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GENERATION OF IN VITRO B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA- SPECIFIC T CELL RESPONSES USING DENDRITIC CELLS

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GENERATION OF *IN VITRO* B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA- SPECIFIC T CELL RESPONSES USING DENDRITIC CELLS

by

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ABSTRACT

Generation Of *In Vitro* B-Cell Chronic Lymphocytic Leukaemia-Specific T Cell Responses Using Dendritic Cells. RV Goddard.

Immunotherapy using dendritic cells has shown encouraging results in both haematological and non-haematological malignancies. In this study, monocyte-derived dendritic cells from patients with B-cell Chronic Lymphocytic Leukaemia were generated by culture in Interleukin-4 and Granulocyte Macrophage-Colony Stimulating Factor. Lysate-pulsed autologous dendritic cells were used as antigen presenting cells in co-culture with autologous B-cell Chronic Lymphocytic Leukaemia T-cells. B-cell Chronic Lymphocytic Leukaemia T-cells stimulated with B-cell Chronic Lymphocytic Leukaemia lysate-pulsed autologous dendritic cells showed a significant increase in cell surface expression of Interleukin-2 Receptor (CD25), Interferon-gamma secretion and cytotoxicity against autologous B-cell Chronic Lymphocytic Leukaemia B-cell targets but not against targets from healthy volunteers. Responses were only stimulated by the B-cell Chronic Lymphocytic Leukaemia B cell lysate. Cytotoxicity was Major Histocompatibility Complex Class II restricted. The addition of maturation agents such as Lipopolysaccharide, Tumour Necrosis Factor-alpha and Polyriboinosinic Polyribocytidylic Acid to monocyte derived dendritic cells was unsuccessful at increasing anti-tumour responses. Pre-treatment of T cells with Interleukin-15 before stimulation by lysate pulsed autologous dendritic cells increased numbers of activated cells, cytokine secretion and specific cytotoxicity to B-cell Chronic Lymphocytic Leukaemia B-cells. Fusion of monocyte derived dendritic cells and B-cell Chronic Lymphocytic Leukaemia B-cells generated both Major Histocompatibility Complex Class I and Class II restricted cytotoxicity to B-cell Chronic Lymphocytic Leukaemia B-cell targets. When B-cell lysates were analysed using reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis, a B-cell Chronic Lymphocytic Leukaemia specific band at 42,000 Dalton and other patient specific bands were observed. Only the 65,000 Dalton and 42,000 Dalton bands were capable of stimulating comparable T cell responses as the whole lysate. The 65,000 Dalton band from normal healthy volunteers showed a dominant peptide that closely matched Human Serum Albumin. The 42,000 Dalton band from B-cell Chronic Lymphocytic Leukaemia patients showed a possible match with Human Actin.

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LIST OF ABBREVIATIONS

B-CLL =B-cell chronic lymphocytic leukaemia
MHC= major histocompatibility
TPA= 12-o-tetradecanoyl-phorbol-13-acetate
$DiOC_{18}$=3,3'-dioctadecyloxacarbocyanine
VEGF= vascular endothelial growth factor
TRAF= tumour necrosis factor-associated factor
ITAM= immunoreceptor tyrosine activation motifs
ICAM= Intercellular adhesion molecule
V(D)J= variable diversity joining recombination
ELISA= Enzyme linked immunoglobulin sandwich assay
ALL= acute lymphoblastic leukaemia
RPMI= Roswell Park memorial institute
cAMP= cyclic adenosine monophosphate
EBV= Epstein-Barr virus
FISH= Fluorescence in situ hybridisation
RBI= Retinoblastoma suppressor gene
ATM= Ataxia telangeictasia mutated
FDX= Ferredoxin
CTL= cytotoxic/cytolytic T lymphocyte
CLIP= class II associated Ii protein
GILT= gamma-interferon-inducible lysosomal thiol reductase
BLOTTO= bovine lacto transfer technique optimiser
GATA= guanine, adenine, thymidine, adenine containing
CTLA= cytotoxic T lymphocyte-associated antigen
STAT= signal transducer and activator of transcription
TAP= transporters associated with antigen processing
TAA= tumour associated antigen
MAGE= melanoma-associated encoding gene
CEA= carcino-embryonic antigen
CML= chronic myeloid leukaemia
GM-CSF= granulocyte macrophage-colony stimulating factor
SDS-PAGE= sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris= Tris(hydroxymethyl)aminomethane
HTLV= human T cell leukaemia virus
GVL= graft-versus-leukaemia
GVHD= graft-versus-host-disease
ADCC= antibody dependant cell mediated cytotoxicity
EDTA=Ethylenediaminetetraacetic acid
TMB= 3,5,3'5'-tetramethylbenzidine
HBSS= Hanks balanced salt solution
HCL= hydrochloric acid
PVDF= polyvinylidene difluoride
DNA= Deoxyribonucleotide acid
NHL= non-Hodgkin's lymphoma
TIL= tumour infiltrating lymphocytes
LAK= lymphokine-activated killer
TGF= transforming growth factor
PBMC= peripheral blood mononuclear cell
PBS= phosphate buffered saline
HCL= hairy cell leukaemia
BCR= B cell receptor
FL= Follicular lymphoma
BSA= bovine serum albumin
PPD= purified protein derivative
PHA= phytohemagglutinin
MLR= mixed lymphocyte reaction
TNF= tumour necrosis factor
PLL= pro-lymphocytic leukaemia
CD= cell surface designation
TBS= Tris buffered saline
ANOVA= analysis of variance
OPA= o-phthalaldehyde
TCRBV= T cell receptor B variable
DC= dendritic cell
HSP= heat shock protein
HLA= human leukocyte antigens
ER= endoplasmic reticulum
APC= Antigen presenting cell
LFA= leukocyte functional antigen
AEP= asparaginyl endopeptidase
R= receptor
Ig = Immunoglobulin
Th1= Type 1 T helper
Th2= Type 2 T helper
IL= Interleukin
IFN= interferon
LPS= Lipopolysaccharide
T-bet= T box transcription factor
TCR= T cell receptor
Cat= cathepsin
NK= natural killer
RDX= radixin
L= Ligand
kDa= Kilo Dalt

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Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award. All the work presented in this study was performed by the author.

Signed: Ruth Goddard

Date: 18/9/2002

Publications

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RV Goddard, S Lewis, AG Prentice, ER Kaminski (1999). Use of autologous lysate-pulsed DCs to stimulate T-cell responses in patients with B-cell chronic lymphocytic leukaemia. British Journal of Haematology:**105 supple 1**, 84.

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RV Goddard, ER Kaminski, JA Copplestone, AG Prentice (2001). Anti-tumour responses are stimulated by components of B-cells chronic lymphocytic leukaemia cell lysates. Experimental Hematology: **29 supplement 1**, 16.

Oral presentations

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- 4th Congress of European Haematology Association, Barcelona, June 1999
- 41st Annual American Society of Haematology meeting, New Orleans, December 1999.
- 40th Annual British Society of Haematology meeting, Bournemouth, March 2000.
- 2nd Edward Jenner Institute for Vaccine Research Conference, Oxford, September 2000.
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1. INTRODUCTION

1 The Immune system

The immune system is divided into the innate and the adaptive compartments. The innate immune system is the first line of defence of the human body against pathogens. If an infection is able to overpower these defences then the adaptive immune system is activated. The adaptive immune system is capable of producing a specific reaction to the pathogen and maintaining an immunological memory of that pathogen so that a more effective response can be generated upon re-infection. The most important cells of the immune system are the white blood cells or leukocytes. Leukocytes consist of either cells which form a major part of the innate immune system (natural killer cells, neutrophils, polymorphs, monocytes, and macrophages) or lymphocytes (T and B cells) which mediate the adaptive immune response. The innate system and the adaptive system do not act in isolation of each other. Antigen uptake, processing and presentation is one of the main ways for phagocytes to activate lymphocytes. Once activated the lymphocytes secrete either lymphokines or antibodies, which in turn aid the phagocytes to destroy the infectious agent more effectively.

1.2 B lymphocytes (B-cells)

B-lymphocytes possess three functions: (i) antigen presentation by presenting peptides upon the numerous Major Histocompatibility Complex (MHC) class II molecules on their cell surface, (ii) antibody recognition of foreign proteins and (iii) by effector cell

function through antibody production. Some but not all B-cells will express the following markers; cell surface designation (CD)1, CD4, CD5, CD6, CD9, CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD38, CD40, CD53, CD54, CD72, CD73, CD74, CD79, CD80, CD81, CD82, CD83, CDw84, CD86, CD138, CD139, CD150, CD178, CD179, CD180, CD229[11].

1.2.1 B-cell development

B-cells initially develop from lymphoid stem cells in the haemopoietic tissue of the foetal liver. However, during the neonatal period the production of B-cells moves to the bone marrow where it continues in adult life. B progenitor cells express markers such as CD10, CD19, CD38 and MHC class II. After re-arrangements of the heavy chain gene pre-B cells emerge expressing μ heavy chains in the cytoplasm and CD20 on the cell surface and CD10 expression is lost. Proliferating pre-B cells give rise to smaller pre-B cells. Light chain gene re-arrangement occurs and the resulting immature B-cell expresses assembled surface immunoglobulin and CD21. Loss of CD38 expression then occurs. The resulting mature B-cell can be stimulated to secrete soluble immunoglobulin (antibody) by clonal selection driven by the presence of a pathogen's antigen within the body. However, B cells which bind cell-associated self antigens can undergo apoptosis by clonal deletion or undergo receptor editing and further recombine Ig genes. Initially the antibody will be an IgM type. However in further response to the antigen, the type of immunoglobulin changes (class switching) and B-cells of that clone become either plasma cells or memory B-cells. Although the structure of the antibody changes the antigen specificity rarely does. Plasma cells regain CD38 expression and show a new marker, PCA-1, on their cell surface [12].

1.2.2 Antibody production

B-cells' definitive feature is their ability to produce immunoglobulins which, with the

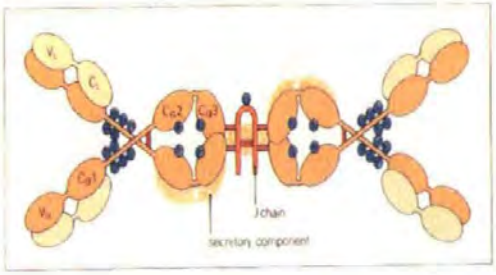
addition of transmembrane section and cytoplasmic tail, can act as unique antigen receptors called B cell receptors (BCR). The immunoglobulin (Ig) molecule is made up of four protein chains, two light chains and two heavy chains (Figure 1). Each chain consists of a variable region at the amino-terminal end referred to as V_L and V_H. Differences in the variable regions create a plethora of antigen specificities (idiotypic) enabling the B cells to recognise many pathogen antigens. The remaining part of the molecule has a constant structure. The light chain is called the CL region. The heavy chain is further divided into 3 globular regions, C_{H1}, C_{H2}, C_{H3} stabilised by intra-chain disulphide bonds. Between C_{H1} and C_{H2}, the two heavy chains are linked by two disulphide bonds at the hinge region. This allows each variable region to bind antigen independently as it provides spatial flexibility [3].

There are five different classes of immunoglobulin (Ig) produced in humans; IgG, IgM, IgA, IgE, IgD. They vary in structure, particularly in the constant regions of the heavy chain amino acid structure. Hence each class of Ig's heavy chain is designated as γ, μ, α, ε, δ and is highly conserved within each class of Ig (isotypic). IgM is a pentameric version of the basic Ig structure with a central J chain. The two heavy chains (μ-chains) have an additional constant region ($C\mu4$) with a penultimate cysteine enabling binding to the J chain or other $C\mu4$ chains. The basic Ig structures are held together by disulphide bonds between constant heavy chain regions 3 ($C\mu3$). IgM secretion is confined to the intra-vascular pool and predominates in early responses. IgD has a high concentration of carbohydrate due to variations in amino acid sequence of the three constant δ-chains. IgD is found in human serum at low concentrations and is susceptible to proteolysis. IgE contains a fourth constant domain on the heavy chain ($C\varepsilon4$). The hinge region is organised differently with $C\varepsilon2$ placed between two disulphide bonds. The Fc portion (released by

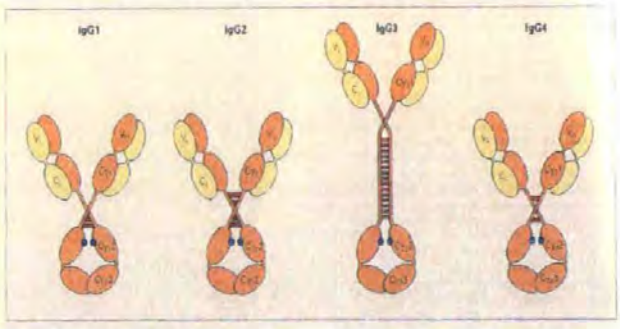
digestion with papain) of the IgE binds mast cells. IgG and IgA are further subdivided into sub-classes; IgG1, IgG2, IgG3 and IgG4 and IgGA1 and IgGA2. IgG sub-classes differ in their hinge regions by the number of inter-heavy chain bonds. IgG is the major Ig found in serum and is present equally in intra-vascular and extra-vascular pools. IgGs are the major antibodies involved in secondary responses and anti-toxin function. IgA possesses an additional C-terminal octapeptide with a penultimate cysteine residue that can bind to a J chain. IgA is the main component of secretions such as saliva, colostrum, milk, tracheobronchial and genito-urinary secretions. It is found in serum mainly in a dimeric form. Secretory IgA, found in seromucous secretions, can occur in either subclass as a dimeric form binding the secretory component by cysteine residues in $C\alpha1$ and $C\alpha2$ [4].

