 1425
20 June 2004

THROUGH: **Mohamed M Al Turki, CCRP**
P.P Co- Director
Office of Research Affairs

FROM: **Ghada A. Al Hawsawi, HRA**
Proposal Processing Section
Office of Research Affairs

REF: ORA/0871/25

SUBJECT: **Proposal # 2040 010**
Investigation of M-Phase Phosphoprotein (MPP11) As a Novel Target for Leukemia T- Cell Immunotherapy

---

The above-referenced proposal was presented and discussed at the Basic Research Committee (BRC) and the Research Ethics Committee (REC) meetings on 27 Rabia I 1425/16 May 2004 and 20 Rabia II 1425/08 June 2004, respectively.

The BRC recommended the proposal for approval as submitted and would like to forward the attached external reviewer's comments to the investigators for information. However, the REC recommended the proposal for approval provided the Consent Form is revised according to the attached copy.

Please forward a revised consent form to the Office of Research Affairs (ORA) at your convenience, but not later than two months.

Attachment: consent form with correction
Bookmark

cc: Chairman, Basic Research Committee
Chairman, Research Ethics Committee

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AND RESEARCH CENTRE

المملكة العربية السعودية

مستشفى الملك فيصل التخصصي
ومركز الأبحاث

RESEARCH CENTRE
OFFICE OF RESEARCH AFFAIRS

MBC: 03 Fax: 27894 Ext.: 32937

INTERNAL MEMO

TO:	**Said Dermime, PhD** Scientist, Tumor Immunology Unit Department of Biological and Medical Research	**DATE:**	22 Muharram 1425 13 March 2004
THRU:	**Mohamed Al Turki, CCRP** Co-Director Office of Research Affairs	**REF.:**	ORA/0164/25
FROM:	**Weaam Al Jassim, RPh** Proposal Processing Section Office of Research Affairs		
SUBJECT:	**Project # 2030006** Use of Human Dendritic Cells as Potential Adjuvant for Generation of Specific Immune Responses to the Tumor-Associated Antigen Wilms Tumor (WT1) In The Saudi and Middle East Populations.		

The Compliance Report in relation to Consent Forms of the above-referenced project was discussed at the Research Ethics Committee (REC) meeting on 2 March 2004.

It is my pleasure to inform you that the committee accepted your reply and recommended the continued approval of the project. The committee would like to remind the investigators that according to the RAC policy, copies of the signed Consent Forms should be kept in the Medical Records of all research subjects.

Please submit a Progress/Final Report by 2 February 2005. The Report should be reviewed and accepted by the Committees by 2 March 2005. The Progress/Final Report Form is available at the Office of Research Affairs, Research Centre, Room 304, MBC 03, extension 32937/24528 or at http://rckfshrc.edu.sa/rac/.

Thank you for your cooperation.

cc: Chairman, Research Ethics Committee
Chairperson, Department of BMR