Figure 1: Antibody basic structures

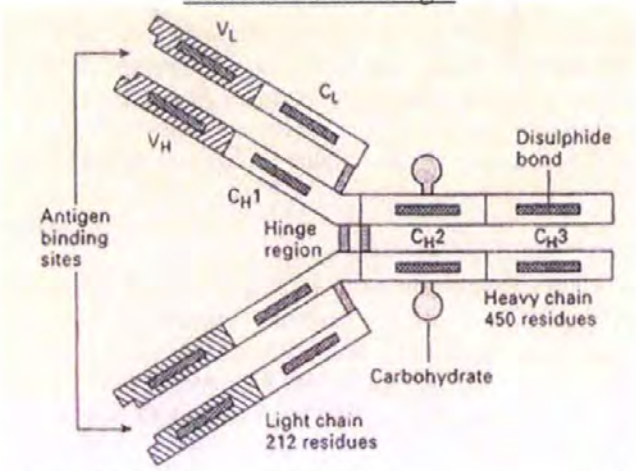
Soluble IgA



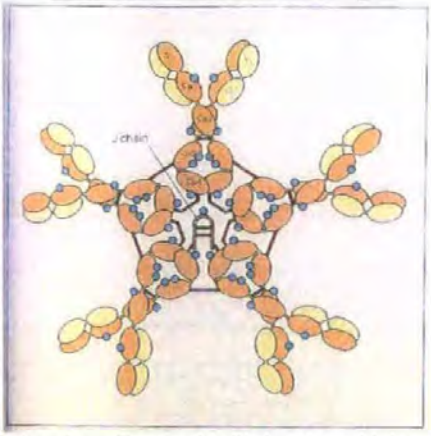
IgG sub-classes



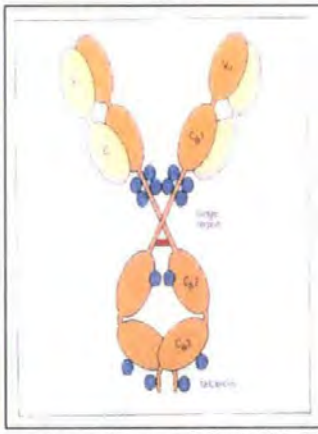
Basic structure of IgG



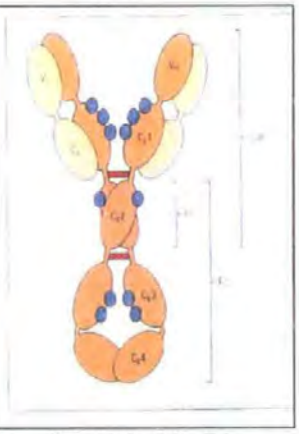
Pentameric structure of IgM



IgD



IgE



Taken from Turner M, Molecules which recognise antigen. In: Roit IM, Brostoff J, Male DK, Eds. Immunology 2nd Edn. London: Gower medical publishing. 1989.

1.2.2 Importance of T cell help

Although B-lymphocytes express receptors for lymphokines and chemokines, contact-mediated signals from T-cells are required to produce a highly effective humoral response [5]. Firstly, signalling through the BCR primes the B-cell to be more responsive to signals from T cells [6]. Engagement of MHC class II molecules stimulates early biochemical events and lead to B cell proliferation and differentiation [7]. MHC class II engagement by T-cells enhances both BCR signalling via 2 BCR co-receptors (CD19 and CD22)[8] and CD40 [9] signalling by co-localisation with cholesterol and glycosphingolipid enriched membrane microdomains [10]. Direct signalling through CD40 occurs by ligation with CD154, a trimer Tumour Necrosis Factor (TNF) family member, expressed on activated T cells. CD40 engagement can stimulate proliferation, antibody secretion, cytokine production, increased ability for antigen presentation, isotype switching, development of germinal centres and establishment of humoral memory responses [11]. These many complex roles of CD40 engagement rely on the complicated interaction of CD40 with TNF-associated factors (TRAF's) [12]. Engagement of CD134-ligand (CD134L) (OX40L) is thought to play a role in the secondary antibody response and immunoglobulin isotype switching [13]. Signalling through enhanced adhesion molecules such as intercellular adhesion molecule –1 (ICAM-1) [14], CD81 [15] and CD22 [16] have been shown to amplify activation signals from T helper cells. Engagement of CD72 by its ligand CD100 enhances B cell activation via CD40 and inhibits IgG production but not initial IgM production [17]. CD27, a tumour necrosis factor receptor (TNF-R) family member, expressed by a subset of memory B cells is ligated by CD70 expressed by T lymphocytes at the later stages of their activation [18]. CD27-CD70 interactions are particularly important for the development of antibody secreting plasma cells [19].

Due to thymic selection of T cells, self-reactive T cells are more tightly regulated than B cells. Hence any self-reactive B cells cannot become activated and produce high-affinity autoreactive antibodies without T cell help.

1.3 B-cell Chronic Lymphocytic Leukaemia (B-CLL)

B-cell chronic lymphocytic leukaemia is one of the most common types of leukaemia among adults in Europe and North America [20]. B-CLL affects twice as many males as females. The median age at diagnosis is between 65 and 70 years. B-CLL is characterised by the accumulation of a clone of malignant B-cells in lymphoid tissue, the bone marrow and the peripheral blood. The first indication of the disease is often a consistent lymphocyte count of higher than 4.5. x 10^9/L in peripheral blood samples (often taken for other clinical reasons). The phenotype of these B-cells includes the expression of pan-B-cell associated cell surface markers such as CD19, CD20, CD40, CD45RA, MHC class II DR and CD37. The B-CLL cells also express high levels of CD5, a molecule that is usually found upon the surface of T cells but also on a small subset of peripheral B-lymphocytes [21] and CD23 which are not usually expressed on other B-cell lymphomas. B-CLL cells usually express low levels of CD21, MHC class II DP, CD1c and surface immunoglobulin. B-CLL cells sometimes express CD25. However, CLL cells can be differentiated from pro-lymphocytic leukaemia (PLL), mantle cell lymphoma or hairy cell leukaemia (HCL) by the absence FMC7 and CD10 [22], from acute lymphoblastic leukaemia (ALL) and follicular lymphoma (FL) by the absence of CD22, CD35, CD79b, CD10 and from myeloma by the absence of CD38 [23].

The B cells that accumulate in CLL patients are functionally inactive as they do not respond to B cell stimuli such as lipopolysaccharide (LPS), Epstein-Barr virus (EBV) proteins and anti-IgM antibodies. CLL B cells do not act as good stimulator cells in an

allogeneic mixed lymphocyte reaction and function as poor antigen presenting cells [24]. The CLL clone persists within the periphery due to its failure to respond to apoptotic stimuli rather than increased proliferation of B-cells. In the majority of patients the CLL cells are in the quiescent G_0 phase of the cell cycle [25]. Hypomethylation of the *bcl-2* promoter region results in high levels of *bcl-2* protein in 85% of patients. *bcl-2* is one of the proteins that control the apoptosis caspase cascade. Therefore, the high expression of *bcl-2* has been linked along with other factors to the B-CLL cells' ability to resist apoptosis.

Chromosome abnormalities were originally shown in 50% of B-CLL patients. However with the adoption of fluorescence *in situ* hybridisation (FISH) this has risen to 80% of B-CLL patients. Deletion of the retinoblastoma suppressor gene (RBI) at 13q14 is one of the most common chromosome abnormalities. However, RBI has not been shown to be involved in the pathogenesis of B-CLL. Patients with only a single deletion at 13q have a better average survival than patients with other chromosome abnormalities [26]. The next most common chromosome abnormality is deletions at the 11q21-23 region disrupting the ataxia telangeictasia mutated (ATM), radixin (RDX) and FDX genes. This genetic abnormality may be responsible for familial CLL as germline mutations in the ATM gene have been identified. Deletions at 11q usually correlate with a poor prognosis for CLL patients [27]. Trisomy or partial trisomy of chromosome 12 is another common abnormal karyotype found in B-CLL. Trisomy 12 is associated with atypical cell morphology, bright expression of surface immunoglobulin, CD20 and FMC7 and absence of CD23, higher proliferative rate, advanced disease and poor prognosis [28]. Inactivation by the p53 tumour suppressor gene as a result of a mutation at chromosome17p is associated with resistance to chemotherapy. Decreased p53 function can result in an increase in B-cell proliferation and prolonged cell survival. Therefore it is unsurprising that it is closely associated with

advanced disease and a more aggressive form of the disease i.e. those patients with Richter's transformation [7]. Translocations of chromosome 14 at 32q have been observed in B-CLL. The Immunoglobulin heavy chain is encoded at 32q. However, the frequency of these translocations amongst B-CLL patients is dependent upon the laboratory technique used. The most common translocation is from 14q32 to 11q13. The immunoglobulin heavy chain and bcl-2 genes become adjacent. This translocation is more commonly found in mantle cell lymphoma. Hence high frequency of translocation 14q32 to 11q13 may be a result of misdiagnosis of mantle cell lymphoma as B-CLL [7].

B-CLL is an indolent disease but as the disease advances there is a progressive enlargement of the spleen and lymph nodes. Lymphoid cells accumulate in the bone marrow through diffuse interstitial infiltration resulting in the complete filling of the inter-trabecular space. Anaemia, thrombocytopenia and netropenia will develop due to failure of the bone marrow or because of splenic pooling or hypersplenism. Autoimmune haemolytic anaemia, thrombocytopenia or both (Evan's syndrome) can occur due to polyclonal autoantibody production by normal non-malignant B cells. Hypogammaglobulinaemia occurs due to the disordered immune function of B-CLL patients. B-CLL patients are more susceptible to infections such as *S. pneumoniae, S. aureus, E. coli,* or *Herpes zoster* and have a higher incidence of solid tumours probably also because of the disordered immune function.

Although chemotherapy can induce partial or complete remission [29], long-term disease-free survival is unusual. Therapy for CLL is given generally with palliative intent rather than curative. The patients age, quality of life and immune status all influence choice of treatment. Currently patients in the earlier stages of disease are reviewed regularly and only receive treatment when the disease progresses. For most B-CLL patients chlorambucil

(an alkylating drug) is the front line treatment. In 50% of patients chlorambucil produces a reduction in lymphocytosis, an improvement in haemoglobin and platelet count and shrinkage of lymphadenopathy and splenomegly. In these patients chlorambucil delays the rate of disease progression but does not increase overall survival. The treatment is often discontinued when lymphocyte counts have returned to normal ranges. Patients can often develop resistance to chlorambucil. Corticosteroids alone such as prednisolone are only partially effective. However, they may inhibit the infiltration of lymphocytes into the bone marrow prior to treatment with an alkylating drug. Combination therapy of alkylating drugs and steroids increase response rates to 70% but not increase overall survival. Purine analogues such as fludarabine have widened treatment options for patients no longer responsive to chlorambucil. Early results are promising as treatment with fludarabine has been shown to increase complete remission rates, overall response rates and prolong disease free-survival [130]. However no change in overall survival rates has been seen. Recent trials of bone marrow transplantation or monoclonal antibodies are promising [131]. New treatment modalities for this disease are required and trials of bcl-2 antisense have begun. In view of the fact that B-CLL is a tumour of the immune system it seems challenging and logical to attempt to harness that system to treat this disease.

1.4 T-cells

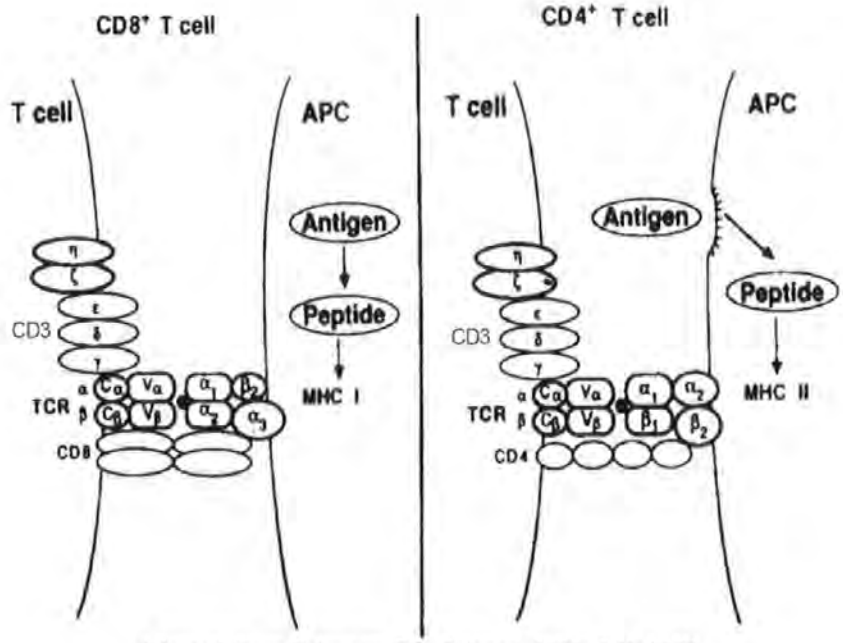
T cells are critical in developing cell-mediated immune responses.

1.4.1 T cell receptor (TCR)

There are 2 types of TCR. TCR1 consists of a γ-receptor chain encoded by chromosome 13 and a δ-receptor chain encoded by chromosome 14. TCR2 consists of a 45kDa α-receptor chain encoded by chromosome 14 and a 40kDa β-receptor chain encoded by chromosome 7 joined by a disulphide link near the cell membrane (Figure 2). Each chain contains a

variable domain and a constant domain maintained by intra-domain disulphide bonds [32]. TCR are generated by a series of somatic site-specific deoxyribonucleotide acid (DNA) recombination reactions collectively termed variable diversity joining (V(D)J) recombinations. The formation of the receptors 2 protein chains is dependent upon three recombination events; the recombination of V, D and J genes to form the variable domain of the α chain and the joining of V and J to form the variable region of the β chain. Each variable region then associates with the recombined constant domains. This generates a large repertoire of T cells which can recognise many antigens. Rearrangement is tightly regulated and TCR genes are only fully assembled in T cells [33].

Figure 2: TCR interaction with MHC



Adapted from Schwartz RH, Science. 1990; 248:1350.

The development of TCR1 positive T cells is separate from TCR2 positive T cells. TCR1 cells do not express either CD4 or CD8. TCR1 cells are capable of recognising antigens such as tetanus toxin (TT), *staphylococcal enterotoxin* A, mycobacterial antigens

and heat shock proteins (HSP). TCR 1 cells can recognise highly polymorphic antigens such as tumour idiotypes from B cell lymphoma. Recognition of antigen does not occur using the MHC system but by recognising intact antigens by HSP 70 [34].

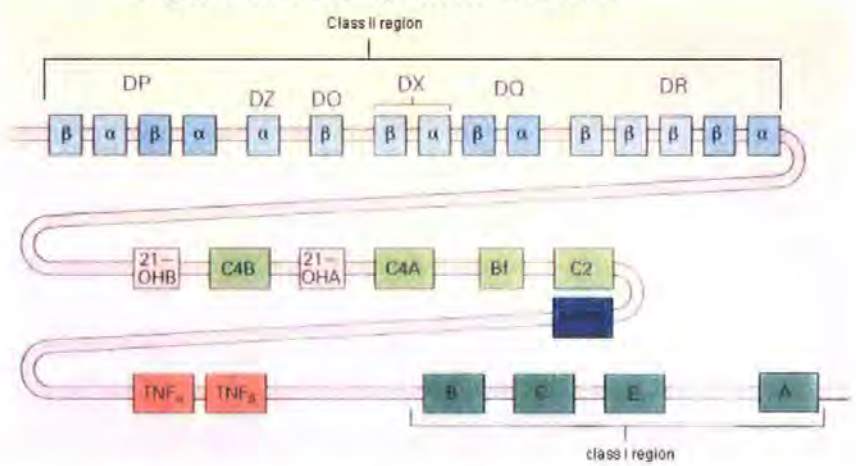
Most T cells in the periphery have the TCR2 receptor. Each heterodimer is noncovalently linked to invariant chains of the CD3 complex (CD3δ, CD3γ and CD3ε homodimer) and ζ-homodimer. Within seconds of antigen receptor cross-linking the phosphotyrosine content of Src family of non-receptor protein tyrosine kinases (PTKs) increases perhaps by autophosphorylation at the Src homology 1 (SH-1) site. In resting lymphocytes key Src PTKs are kept in poorly phosphorylated states by phosphotyrosyl phosphatases such as those found on CD45. Src PTKs phosphorylate the immunoreceptor tyrosine activation motifs (ITAMs) of the invariant chains until binding with SH2-containing proteins occurs. Zap contains two tandem SH-2 domains before the catalytic domain. Zap is recruited to ζ and ε subunits of TCR-CD3 complex by interaction with the activated ITAM's. This interaction in turns activates Zap[35].

1.4.2 Major Histocompatibility Complex (MHC)

The TCR2 molecule is responsible for the recognition of antigens presented in the context of another molecule, the human leukocyte antigen (HLA), coded for by the Major Histocompatibility complex (MHC). Three major sets of molecules are encoded within this region on chromosome 6, class I, class II and class III. MHC class II molecules are made up of two polypeptides, an α-chain and a β-chain each with two domains. Genes within the MHC class II region that encode for many genes are arranged into three groups, DP, DQ and DR and contain genes for at least one α-chain and one β-chain. MHC class I molecules are made up of an α-chain consisting of three domains and β_2-microglobulin (Figure 4). Genes within the MHC class I region encode for twenty genes including the classical

Human Leukocyte Antigens (HLA) -A, -B, -C, and -E heavy chains (Figure 3). Class III genes encode complement components involved in the cleavage of C3. Other genes reside in this region such as TNF and HSP 70 [36]. At each gene locus there are many different alleles within the human population. Since any region of A, B, C, D-related, or E may be linked together and with two non-identical chromosomes the number of haplotypes is very large. HLA haplotypes do not associate in a completely random manner. Paired specificities have been discovered with linkage disequilibrium e.g. if 16% of the population have HLA-A1 and 10% of the population have HLA-B8, then 1.6% of the population would be predicted to possess both HLA-A1 and HLA-B8 if the association was random. However, 8.8% of the population carry HLA-A1 and HLA-B8 genes [37].

Figure 3: Arrangement of MHC

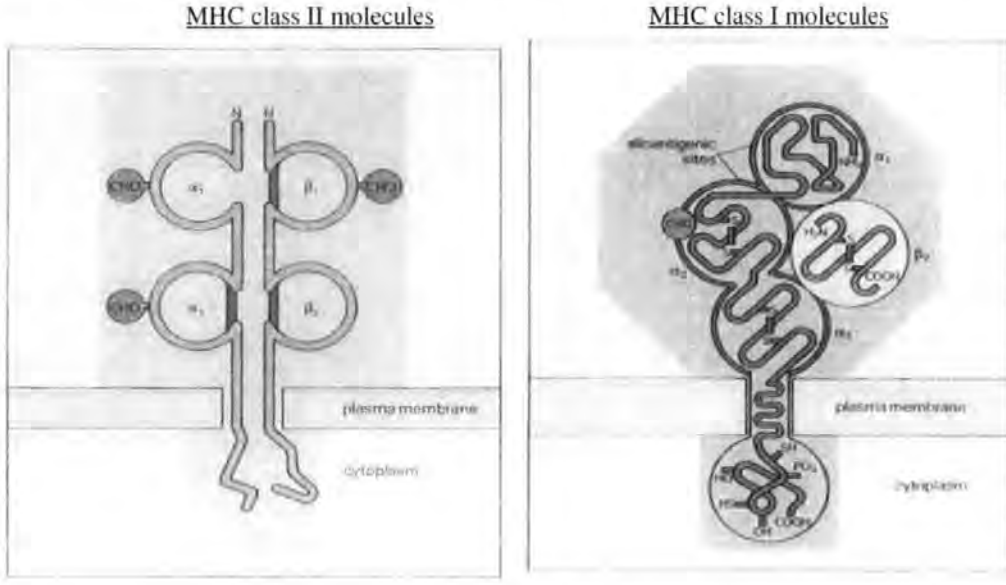


Taken from Owens M. Major Histocompatibility complex. In: Roit IM, Brostoff J, Male DK, Eds. Immunology 2nd Edn. London: Gower medical publishing. 1989. and Parham Nature. 1990; 348: 674

The crystal structure of MHC class I molecule revealed a groove on its surface constructed by 2 domains of the α chain (α1 and α2) forming the sides and a β-pleated sheet forming the floor. Along the groove there are 6 binding sites for the antigen peptide [38] which is usually 8 or 9 peptides long. Deep and highly conserved pockets at each end bind the carboxyl and amino termini by hydrogen bonding [39]. A deep polymorphic pocket

in the middle of the groove plays a major role in allele-specific peptide binding [40]. The MHC class II crystal structure has now been described and it also contains a peptide binding groove which binds peptides usually 13 or 14 peptides long [41]. Therefore MHC-binding motifs can now be predicted to bind certain HLA alleles [42].

Figure 4: Schematic representation of MHC class II and MHC class I



Taken from Roitt, Brostoff, Male. Immunology chapter 4, 2nd Ed. Gower medical publishing. 1989.

CD4 or CD8 become intimately associated with the TCR and can enhance the binding of the TCR by binding nonpolymorphic portions of MHC molecules [43] or by affecting signal transduction [44]. T cells which have CD4 on their cell surface recognise antigens by engagement with MHC class II molecules. T cells which are positive for CD8 recognise antigens by MHC class I molecules. In general, CD8 positive T cells are responsible for cell-mediated killing and CD4 positive T cells provide T cell help in the form of cytokine and chemokine secretion. There is a minority population of CD8 positive T cells involved in cytokine secretion and a CD4 positive population of T cells that can

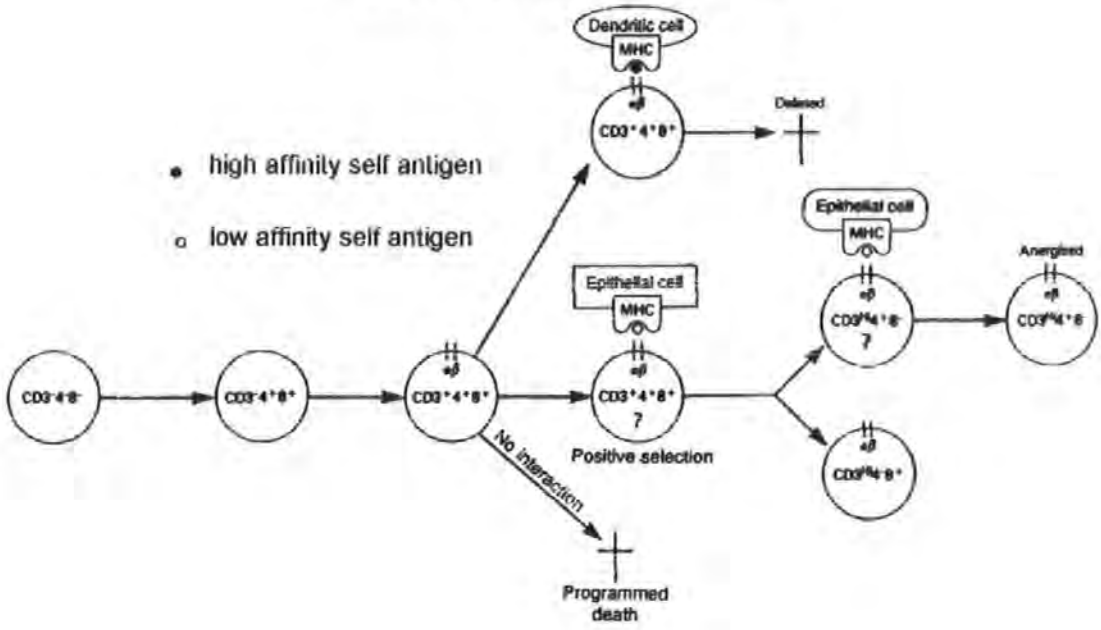
mediate cell cytotoxicity.

Peripheral T cells that traffic through lymph nodes and the vasculature are susceptible to chemokine signals to attract them and arrest their movement. T cells in this state have a 'hand-mirror' shape, with the nucleus pushed into the leading edge of the cell and the cytoplasm concentrated in a thin 'handle-like projection that lags behind as a uropod. These highly polarised T cells have Integrins, TCR and co-receptors, CD43, CD44, cytoplasmic organelles (such as endoplasmic reticulum (ER)/Golgi and microtubule-organising centre) and secretory vesicles all located in the uropod region [45]. Chemokine receptors CCR2 and CCR5 are found at the leading edge of the T cell.

1.4.3 T cell development

T lymphocytes develop in the thymus from a few progenitor stem cells which originate from the fetal liver or adult bone marrow. Initially the thymic T cells differentiate from stem cells by interaction with the thymic epithelial microenvironment. The thymocytes become organised into cortical and medullary regions within the thymus. T cell maturation occurs in three stages starting in the thymic cortex and maturing in the thymic medulla until they emerge as peripheral T cells (Figure 5).

Figure 5: T cell development



At the earliest stage, in the thymic cortex, the cells undergo gene re-arrangements of the T cell receptor (TCR) β chain. The TCR-associated complex CD3 is expressed in the cytoplasm after the gene re-arrangements[46]. The TCR2 thymocytes differentiate further and molecules such as CD1, CD4 and CD8 are expressed upon the cell surface simultaneously. It is at this stage that the T cells are educated by the thymic epithelial binding of the MHC and self-antigens. Firstly positive selection ensures that TCR receptors that bind "self MHC" haplotypes with low affinity "self antigens" on thymic cortical epithelial cells receive a positive signal for further differentiation. Thymocytes which do not bind MHC, because of errors in TCR rearrangement, undergo programmed cell death. Cells which bind "self MHC" and show high affinity binding to "self-antigens" on thymic DCs (DCs) are deleted [47]. During the final stage of maturation the mature thymocytes loose CD1 molecules from the cell surface and become either CD4 positive T cells or CD8 positive T cells [46]. Single positive cells that bind to "self-antigens" presented by thymic epithelial cells in the context of "self-MHC" are anergised [47]. Hence by negative selection

T cells with the ability to recognise foreign antigens in the context of "self MHC" travel to the periphery.

Once T cells are activated by antigen presenting cells (APCs) and become effector T cells they migrate to non-lymphoid organs and migrate to sites of infection directed by integrins and inflammation related cytokines and chemokines. T-cells with high-affinity TCRs become dominant in the primary response and are specifically selected as memory T cells [48]. A small subset of these cells may persist as memory effector T cells capable of launching an *in situ* secondary response to re-infection [49]. However, T cells that receive only a short stimulation proliferate but do not acquire effector function of cytokine secretion or cytotoxicity. These non-polarised cells retain their ability to home into the lymph nodes and become a different subset of long-term memory T cells which have no defined effector function until the establishment of secondary responses [50]. In this way T cell memory is able to establish more rapid and effective responses to a repeat infection whilst still retaining the flexibility in the nature of that response.

1.5 Antigen presentation

The mechanism for the initial formation of the T cell-APC contact is not clear but may involve villus/villus contact [51] with leukocyte functional antigen-1 (LFA-1) or CD2 mediating adhesion [52]. Once initial contact has been made a stop signal occurs to enable the T cell to resist chemokine gradients that would otherwise stimulate the T cell to continue chemotaxis [53]. The stop signal is raised intracellular calcium and this coincides with the T cell becoming a more spherical shape [54]. The nature of the calcium signal is related to the antigen encountered on the MHC[55]. T cell activation is generated at the immunological synapse, a specialised area of contact between T cells and APC [56]. Small TCR-MHC clusters form with CD4 co-clusters and strong calcium signals are recorded.

The CD3 coalescence directs clustering of TCR molecules to form the central supramolecular activating complex with a surrounding peripheral supramolecular activating complex of ICAM-LFA-1 ligand pairs[57]. Exclusion of CD45, CD43 and ICAM from the MHC-CD3 complex[58] stabilise the central supramolecular activating complex. Reorganisation of the microtubule-organising centre mediated by CD3 occurs following antigen recognition[59].

The maintenance of the central supramolecular activating complex requires co-stimulation by engagement of CD28 at this point[60]. Both CD28 and CD152 are immunoglobulin supergene family glycoproteins expressed as homodimers that play a major role in co-stimulation of T cells by signal transduction via cytoplasmic tails with tyrosine- containing motifs [61]. T cells express CD28 on the cell surface at relatively constant levels apart from small fluctuations which occur during T cell activation [61]. CD28 enhances T cell activation by increasing proliferation, cytokine secretion and T cell survival in the presence of TCR stimulation that would otherwise be insufficient for T cell proliferation[61]. CD28 also upregulates CD40L which is vital for the development of fully functional T cell effector cells[62]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (CD152) plays an inhibitory costimulatory role in regulating T cell activation. Although both CD28 and CD152 bind CD80 and CD86 on APCs, CD152 has 100-fold higher avidity for CD86 than CD28[63]. The CD152 inhibitory signal will predominate when T cells encounter antigen on APCs [64]. The majority of CD152 is localised within the peri-nuclear Golgi even at times of maximal expression, 36-48 hours after T cell activation [65]. Intracellular CD152 traffics to the cell surface at the site of the TCR-APC interface and is quickly endocytosed [65]. Hence the inhibitory effect of CD152 can be controlled by tightly restricted expression at the cell surface as it can compete with CD28 for its ligand and

downregulate a T cell response[64]. Naïve T cells do not express CD152.

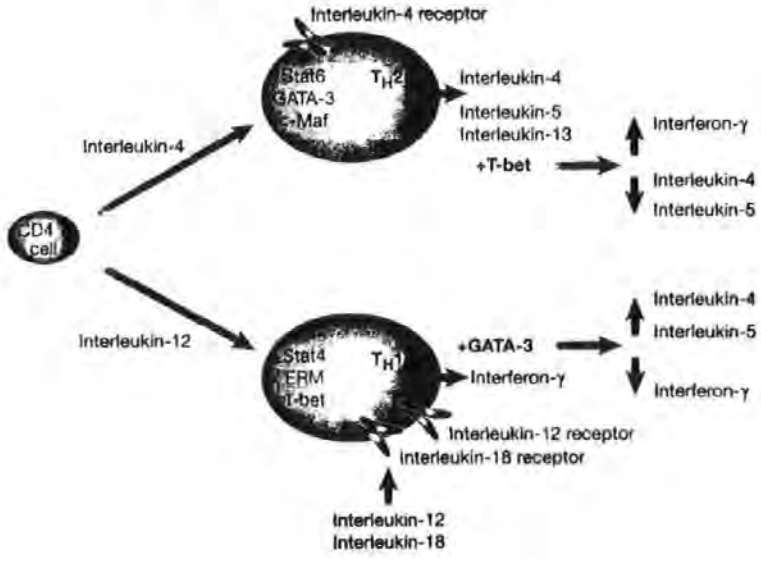
The formation of the supramolecular activating complex allows high binding avidities to be achieved by summation of a variety of independent avidities [60]. Signal transduction molecules such as protein kinase Cθ migrate to the central supramolecular activating complex [66]. Once the T-cell coupling signal has progressed, the TCR is down regulated. $CD8^+$ T cells, after stimulation, internalise class I MHC ligands and CD28[67]. Serial triggering of additional TCR by low affinity peptide-MHC complexes results in sustained signalling whilst the T cell and the APC maintain cellular contact [68]. Eventually the T cell disengages from the APC having become fully committed to effector function. However, the early T-cell-APC contact is highly dynamic as it can be disrupted by APC loaded with higher antigen concentrations[69], increased levels of T-cell cAMP, modifications of the T-cell cytoskeleton[70] or the presence of collagen[71]. Prolonged contact at lymph nodes may be facilitated by the lack of collagen.

1.6 Cytokine secretion

There are principally two phenotypes of cytokine secreting T cell. Type 1 T helper cells (Th1 cells) provide protection against intracellular pathogens such as bacteria and viruses and are also implicated in organ specific autoimmune disease [72]. Type 2 helper T cells (Th2 cells) are specialised to eradicate parasites such as flatworms and roundworms and are involved in allergic reactions [72]. One of the defining features of Th1 versus Th2 T-cells is the range of cytokines that they secrete [73]. Conversely the microenvironment of cytokines can affect the differentiation of any one particular naïve T cell into one Th type or the other [73]. Figure 6 illustrates that development of Th1 type response is driven by interleukin-12 (IL-12) by stimulating the production of interferon–γ (IFN-γ) [74]. Increased IFN-γ secretion up-regulates receptors to IL-12 and inhibits the growth of Th2 T cells [74].

Signal transducer and activator of transcription-4 (Stat-4) [75], ezrin, radixin and moesin family (ERM) [76] and T box transcription factor (T-bet)[77] are all involved in Th1 differentiation. IL-4 on the other hand induces Th2 responses by the production of IL-4, IL-5 and IL-13 through activation of Stat-6 [75], c-Maf [78] and guanine, adenine, thymine, adenine containing (GATA-3) [79]. IL-4 down-regulates the expression of IL-12 receptors and so inhibits the growth of Th1 type T cells[73]. Th1/Th2 cytokine profiles become more fixed as T cells are exposed to repeated or sustained culture conditions. However, even committed Th2 cells can be converted to Th1 cells by T-bet [77]. A sustained TCR engagement is required not only to stimulate naïve T cells but also for proliferating cells to differentiate into effector T cells especially the Th2 type [80, 81]. Long-term clones reach a point when they are fully committed to one or other Th phenotype[77].

Figure 6: Th1/Th2 T cell phenotypes



Taken from O'Garra A, Nature. 2000; 404: 720.

1.7 Cell mediated killing

Generation of antigen-specific T cells that express CD8 is thought to require two

signals. Firstly, the engagement of the TCR by the peptide-MHC class I complex and secondly, a co-stimulatory signal stimulating CD28 on the T cell by engagement of CD80 or CD86 on the APC [61]. Ligation of CD28 provokes the release of IL-2 from antigen specific $CD8^+$ T cells which is necessary for the acquisition of cytolytic function [82]. CTL generation is often dependent upon T cell help supplied by $CD4^+$ T cells. Stimulation of cytotoxicity can be triggered by a single peptide-MHC I complex. There is rapid localised calcium release at the uropodium when cytotoxic T lymphocytes encounter target cells [83].

$CD8^+$ cytotoxic T cells kill targets by the perforin pathway. When effector-target cell contact is made the Golgi apparatus is re-orientated [87]. Perforin normally stored in the cytoplasmic granules is exocytosed by the cytotoxic T cell [85]. Perforin inserts into the plasma membrane of the target cell and forms pores in the target membrane by polymerisation [84]. Polarised secretion of a family of eleven granule-stored serine proteases (granzymes) occurs [84]. Granzyme B has clearly been linked to promote DNA fragmentation by identification of substrates including pro-caspases [84]. The eleven granzymes have one of four specificities; tryptase cleaving after arginine or lysine , asp-ase (cleaving after asparagine), met-ase (cleaving after methionine or leucine) and chymase (cleaving after phenylalanine, tyrosine or tryptophan) [84]. The proteolytic activity is associated with the induction of DNA fragmentation and apoptosis. Perforin mediated cytotoxicity is common when there are high levels of IL-2 [85]. The Perforin and granzyme killing pathways' principle function is to eliminate parastized, infected cells that resist apoptosis due to pathophysiological changes. Hence it is the perforin mechanism that is linked to killing of virus-infected and tumour cells [86].

An alternative pathway mediated by Fas-FasL (CD95/CD95L) is also involved in cytotoxicity. Fas-FasL killing has a primary function of control of normal cell renewal by

inducing apoptosis of actively proliferating cells [87]. Interleukin-1 converting enzymes are activated within the target cell after attack by a cytotoxic effector cell [87]. It is well established that $CD4^+$ T cells express cytotoxic activity. Cell-mediated cytotoxicity is associated with Th1 cells. CD4-mediated target cell death is by DNA fragmentation. $CD4^+$ T cells preferentially kill their targets by Fas-FasL pathway but can also kill by the perforin pathway. $CD4^+$ T cells preferentially lyse MHC-class II cells such as APCs. $CD4^+$ T cells are capable of killing tumour cells by bystander lysis [88].

1.8 Antigen presenting cells (APC)

B cells, monocytes, macrophages and DCs are capable of presenting antigens to both B and T cells. Both B-cells and T-cells express reciprocal adhesion molecules such as ICAM-1 (CD54) and LFA-1 (CD11a-CD18) and thus, by establishing close cell-cell contact, facilitate antigen presentation and T cell activation [89]. CD40 has been shown to play a crucial role in enhanced antigen presentation by B-cells to T-cells [90]. Activated B cells can induce proliferation and differentiation of un-activated B cells by means of the CD134L-CD134 (OX40) interaction [91]. The interaction of CD137 on activated T cells and CD137L on B cells delivers important co-stimulatory signals to the T cell [92].

1.8.1 Dendritic cells (DCs)

Dendritic cells are easily identifiable under the microscope by their dendrite cellular processes around the cell lamellae. Dendritic cells vary in their differentiation state and cellular origin. The first dendritic cell to be described was the Langerhans cell. These are naïve skin DCs. Their primary function is antigen uptake and they are highly adapted to that. Langerhans cells express Lag antigens, and Langerin or Birbeck granules can easily be observed [93]. Follicular DCs do not originate from the bone marrow but retain antigen for longer periods and re-stimulate B cells within the B lymphoid follicle. In general, DCs of

myeloid origin associate with T cells within the lymph nodes to initiate proliferation and activation of T and B cells. These cells are MHC II$^+$, CD4$^+$, CD33$^{strong+}$, CD123^{dim+}, CD13$^{strong+}$, CD29$^+$, CD58$^+$, CD5$^+$, CD2$^+$ and CD86$^+$ and produce large amounts of IL-1β, IL-6, IL-12 TNF-α and IL-8 upon stimulation [94]. Thymic or lymphoid DCs have an altogether different role in the deletion of maturing T cells [95]. They possess unique markers CD8α [96] as well as CD11c$^+$, DEC205$^+$, CD11b^{dim}, 33D1$^-$ and CD4$^-$ [93]. Thymic DCs also have a mature phenotype although the maturation stimulus is unclear it is not related to pathogens or inflammatory stimuli like myeloid DCs. A further type of dendritic cell, plasmacytoid DC, was identified as the dendritic cell present at inflamed lymph nodes surrounding high endothelial venules[97]. They are distinct from monocytes and myeloid DCs in that they are CD123$^+$, CD45RA$^+$, CD4$^+$, IL-3R$^+$ and CD11c$^-$ and CD1a$^-$ and present in low levels in peripheral blood[98]. When cultured *in vitro* in the presence of CD40L myeloid and plasmacytoid DCs mature into DC1 and DC2 which selectively trigger Th1 and Th2 responses respectively[99]. Although the degree to which the responses are polarised is also dependent upon the maturation state of the DC and the DC: T-cell ratio[100]. Thymic and plasmacytoid DCs appear to share a common lymphoid precursor which is CD11c$^-$, CD1a$^-$ and IL-3R$^+$ whereas, interstitial DCs and Langerhans cell derived DCs appear to share a myeloid precursor which is CD11c$^+$, CD1a$^+$ and IL-3R$^-$ [93].

Dendritic cells are a discrete population of "professional APC". They are referred to as such because they are capable of stimulating both a primary and secondary immune response. In fact DCs secrete specific chemokines such as CCL18/DC-CK1, CCL19/ELC, CCL22/MDC and CCL17/TARC that attract naïve, recently activated and memory T cells that express CCR7 and CCR4[101, 102]. They present antigens in the context of MHC class I and II and express the vital co-stimulatory molecules such as CD80, CD86 and CD40.

Immature resident DC's are present in the peripheral tissues where they are highly specialised for antigen uptake by micropinocytosis via aquaporins 3 and 7, clathrin-mediated endocytosis, specific uptake by DEC205, mannose receptor, Fc receptors, heat shock proteins by CD91 and phagocytosis by CD36 through CrkII-Dock180-Rac1 complex [103]. Immature resident DC's spontaneously migrate at a low rate to the draining lymph node. MHC class II complexes on these cells are not stably expressed on the cell surface but accumulate in lysosomes [104]. These DC's have not received danger signals and are not mature hence they do not induce effector T cell responses. These DCs have a low density of antigen and low levels of co-stimulation molecules and have been shown to stimulate regulatory T cells to secrete IL-10[105]. Once antigens and "danger signals"[106], in the form of cytokines and chemokines, have been detected, the DCs migrate to the closest draining lymph node. Monocytes are recruited from the blood and rapidly differentiate into DCs[107]. These replacement DCs are stimulated to continue antigen sampling in the periphery by the secretion of chemokines by the originally stimulated DCs[108]. During this migration the dendritic cell becomes a highly specialised antigen-presenting cell and secretes a different set of chemokines[108]. Mature DCs have high levels of MHC molecules on their cell surface but low rates of new MHC biosynthesis thus making them insensitive to new antigens [104]. Macropinocytosis and phagocytosis is down-regulated by mature DCs and this mechanism involves a small GTPase, Cdc42 [103]. *In vitro* studies have demonstrated that DCs mature in response to inflammatory stimuli such as LPS[109] and TNF-α[110] and T cell feedback signals such as CD40L[111].Once an interaction between DCs and lymphocytes has been formed the dendritic cell matures further in response to signals from the lymphocytes. Activated DCs express the lectin DC-SIGN which binds ICAM-3 and hence stabilise the DC-T cell synapse [112]. The kind of response stimulated is dependent

upon the nature of the antigen and the adjuvant properties of any microbial products, upon the receptor through which DC maturation agents signal, upon the origin of the responding DC subset, upon the microenvironment in which the antigen is encountered and upon the cytokines released by neighbouring cells[93]. The evolution of an immunodominant T cell is due to interclonal competition for APCs. The competition has been decreased experimentally by increasing the number of available antigen loaded DCs [113]. Hence a more common pathogenic antigen will stimulate a broader spectrum of T cells. Eventually the DCs will die. Interaction between DCs and naïve $CD4^+$ T cells results in the DCs' disappearance from the lymph node[114].

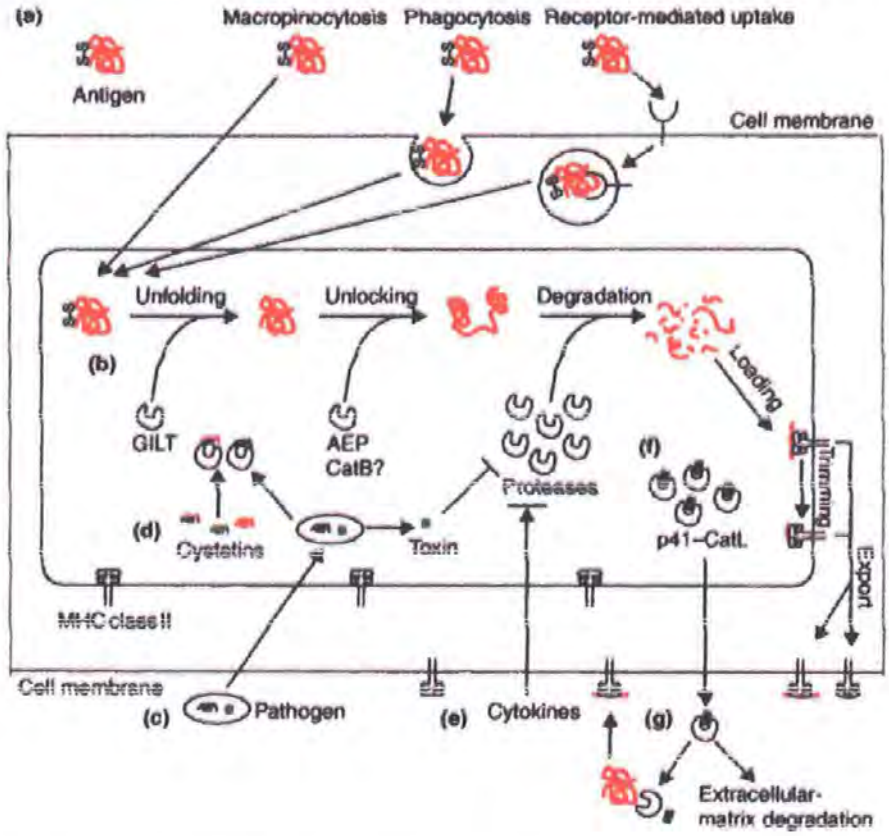
1.9 Antigen processing

1.9.1 MHC class II antigen processing

Exogenous proteins are taken up into APCs by phagocytosis, by fluid-phase endocytosis or by specific receptors such as the mannose receptor [115]. Such receptors target and concentrate the antigen into the intracellular compartments for processing and interaction with class II molecules and hence can recognise rare antigens [116]. The invariant chain Ii directs class II molecules into the endocytic pathway and protects them from binding peptides before reaching the endosomal compartment (as illustrated in Figure 7). The Ii is proteolysed in a sequential multi-step process to form a class II associated Ii peptide (CLIP) which is replaced by peptide antigen [116]. Exogenous antigen is unfolded by exposure to acidic pH. Unfolding is also aided by γ-IFN-inducible lysosomal thiol reductase (GILT) which reduces the inter-molecular and intra-molecular disulphide bonds and tertiary protein structure [117]. Antigen is unlocked by initial cleavage by endopeptidases and is attributed to the cysteinyl protease asparaginyl endopeptidase (AEP). AEP has been shown to be essential in proteolysis of carboxy-terminal domain of tetanus toxin antigen in order to

order to produce antigenic peptides associated with tetanus toxin T cell responses [118]. Specific exopeptidases further degrade the protein into peptides by trimming protein residues from the ragged amino or carboxyl termini. The endocytic proteases most commonly found in APCs are the aspartic protease cathepsins (Cat) D,B,F,H,L,S,Z and AEP [119]. These proteases are synthesised in the endoplasmic reticulum (ER) as proenzymes with a propiece that occupies the active site. The propiece is removed by autocatalytic or paracatalytic enzyme action and the mature active form of the enzyme is generated [120]. The final step of Ii proteolysis was mediated by Cat S in B cells and DCs whereas Cat L performed this final cleavage in cortical thymic epithelial cells [116]. Once Ii proteolysis is complete the peptide is loaded onto the MHC class II molecule. Long polypeptides can bind with the immunogenic epitope anchored in the MHC groove. The T cell epitope is thus protected whilst the peptide is trimmed. Factors which influence antigens presented by class II molecules can involve the antigen, its glycosylation state, its interaction with internalisation receptors and its early binding of MHC class II molecules [121]. Other factors which regulate the class II restricted peptides are the cathepsin genes expressed by each APC e.g. Cat S, Cat F and Cat Z are found in bone-marrow derived APCs (B cells, DCs and macrophages) whereas Cat L is found in macrophages and cortical thymic epithelial cells [122]. The strength of proteolysis in the endocytic compartments is regulated by factors such as cytokines (IFN-γ, IL-6, TNF-α, IL-1b and IL-10), pathogen products (Bm-CPI-2 secreted by the nematode *Brugia malayi* and toxin secreted by *Helicobacter pylori*) and endogenous competitive inhibitors (propiece of the enzyme, cystatin inhibitor family and p41) [116].

Figure 7: Classical MHC class II antigen presentation pathway



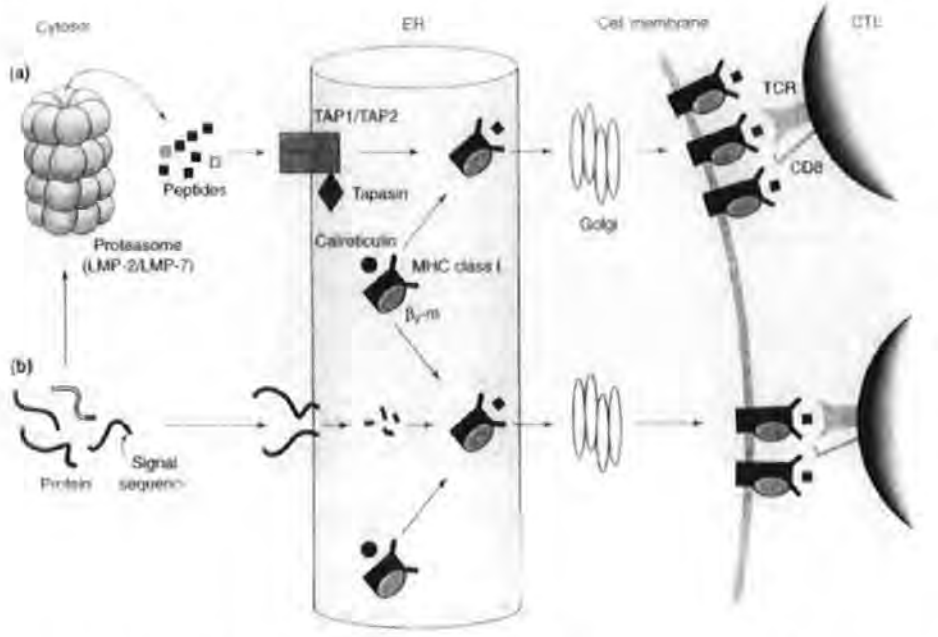
Taken from Lennon-Dumenil, et al. Curr Opin Immunol. 2002; 14: 15-21.

1.9.2 MHC class I antigen processing

MHC class I pathway (Figure 8) is involved in antigen presentation of endogenously synthesised proteins to cytotoxic T lymphocytes (CTLs). Antigenic peptide fragments are generated by the multicatalytic proteasome complex. The 20S proteasome core is barrel shaped and composed of four stacked rings of seven subunits each with two outer α rings and two inner β rings. The cleavage of peptide bonds is performed by β1, β2 and β5 with their active face inside the proteasome lumen. Assess to the catalytic lumen is controlled by 19S cap made up of fifteen different sub-units. IFN-γ treatment stimulates the replacement of β1, β2 and β5 with subunits lymphoblastoid type of latency-2 (LMP-2), MACL1 and LMP-7 to form the immunoproteasome and thus changing the cleavage preferences [123]. The peptides are shuttled into the ER by a nucleotide triphosphate (NTP)-dependent

heterodimeric complex composed of the transporters associated with antigen processing (TAP), TAP1 and TAP2 [124, 125]. The TAP1/TAP2 complex selects peptides of a distinct length (8-10 amino acids) and according to the binding affinities of the 3 N-terminal and C-terminal end amino acid peptide residues [126, 127, 128, 129]. The expression of TAP1 and TAP2 can be increased by IFN-γ stimulation [130]. Peptides can also be processed by the TAP independent pathway which involves proteolysis of hydrophobic signal peptides from proteins translocated directly into the ER [131, 132]. TAP is physically associated with MHC class I molecules and mediates the loading of peptides into the MHC class I binding cleft either directly or via chaperone molecules such as tapasin [133,134]. Export of the MHC class I –β_2-microglobulin-peptide complex is regulated by the chaperone molecule calreticulin. The final trimeric complex of MHC class I heavy chain, β_2-microglobulin and peptide is transported to the cell surface by the trans-golgi complex [135, 136, 137]. At the cell surface the peptide is presented in the context of a specific MHC class I molecule and interacts with the clonotypic T-cell receptor.

Figure 8: Classical MHC class I antigen presentation pathway



based upon Figure 1 from Seliger B, Maeurer MJ, Soldano Ferrone, Immunology Today. 1997; 18:293.

1.9.3 Cross-priming

The term cross-priming was first described by Bevan[138] and describes the ability of host APCs to prime cytotoxic T lymphocyte responses against minor histocompatibility antigens captured from foreign donor cells. It is primarily used to describe the uptake and re-presentation of cell associated antigens in the class I pathway [139] but has more recently been applied to the class II pathway as well [140]. Cross-presentation has been shown to be involved in tolerogenic responses (cross-tolerance) by inducing tolerance to parenchymal antigens [140, 141] and immunogenic responses (cross-priming) by inducing CTLs to tumours [142, 143], grafts [144] and DNA-encoded antigens [145]. The cell type responsible for cross-presentation of antigens has not been isolated *in vitro* it has been found that macrophages [146, 147], DCs [148] and B cells [149] can all be induced to cross-present exogenous antigens of high concentration. A possible candidate for the cell responsible for cross-presentation was isolated from a genetically modified tumour model and this cell bore characteristics of both DCs and macrophages [150]. Dendritic cells have been shown to be able to capture apoptotic cells and cross-present class-I-restricted antigens whereas macrophages could capture apoptotic cells but not cross-present the antigens [148]. Dendritic cells express $\alpha v\beta 3$ integrin which may play an important role in this process [151].

Several heat-shock proteins, HSP70 [152], HSP90 and gp96 [153] have been shown to immunise CTL responses that are specific for antigens derived from donor cells. The HSPs act as chaperones, carrying precursors of cellular peptide antigens that can be presented by any MHC haplotype . However, proteins such as gp96 can be absent and cross-presentation will still occur [154].

For CTL development by cross-presentation, antigen has to be cross-presented by

the same cell to $CD8^+$ and $CD4^+$ T helper cells[155]. This implies either a three cell cluster enabling short range soluble signals such as IL-2 or a sequential interaction with the $CD4^+$ T cell which modifies the APC enabling it to stimulate the $CD8^+$ effector cell. CD40 has arisen as one of the molecules that could mediate this sequential stimulation[156]. Other signals provided by virus infection can transform the APC into a CTL priming cell [157]. Hence some CTL responses require $CD4^+$ help and others do not.

1.10 Tumour antigens

Several human (MHC) HLA class I-restricted tumour-associated antigens (TAAs) have been isolated. They can be segregated into the following groups.

a) Oncofetal antigens

TAAs encoded by normal, non-mutated genes may be persistently expressed by tumour cells and encode differentiation antigens associated with an earlier fetal stage which are silent in normal adult cells [158]. Of the twelve members of the melanoma antigen-encoding gene (MAGE) family six are expressed in melanoma, head and neck cancer, non-small cell lung cancers and bladder carcinomas[159, 160]. MAGE-1 expressed on testis is not presented in terms of MHC class I [158]. Therefore it has potential as MHC class I specific cytotoxic T cells will be unable to recognise it on the testis. Other oncofetal antigens include BAGE [161], GAGE[162] in melanoma, α-fetaprotein in hepatic carcinoma[163], Carcino-embryonic antigen (CEA) in colonic cancer [168].

b) Tissue-specific differentiation antigens

These are non-mutated antigens specifically expressed by a specific cell type whose expression levels may change in individuals with cancer. Melanoma antigens from melanocytes such as tyrosinase [164], gp100 [165], Mart-1/melan A, [166] were some of the

first antigens to be described. The membrane tyrosinase kinase receptor her-2/neu normally expressed in epithelia has been associated with breast and ovary cancer [158].

c) Neoepitopes

These antigens are generated by point mutations in ubiquitously expressed genes such as CDK4, β-catenin [167] and MUM-1/L33-B.

d) Point mutations in oncogenes

Point mutations in oncogenes are usually found in carcinogen induced tumours such as the tumour suppressor gene p53 or K-Ras codon 12, 13 or 61. p53 mutations have been associated with human colorectal cancer and human lung carcinomas [168]. K-Ras has been associated with patients with gastrointestinal cancer [158].

e) Recombined proteins

Fusions between 2 proteins results in production of new antigen such as the bcr/abl fusion gene found in CML patients.

f) Viral epitopes

After infection with certain viruses, genes of the virus express cellular oncogenes which cause growth and uncontrolled cellular division hence resulting in malignant transformation. Viruses associated with cancer such as Epstein-Barr Virus (EBV) in lymphomas, Human T cell leukaemia virus-1 (HTLV-1), Hepatitis B virus in hepatocellular cancer and papilloma virus in cervical cancer all express viral epitopes [158].

g) Idiotypic epitopes

The malignant B cell in B cell lymphomas secrete immunoglobulins of the same idiotype which can act as antigens. The malignant T cell clone in T cell lymphoma all bear TCR of the same idiotype [158].

h) Mucin rich epitopes

Tn is a blood group-related carbohydrate epitope consisting of a-N-acetyl-galactosamine linked to the hydroxyl group of serine or threonine in glycoproteins. TAG-72 is a common glycoprotein marker that results from the addition of sialic acid on to Tn and is found in gastrointestinal, breast and ovarian carcinomas. Thomse-Friedenreich antigen (T-antigen) is generated by the addition of β-linked galactose to the Tn and is found on epithelial tumours particularly colon cancer. Normally the expression of these antigens is masked by the additional terminal sugar moiety [168]. MUC1 gene mucin is found in breast, pancreatic and ovarian cancer. A mucin is encoded that is heavily glycosylated in normal cells and exposed in malignant cells revealing a glycoprotein which is antigenic [169].

1.11 Recognition and presentation of tumour antigens

Cell surface expression of MHC class I molecules is reduced on human tumours [170. 171]. Loss of functional LMP-2, LMP-7, TAP1 and TAP 2 has been shown, in tumour cell lines from small-cell lung carcinoma [172], Burkitts lymphoma [173] and hepatocellular carcinoma [174] and in surgically removed malignant tumours [175], to correlate with low surface expression of MHC class I. However, in some human prostate carcinoma cell lines, despite high cell-surface expression of MHC class I, there are low levels of TAP2 mRNA [176]. Down-regulation of TAP molecules, impairing the assembly of MHC class I molecules in the ER, resulted in low expression and stability of tumour antigen bearing complexes on tumour membranes. TAP defects therefore offer an explanation of how tumour cells may escape MHC class I restricted CTL-mediated recognition [177]. Some tumour epitopes are processed more efficiently such as MAGE-A3 by the immunoproteasomes and others are not such as Tyrosinase, gp100, Melan-A. Cells unable to catabolise the tumour proteins to

expose MHC class I binding epitopes do not express these epitopes on the cell surface and so evade reactive T cells to those epiotopes [123]. Cells with TAP abnormalities show enhanced lysis by natural killer (NK) cells. Therefore, TAP-independent loading of antigens should still occur. Other methods of antigen presentation such as MHC class II antigens, nonpolymorphic CD1 molecules, minor histocompatibility antigens such as TL and heat shock proteins may be the remaining anti-tumour immune surveillance mechanisms.

1.12 Immunotherapy

Early attempts at cancer immunotherapy were relatively crude, such as injecting patients with killed tumour cells or adjuvants such as *Bacillus Calmette Guerin* [178] and *Corynebacterium parvum*. That the immune system had the potential to eradicate residual leukaemia became apparent during allogeneic bone marrow transplantation. The graft-versus-leukaemia (GVL) effect of the incoming donor immune system was a consequence of graft-versus-host disease (GVHD) [179, 180, 181]. GVL is based upon direct donor T cell recognition of the recipient's allogeneic MHC molecules. This would also encompass recognition of recipient 'self' peptide sequences held within the recipient's allogeneic MHC molecules. Amongst the donor T cells stimulated by allogeneic differences in MHC would be a small proportion of T cells able to recognise antigens on the tumour cells. $CD4^+$ T cell allo-recognition of MHC class II molecules would provide T cell help for responses to tumour peptides which had previously been inhibited due to lack of presentation of tumour antigens in the context of MHC class II. The background cytokine profile generated by a large anti-MHC response would influence cells such as DCs and natural killer cells too [182]. T cell depletion of the donor bone marrow has decreased GVHD but also increased the relapse rate and engraftment failure of HLA-matched allogeneic bone transplantation.

Donor lymphocyte infusions have now become a more effective treatment for patients after allogeneic bone marrow transplantation in CML, but not so for other haematological malignancies such as AML, myelodysplasia and multiple myeloma [183]. Analysis of the T cells from successful allogeneic transplants that had received $CD8^+$ depleted donor lymphocyte infusions have found HLA class I–restricted CTLs that recognise broadly expressed minor histocompatibility antigens [184]. Reduced intensity conditioning treatments prior to transplant has also resulted in less GVHD in allogeneic MHC-matched setting in CML [185].

Initial attempts at vaccination with autologous or allogeneic tumour cells were largely unsuccessful [186]. Identification of tumour antigens such as MAGE, BAGE and GAGE advanced the prospects of vaccination for immunotherapy [187]. More tumour associated peptide sequences were determined by peptide elution [188], motif analysis [189] and anti-idiotype antibodies [190, 191, 192]. As a result anti-idiotypic antibodies became useful as therapeutic agents [193]. Peptide vaccination to target antigens such as MUC-1 showed strong responses [194]. However, peptide vaccines were susceptible to proteolytic digestion. Peptides were designed with a pan-reactive DR epitope, a CTL activating epitope and a fatty-acid moiety in order to overcome this [195]. Heat-shock proteins offered a mechanism by which peptides could be isolated and more effectively used as a vaccine. The heat-shock protein would have non-covalently bound peptides generated from the tumour cell. Vaccination with tumour derived HSP-peptide complexes has shown protective immunity in animal studies [196, 153].

The isolation of tumour associated antigens made it possible to use the humoral arm of the immune system as therapy. There are two main mechanisms by which antibodies can mediate tumour lysis. Complement–fixing antibodies bind to the tumour cell membrane

and promote the attachment of complement components that create pores in the tumour cell membrane and disrupt the cell by loss of membrane integrity. Antibody–dependent–cell-mediated cytotoxicity (ADCC) occurs when antibodies usually of the IgG class form an intercellular bridge by binding the variable region to a tumour antigen on the tumour cell and binding effector cells such as NK cells macrophages and granulocytes, by Fc receptors such as CD64, CD32 and CD16. *In vitro* and *in vivo* ADCC has been shown to be more influential in tumour lysis than complement fixation [163]. However, ADCC effector cells have shown poor infiltration of large tumour masses and loss of the tumour antigen frequently interferes with antibody therapy [163]. Anti-idiotypic responses have been generated in non-Hodgkin's lymphoma (NHL), lymphomas and B-cell neoplasms such as multiple myeloma [3]. Analysis of differentiation specific cell surface molecules lead to the development of Campath-1H (target is CD52), Rituximab (target is CD20) and Bexxar (target is CD20) which are now being used in the treatment of B-cell malignancies including B-CLL [197]. Bispecific antibodies were generated which bound target cells with one domain and effector cells with the other [198]. More effective than this was the grafting of effector cells with a chimeric receptor, consisting of a heavy and light chain variable region of a monoclonal antibody (that binds a tumour associated antigen) and an intracellular signalling domain [199, 200, 201, 202].

Adoptive cellular immunotherapy was demonstrated in animal models. Tumour specific syngeneic T cells were transferred into hosts bearing tumours. These experiments showed that complete tumour elimination required an extended presence of transferred T cells within the host. Tumour reactive lymphocytes that had infiltrated solid tumours (TIL) were isolated and expanded *ex vivo* with IL-2, tumour or both. TILs were re-infused into patients. These TIL were able to home to the tumour and mediate a significant effect in

melanoma [163]. Recently DCs are being used to generate T cell clones that could be re-infused [203].

Lymphokine-activated killer (LAK) cells have been activated *in vitro* by IL-2 treatment. They are non-MHC restricted in their tumour killing. Clinical studies with infusion of LAK cells and IL-2 induced remissions in patients with malignant melanoma and renal cell carcinoma. However, toxicity levels were very high [204, 205]. It was found that lower doses (less toxic) of IL-2 were as effective and that LAK cells did not add to the efficacy of IL-2. Systemic administration of cytokines was complicated by their widespread and varying functions. Cytokines considered for immunotherapy included IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL12, TNF-α and β, interferon (IFN) α,β, and γ and granulocyte macrophage- colony stimulating factor (GM-CSF) [3]. Systemic immunotherapy with cytokines such as IFN-α and IL-2 in renal carcinoma [206],[207] and melanoma[208] have yielded modest responses rates with low complete remission rates. IFNs have shown disappointing results in solid tumours but unexpected results in hairy cell leukaemia, Kaposi's sarcoma and as a maintenance treatment for multiple myeloma. TNF has shown to be inactive and toxic [3]. These studies indicated that cytokine therapy alone was susceptible to immunosuppression by the tumour and was best used in combination with other treatments [209].

In transforming growth factor-β (TGF-β) insensitive tumours, i.e. ones which do not have a growth response to TGF-β, neutralising TGF-β by antisense *in vitro* and *in vivo* inhibited the growth of malignant cells [210]. Irradiated tumour cells were vaccinated into a rat glioma model and found to be moderately effective. However, when the tumour cells were also modified genetically to secrete low levels of TGF-β, effector cells were generated with much higher lytic ability [211]. Antibodies neutralising TGF-β showed an increase in

NK activity in a mouse/human breast cancer model [212]. The positive effect of IL-2 on the stimulation of CTLs coupled with anti-TGF-β antibodies has shown promising results [213].

In order to ensure presentation of tumour associated antigens in the context of MHC class I molecules, vaccinia virus, fowlpox virus and adenovirus have been used as viral vectors to encode tumour associated antigen genes. Vaccinia virus encoding CEA antigen has generated antibody and cellular responses in colorectal cancer patients [214]. Anti-tumour responses have been observed after vaccination with tumour cells that have been adenovirally transduced with cytokines such as GM-CSF [215]. DNA vaccination has also been used as a vaccination method for ensuring tumour antigen entrance into the cell [216]. In B-cell lymphomas idiotype specific DNA sequences have been used to immunise patients [217]. Cytokines such as IL-12 have been combined with DNA vaccines and show enhanced responses [218]. Tumour cell loss of MHC class I antigen expression is thought to be an escape from tumour surveillance [219]. Gene transfer of tumours with HLA class I molecules has been shown to elicit clinical responses [220].

The recent marked growth in our understanding of the immune system and its interaction with malignant disease includes the mechanism by which antigen is presented to T lymphocytes [221,222]. Dendritic cells are now known to be essential for the initiation of primary immune responses and are particularly efficient at capturing and presenting antigens to naive T-cells [223, 224, 225]. By priming them *in vitro* with tumour antigens, these "professional" antigen-presenting cells (APCs) may bypass the state of inertia in which the immune system appears to co-exist with most tumours [226]. Studies of dendritic cell vaccines in both animal models and man have demonstrated the generation of anti-tumour immune responses [273, 227, 228]. Reliable methods for generating immature DCs from peripheral blood mononuclear cells have facilitated their use in immunotherapy [229,230]. *In*

vitro studies in man have demonstrated that DCs loaded with tumour antigens can induce CTL responses against melanoma [231.232.233], chronic myeloid leukaemia [234.235.236], acute myeloid leukaemia [237] and pancreatic cancer [238]. *In vivo* studies using DC loaded with tumour antigens have demonstrated encouraging clinical anti-tumour responses against; B-cell lymphoma [239], melanoma [240], myeloma [241], parathyroid carcinoma [242], prostate cancer [243. 244. 245] and renal carcinoma [246]. Tumour infiltration by DCs has been shown to be a good prognostic factor in colorectal adenocarcinomas [247], human gastric cancer [248] and papillary thyroid carcinomas [249].

2.18 Rationale for this study

It seems unlikely that an intrinsic "malignant" transformation of the $CD5^+$ B cell is solely responsible for the pathogenesis of B-CLL. The rapid death of these cells by apoptosis *in vitro* and partial reversal of this by some cytokines [250] implies that the development of this disease must be dependent upon co-operative interaction between the malignant cells and other normal components of the immune system. More recently these investigations have intensified along with the growth in understanding of the relationship between different cell types in the normal immune response, both by direct cell-cell interactions and via signalling proteins [251. 252]. From the earliest descriptions of T cell numbers and phenotype [253. 254] to recent findings of highly specific abnormalities of intrinsic T cell function and T cell-$CD5^+$ B-cell interactions [255. 256. 257], T cell dysfunction has thought to play a role in B-CLL. Hence although conventional therapies for B-CLL do offer temporary relief, the disease persists. B-CLL is essentially a malignancy of a cell of the immune system (B-cells). It seems logical that the final correction of such a disease will be held in the manipulation of the immune system. *In vivo* the interactions of B cells, T cells and APCs are so complex that we need a much greater understanding of them before

we can fully understand a disease such as B-CLL. Recent leaps forward in dendritic cell knowledge opened up the possibility of being able to crudely alter the balance between anergy and anti-tumour responses. This study evaluated whether *in vitro* B-CLL specific T-cell responses could be generated using autologous tumour cell lysate-pulsed dendritic cell. By manipulation *in vitro* of the presentation of possible B-CLL specific antigens and their subsequent identification we sought to further the understanding of the complex nature of this malignancy. This study aimed to define some parameters *in vitro* that would provide valuable insights into the correct immunotherapy approach using DCs.

2. MATERIALS AND METHODS

2.1 Patients

Local Research Ethics Committee permission and individual informed consent were obtained for these studies. A group of thirty-two patients, who were either untreated or who had not received treatment in the last 6 months, were selected for the study. Patient details are given in Table 1. Another group of ten healthy volunteers were used as a control. Protocols for isolation of cells from the blood of patients and healthy volunteers were identical. Selection of patients for specific experiments was at random.

Table 1: Patient profile

Patient	IWCLL Stage	WBC count x 10^9/L	Previous treatment
1	A/0	25	NONE
2	A/0	61.7	NONE
3	A/0	18	NONE
4	A/0	14.9	NONE
5	A/0	23.2	NONE
6	A/0 previously A/1	9.8	Chlorambucil
7	A/0 previously CIII	13.5	Chlorambucil
8	A/0 previously CIII	15	Chlorambucil
9	A/I	57	NONE
10	B/II	119.2	NONE
11	C/IV	118	NONE
12	C/IV	122	Chlorambucil and Fludarabine
13	C/IV	167	Chlorambucil and Splenic radiotherapy
14	A/0	20	NONE
15	A/I	26.4	NONE
16	B/I	16	Chlorambucil and Cyclophosphamide
17	A/0	12.1	1 course Chlorambucil
18	C/III	162	NONE
19	A/0 previously A/II	8.6	Splenic radiotherapy and Chlorambucil

Patient	IWCLL stage	WBC count x 10^9/L	Previous treatment
20	C/III	163	2 courses Chlorambucil
21	A/0	45	NONE
22	A/0	12.7	NONE
23	C/III	163	Splenic radiotherapy and Chlorambucil
24	A/0	27.7	NONE
25	C/III	90.5	Chlorambucil
26	B/I	223	Prednisolone
27	B/II	74.1	Chlorambucil and prednisolone
28	B/I	56.4	2 courses of Fludarabine, 1 x CHOP, 1 x Campath + chlorambucil + prednisolone
29	A/I	25.5	Chlorambucil
30	C/IV	98.7	Chlorambucil
31	A/0	15.6	NONE
32	A/II	26.6	NONE

2.2 Immunophenotyping.

The following monoclonal antibodies were used for immunophenotyping studies of DC and effector cells in the cytotoxic assays. CD4-FITC (Serotec, Oxford, UK), CD8-PE (Serotec), CD3-FITC (Serotec), CD16-FITC (Serotec), CD56-FITC (Serotec), HLA-DR-PE (Serotec), CD83-FITC (Immunotech, Coulter, Luton, UK), CD40-PE (Serotec), CD86-FITC (Serotec), CD14-PE/CD45-FITC (Becton Dickenson, Oxford, UK), CD11c-PE (Serotec), CD20-PE (Serotec), CD5-PE (Serotec), CD19-FITC (Serotec), CD1a-FITC (Serotec), anti-IgG1-PE and anti-IgG1-FITC (Serotec). Cells were washed twice in Phosphate buffered saline (PBS) and then twice in PBS + 0.05% Bovine serum albumin (BSA) (Sigma). Directly conjugated antibodies were added at 10µl per 10^6 cells and incubated for 15 minutes at room temperature. Cells were washed twice in PBS and twice in PBS + 0.05% BSA. Positive antibody binding was assessed in terms of gates set at 2% of

relative isotype controls using an Epics Elite flow cytometer (Coulter).

2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated using a well established single step centrifugal technique[258, 259, 260]. Equal amounts of blood and RPMI 1640 + 2mM Glutamine (Sigma, Dorset, UK) + 500U/ml Penicillin (Sigma) + 500 µg/ml Streptomycin (Sigma) were diluted. 10ml of Lymphoprep (9.1% w/v Sodium Diatrizoate and 5.7% w/v polysaccharide) (Nycomed, Robbins Scientific, UK) was added to fresh 20 ml universal (Grenier). 10ml of diluted blood was layered onto the top of the Lymphoprep. Cells were separated by centrifugation in Hereaus Labofuge 200R (Hereaus, UK) swing out rotor for 20 minutes, 2200 rev/min (540g) at 22°C. On removal a middle whitish layer was observed with red blood cells at bottom of universal and yellow serum on top. Cells in the middle whitish layer were removed into a fresh sterile universal. Cells were washed twice in 20ml RPMI 1640 + 2mM Glutamine + 500U/ml Penicillin + 500 µg/ml Streptomycin by centrifugation at 1500 rev/min (252g) for 10 minutes. Cells were resuspended in whatever medium required for culture or further separation procedures. Cells were counted in a 1/10 dilution of 0.04% Trypan blue in a Haemocytometer (Sigma) using light microscopy.

2.4 Depletion of CD19 positive cells from PBMCs

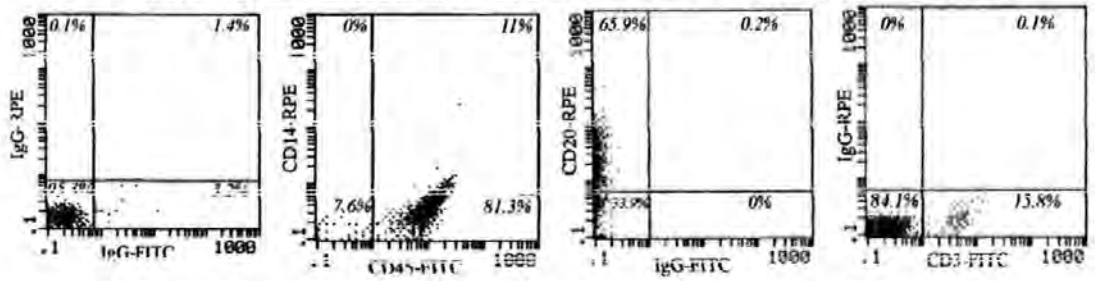
Peripheral blood mononuclear cells (PBMC) were isolated under Class II conditions by density gradient centrifugation from peripheral blood. PBMC's were counted on a haemocytometer in a 1:10 dilution of Trypan Blue (Sigma). Pan B Dynabeads (Dynal, Merseyside, UK) at 4 x 10^8/ml were aliquoted into a conical centrifuge tube so that there was a 4:1 bead to cell ratio. PBMCs were resuspended in cold PBS so that the Pan B Dynabead concentration never fell below 1 x 10^8 beads/ml. Pan B Dynabeads were washed

using magnetic separation on a MP-1 magnet (Dynal) for 2 minutes in cold PBS to remove Sodium Azide preservative. Washed Dynabeads and PBMC's were mixed and incubated whilst rotated for 45 minutes at 4°C. CD19 positive cells were selected by adherence to the tube in closest proximity to the magnet for 3 minutes. CD19 depleted cells were carefully removed with a pipette and used for the isolation of DCs.

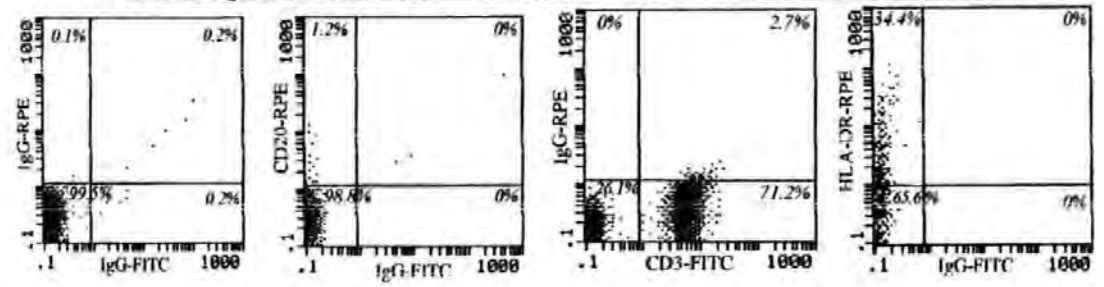
Separation of cell populations by Dynabeads was validated by analysis of cell fractions by flow cytometry (Figure 9). Of particular note is that PBMC from B-CLL patients consist of mainly of B lymphocytes ($CD20^+$) with small percentages of T cells ($CD3^+$) and monocytes ($CD14^+$) (Figure 9A). Depletion of CD19 positive cells with Dynabeads was shown to result in a mixture of cells not containing B cells ($CD20^+$) but consisting mainly of T cells ($CD3^+$) and HLA-DR positive monocyte progenitor cells (Figure 9B). The cells from the first wash on the magnet of the CD19 positive cells contained a mixture of B cells ($CD20^+$) and T cells ($CD3^+$) and so were always discarded (Figure 9C). The cells removed from CD19 positive Dynabeads using Detachabeads were over 99% $CD5^+$ B cells (Figure 9D).

Figure 9: Flow cytometry to illustrate purity of cell separation methods.

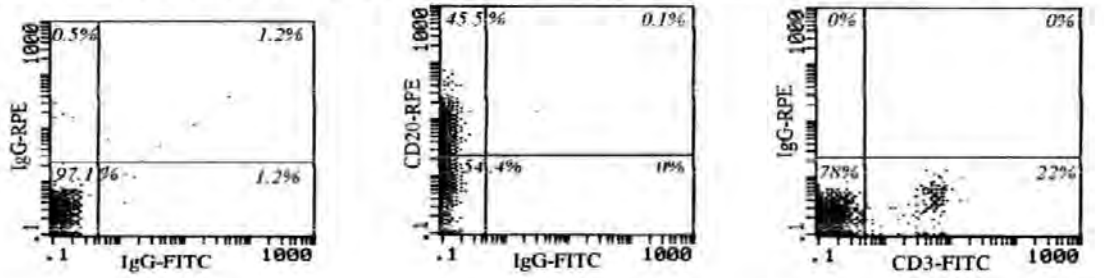
(A) Peripheral Blood Mononuclear Cells isolated using Lymphoprep.



(B) Peripheral Blood Mononuclear Cells not bound to CD19 Dynabead



(C) Peripheral Blood Mononuclear Cells collected from first wash of CD19 Dynabeads



(D) Cells removed from CD19 Dynabeads using CD19 Detachabeads

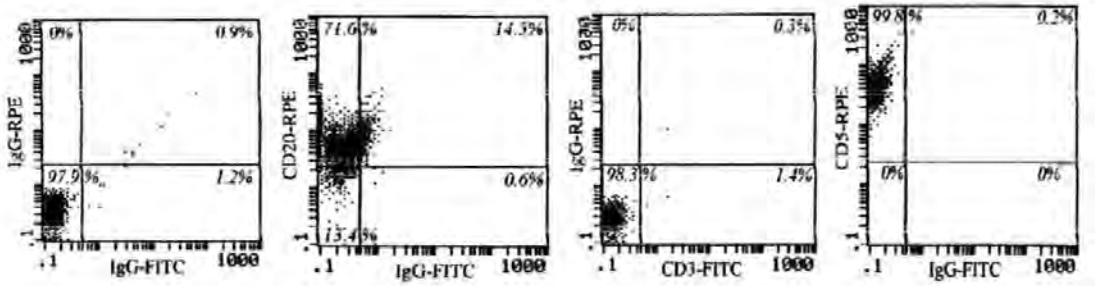


Figure 9: Cells isolated from 1 B-CLL patient (Patient 25) were stained with antibodies and analysed by flow cytometry as stated in Section 2.2.

2.5 Dendritic cell isolation and culture.

PBMC from patients with B-CLL and healthy volunteers were depleted of $CD19^+$ cells using Pan B Dynabeads (Dynal) as stated in Section 2.4. The CD19-depleted PBMC were cultured in a 24 well tissue culture plate (Gibco, Life Technologies, Paisley, UK) at 37°C in 5% CO_2 for 2 hours at a density of $1x\ 10^6$/well. Culture medium consisted of RPMI 1640 (Gibco) + 10% human AB serum + 2mM Glutamine (Sigma, Dorset, UK) + 500U/ml Penicillin (Sigma) + 500 μg/ml Streptomycin (Sigma). Non-adherent cells were removed by 4 rounds of vigorous washing that involved agitation and replacement of the culture medium with a sterile plastic Pasteur pipette. Adherent cells were then cultured in 0.5ml/well culture medium + 800 U/ml GM-CSF (Cambridge Bioscience, Cambridge, UK) + 1000 U/ml IL-4 (Cambridge Bioscience) at 37°C in 5% CO_2 for 6 days. The cultures were fed every 2 days by addition of 0.5ml/well culture medium containing IL-4 and GM-CSF. On day 6, the culture medium was removed and centrifuged. The non-adherent dendritic like-cells were resuspended in fresh culture medium with 800 U/ml GM-CSF and 100 μg/ml IL-12 (Cambridge Bioscience) replaced into the plate and cultured for 16 hours.

2.6 Removal of CD19 positive cells from Dynabeads

The Dynabeads attached to the centrifuge tube described in Section 2.4 were resuspended in 10ml RPMI + 10% AB serum. After 3 minutes incubation on MP-1 magnet weakly positive CD19 cells (first wash) were removed from supernatant with a Pasteur pipette and retained in a universal container (Greinier, UK). CD19 positive B cells attached to Pan B Dynabeads were resuspended in 1ml/4 x 10^8 Dynabeads RPMI 1640 + 10% AB serum. Pan B Detachabeads were added at 100ml/4 x 10^8 Dynabeads. Cells and beads were incubated for 1 hour at room temperature whilst undergoing rotary mixing. After 3 minutes incubation upon MP-1 magnet CD19 positive cells were removed with a

Pasteur pipette and retained in a universal container. Dynabeads were washed in this manner with 20 ml RPMI 1640 + 10% AB serum. CD19 strong and weak positive cells were centrifuged twice at 600 rpm for 3 minutes to remove any Dynabeads or Detachabeads that formed as a pellet. The resulting tissue culture supernatant was centrifuged at 1500 rev/min (252g) for 5 minutes to reveal CD19 positive cells as an opaque white pellet.

2.7 Separation of Granulocytes

Blood was separated using Lymphoprep as stated in Section 2.3. Granulocytes are found in the bottom layer with the red blood cells. This layer was decanted. Red blood cells underwent flash lysis. Cells were centrifuged at 1500 rev/min (252g) for 5 minutes to remove the intact cells in the form of a pellet. Cells remaining were classified as Granulocyte cells, resuspended in RPMI 1640 + Penicillin + Streptomycin + Glutamine + 10% AB Serum and counted using Trypan Blue.

2.8 Preparation of soluble cell lysate.

The $CD19^{+}$ B-cells from the PBMC fraction were removed from Dynabeads using Pan B Detachabeads (Dynal) as stated in section 2.4. On average B-CLL B-cells were 97% $CD5^{+}$ and 92% $CD20^{+}$. B-cells were resuspended in 2 ml of lysis buffer (10mM bicarbonate buffer pH 7.1 and 0.5mM Phenyl Methyl Sulphonyl Fluoride) (Sigma) on ice. The cells were homogenised on ice using a Dounce Homogeniser (Jencons, Leighton Buzzard, UK) and then ultrasonicated on ice using two 10 second bursts with a 15 second rest from a 50W-Vibracell (Sonics and Materials Inc, Jencons). Soluble protein was collected after ultracentrifugation at 55,000 rpm (100,000g) for 1 hour at 4°C. The protein concentration was quantified as stated in section 2.9. Soluble protein lysates were sterile filtered using 0.4μ filters (Nalgene, Marathon Laboratory Supplies, London, UK) and

stored at -70°C. All cell lysates were exposed to $CD19^+$ Dynabeads. Lysates were defined as allogeneic if they originated from a different individual to the effector T-cells. Lysates were defined as non-B-CLL if they were made from B-cells or T-cells from healthy volunteers or cell types unaffected by B-CLL such as granulocytes.

2.9 Protein determination assay

Protein concentration was quantified by the Bradford protein assay method using a protein determination kit (Biorad, Hertfordshire, UK). Duplicate BSA standards or lysate protein samples were diluted 100 µl to 100 µl in the wells of 96 well plate. Standards supplied from Biorad were diluted to 2.9mg/ml. 25 µl of Biorad colour reagent was added to each well. After a 10 minute incubation at room temperature the plate was mixed and read on a Dias spectrophotometer (Dynatech laboratories, UK). A standard curve was generated using EIA Calc software an example of which is shown in Figure 10.

Figure 10: Protein determination standard curve

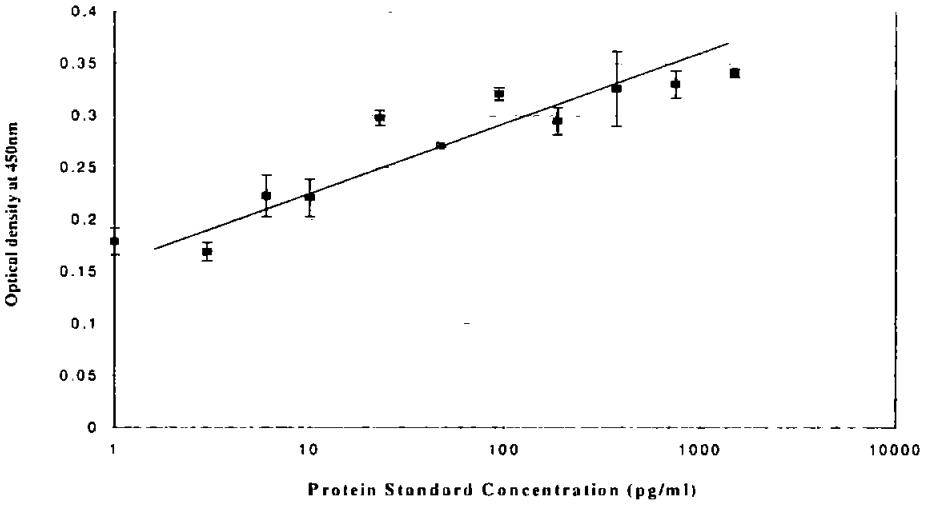


Figure 10: An example of a typical standard curve generated by dilution of BSA standards.

The software programme fits a line by regression analysis. From the equation of that line the optical density measurement of the test lysate wells is used to calculate the concentration in pg/ml. Several dilutions for each lysate were tested to ensure that the protein concentration measured in the lysate was within the optical density range of the standard curve. The calculated concentration was multiplied by its dilution factor to give a total protein concentration for the lysate. When several of the lysate dilutions were within the optical density range of the standard curve, the calculated value was multiplied by its dilution factor and an average lysate total protein concentration was determined.

2.10 Pulsing DCs with Tetanus Toxin and Tuberculin PPD

The known antigens Tetanus Toxin and Tuberculin Purified Protein Derivative (PPD) were used as positive controls to validate the proliferation and ELISA assays. Tetanus Toxin and Tuberculin PPD were added to dendritic cell culture medium at 0.4U/ml and 900U/ml respectively on day 6 of culture and incubated for a further 4 hours at room temperature.

2.11 Pulsing DCs with soluble B-cell lysate.

Dendritic cells were pulsed by the addition of soluble lysate to the culture medium at 100ng/ml per 10^6 cells and incubated for 4 hours at room temperature. Control unpulsed DCs were incubated at this time with lysis buffer except in Patient's 5,7,9,10 and 13.

2.12 T-cell isolation and T-cell cultures.

T-cells were isolated indirectly from the PBMC fraction by depleting adherent and $CD19^+$ cells as stated in Section 2.4 and 2.5. The purity of the T-cells was on average 60% when assessed by flow cytometry using anti-CD3-FITC conjugated antibodies. Lysate-pulsed or unpulsed DCs were aliquoted at a concentration of 10^3 cells per well to 96 well roundbottom tissue culture plates. T-cells were then added to give a T-cell: Dendritic cell ratio of at least 20:1. Cultures destined for cytotoxicity assays were fed with 5 U/ml of IL-2 (Cambridge Bioscience) on days 3,7,10,14 and 17. Dendritic cells in cultures destined for cytotoxicity assays were restimulated by the addition of 100 ng/ml soluble B-cell lysate or lysis buffer on days 7 and 14. Cultures were continued for a total of 21 days or 28 days at 37°C in 5% CO_2 in culture medium + 5% AB serum. Cultures used to assess cytokine secretion or T-cell activation were not fed IL-2 or restimulated with soluble lysate.

2.13 Mixed Lymphocyte Reaction

PBMC were isolated from two mismatched normal healthy volunteers as stated in Section 2.3. Stimulator cells were irradiated with a Caesium source at 30Gy. Stimulators were added to 24 well plates at 3 x 10^5/ well in RPMI 1640 + 10% human AB serum + 2mM Glutamine + 500U/ml Penicillin + 500 µg/ml Streptomycin. Stimulators to act as targets in the final cytotoxicity assay were cultured at 1 x 10^6/ well with RPMI 1640 + 10% AB serum + 2mM Glutamine + 500U/ml Penicillin + 500 µg/ml Streptomycin + Phytohemagglutinin from *Phaseolus vulgaris* (PHA) at 1 mg/ml (Sigma,UK). Responders

were resuspended in RPMI 1640 + 10% human AB serum + 2mM Glutamine + 500U/ml Penicillin + 500 µg/ml Streptomycin at 1 x 10^6/well and added to equal number of wells containing stimulators or not as the case may be. Wells not containing Stimulators were made up to the same volume as those that did, by the addition of appropriate volumes of RPMI 1640 + 10% human AB serum + 2mM Glutamine + 500U/ml Penicillin + 500 µg/ml Streptomycin. Responders and stimulator cells were cultured for 7 days at 37°C in 5% CO_2. On day 3 of culture IL-2 at 5U/ml was added to the MLR. Evidence of clumps of stimulated cells within the MLR but not in wells with responders alone were observed under 10x objective of Hund Wetzlar phase contrast inverted microscope (Wilovert, Jencons) after 7 days.

2.14 Measurement of T cell proliferation (IL-2 Receptor expression).

T-cell activation was measured quantifying cells co-expressing CD3 and CD25 (IL-2 receptor) by double-labelled flow cytometry using the same protocol as 2.2. Flow cytometry was employed using anti-CD3-FITC/anti-CD25-PE conjugated monoclonal antibody (Immuno Quality Products, Mast Diagnostics, Merseyside, UK). Positive antibody binding was assessed in terms of gates set at 2% of anti-IgG1-PE and anti-IgG1-FITC labelled cells. Anti-CD3-FITC (Serotec) and anti-CD25-PE (Serotec) conjugated monoclonal antibodies were added individually to controls to allow for adjustment of compensation.

2.15 Quantitation of cytokine secretion.

Cell-free tissue culture supernatants were harvested on days 1 to 5 and stored at -70°C until required. When convenient, the supernatants were thawed and the concentrations of IFN-γ and IL-4 measured in duplicate by ELISA (Pelkline, Eurogenetics, Hampton, UK). 96 well plates were coated overnight at room temperature by adding 100µl

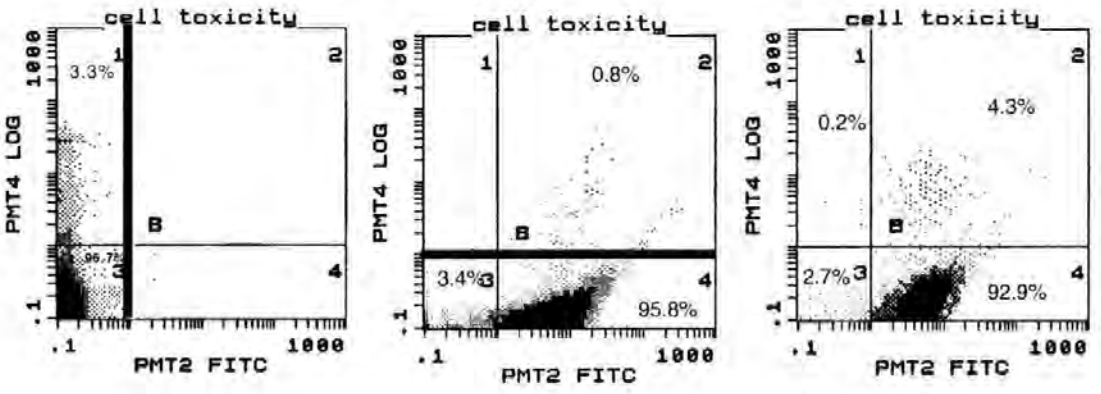
of monoclonal anti-human IFN-γ antibody diluted 1:100 in carbonate/bicarbonate buffer pH 9.6. Plates were washed with 0.2M PBS to remove unbound antibody. Non-specific binding was blocked by 200 µl kit blocking reagent added for 1 hour at room temperature. The 96 well plate was washed with kit washing buffer. 100 µl of IFN-γ standards and test supernatants were diluted in kit dilution buffer and incubated for 1 hour at room temperature. IFN-γ antibody-biotin conjugate was diluted 1:100 and 100µl added for 1hour at room temperature. The 96 well plate was washed with kit washing buffer. Strepavidin-Horseradish Peroxidase conjugate was diluted 1:10,000 and 100µl and was incubated for 30 minutes at room temperature. The 96 well plate was washed with kit washing buffer. 100µl of Substrate solution containing 0.11M acetate buffer pH 5.5 + 0.5 mg/ml 3,5,3'5'-tetramethylbenzidine (TMB) (Sigma, UK) + 0.003 % Hydrogen peroxide was added for 30 minutes in the absence of light at room temperature. The colour change reaction was stopped by addition of 100µl of 1.8 M Sulphuric acid solution. The plate was mixed and read on a Dias spectrophotometer (Dynatech laboratories, UK). A standard curve was generated using EIA Calc software from IFN-γ standards provided. Unknown sample values were calculated from that standard curve. Sensitivity limits for the assays were 2-6 pg/ml for IFN-γ and 0.2-0.4 pg/ml for IL-4.

2.16 Quantification of cell mediated cytotoxicity.

Cytotoxicity was measured by a flow cytometric method, LIVE/DEAD cell mediated cytotoxicity (Molecular Probes, Cambridge Bioscience) [2611]. Target cells were labelled with 4µl per 5 x 10^5 cells of $diOC_{18}$ for 2 hours at 37°C in 5% CO_2 and then washed twice in culture medium. Effector cells were harvested from the tissue culture and placed in flow cytometry tubes (Falcon, Marathon Laboratory Supplies) at the appropriate effector: target ratios. A minimum of 10^4 labelled targets was added. Propidium iodide was added to

each tube. Targets and effectors were gently mixed and centrifuged at 1000 rev/min (112g) for 30 seconds. Targets and effectors were incubated together for 4 hours at 37°C in 5% CO_2. Flow cytometry standard gates were set on unlabelled targets stained with propidium iodide and $diOC_{18}$ labelled targets without propidium iodide as shown in Figure 11.

Figure 11: Gate settings for cytotoxicity controls



A B C

Figure 11: (A) $diOC_{18}$ labelled targets with no propidium iodide set gates for PMT4, (B) unlabelled targets with propidium iodide set gates for PMT2 and (C) targets with no effectors show non-specific cell death.

Non-specific cell death (spontaneous apoptosis) was measured by the cytotoxicity of $diOC_{18}$ labelled targets stained with propidium iodide without effectors (11C). Cytotoxicity was expressed as the number of dead targets (cells staining positive for propidium iodide and $diOC_{18}$) divided by the total number of targets (cells staining positive for $diOC_{18}$). Percentage specific cytotoxicity was measured as follows:

% specific cytotoxicity = (total cytotoxicity - spontaneous cytotoxicity) x 100

A Mixed Lymphocyte Reaction (MLR) was used to validate this system shown in Figure 12.

Figure 12: Mixed Lymphocyte Reaction (MLR) cytotoxicity test validation

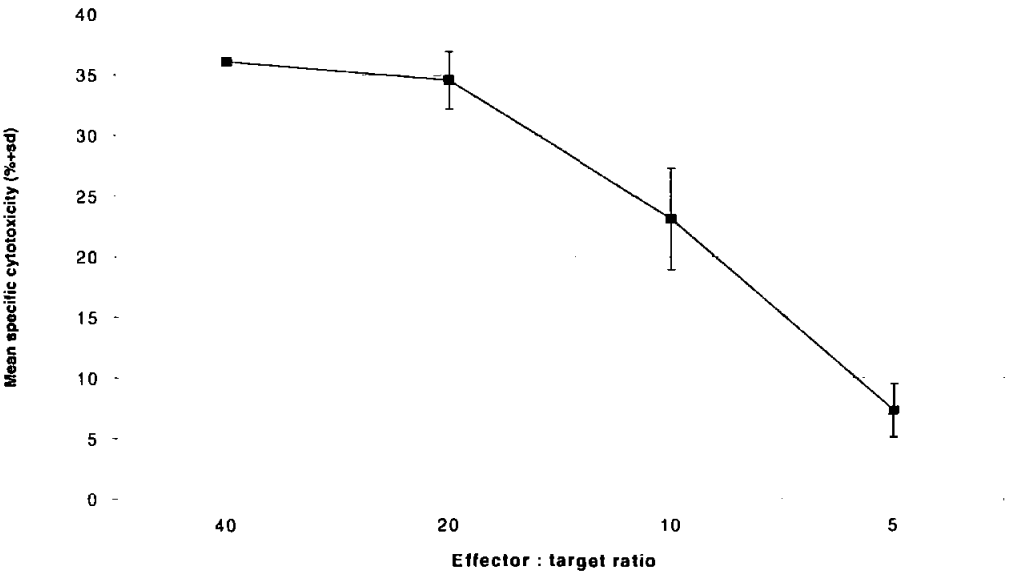


Figure 12: A MLR was cultured between 2 mismatched normal healthy volunteers as stated in Section 2.13. Cells were assessed for cytotoxicity using method outlined in Section 2.16. Results show triplicates for each effector: target ratio. Non-specific cytotoxicity was 22%.

As a control, effectors consisting of unpulsed DCs were used to detect any non-specific uptake of the $diOC_{18}$ dye from target cells by DCs. The B-CLL B-cell targets were 97% $CD5^+$and 92% $CD20^+$. The B-CLL T-cell targets showed binding for CD20-PE < 2%. Allogeneic targets were defined as those originating from another individual to the effector T-cells. Targets were defined as non-B-CLL if they were made from B-cells and T-cells from a healthy volunteer or granulocytes and T-cells from B-CLL patients.

2.16 Antibody blocking studies.

Antibody blocking experiments involved the addition of anti-HLA class I (Serotec), anti-HLA class II (DR, DP, DQ) (Serotec), anti-Pan TCR αβ (Serotec), anti-CD4 (Serotec) and anti-CD8 (Serotec) monoclonal antibodies at 100μg/ml at the commencement of the 4 hour

incubation of effectors and targets.

2.17 Electrofusion

Monocyte derived DCs were isolated as previously described. Electrofusion method was based upon findings of Scott-Taylor *et al* [262]. B-CLL B-cells and DCs were resuspended in 0.3M sodium sucrose solution at a cell density of 5 x 10^5/ml. 0.4ml of B-CLL B-cells and DCs were added to a 0.8ml electro-plated cuvette (Bio-Rad). An exponential pulse of 250V at 25 μFd with a time constant averaging between 3.4 and 4 milliseconds was delivered using Gene Pulser Transfection Apparatus (Bio-Rad). Additional B-CLL B-cells and DCs were pulsed with an exponential pulse of 500V at 25μFd with a time constant of 8.7 milliseconds. B-CLL B-cells and DCs were mixed in electro-plated cuvette but not pulsed as a control for non-specific uptake of membrane fragments. Cells were washed once in Hepes buffered saline solution. Fused cells were separated on 10% w.v. dextran solution 2100 rev/min (500g) for 5 minutes whilst some remained non-separated as a control. The layer containing hybrid cells was washed in Hanks Balanced salt solution (HBSS). Cells in the process of "round-up" were observed in the separated cultures using x20 objective of a Hund Wetzlar phase contrast inverted microscope (Wilovert, Jencons). Cells were resuspended and cultured in culture medium + 10% AB Serum + IL-4 + IL-12 and GM-CSF overnight at 37°C in 5% CO_2. The total viable cell yield was established from the count of cells in an aliquot mixed 1:10 with 0.4% Trypan Blue using a haemocytometer after 16 hours in culture. This was a more accurate estimate of long term survival of the cells as electroporation can make live cells permeable to exclusion dyes such as Trypan Blue [263]. Comparison of the total cell yield in separated and non-separated cultures gave a crude estimation of fusion efficiency. Cell yields and fusion efficiencies can be seen in Table 4. Cells were assessed for the presence of CD86

and CD20 upon the cell surface by flow cytometry after 16 hours in culture as shown in Figure 41. Due to the low yield, fused separated cells exposed to the 2 different voltages were combined and added to T-cell co-cultures.

2.18 Reducing Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was carried out based upon previous methods as stated by Sambrook, Fritsch and Maniatis [264]. Cell lysates were diluted with equal quantities of SDS-gel loading buffer containing 0.05M Tris(hydroxymethyl)aminomethane (Tris),Hydrochloric acid buffered (HCL) pH 6.8 (Sigma) + 1% v/v Glycerol (Sigma) + 2%w/v SDS (Sigma) + 0.1%w/v Bromophenol Blue (Sigma) + freshly added 0.05%v/v β-Mercaptoethanol (Sigma) and boiled for 5 minutes. Cell lysates were loaded onto Tris-HCL Ready gel containing a gradient of 4-15% polyacrylamide (Biorad, Hertfordshire, UK) with Broad Range Molecular Weight Markers (Biorad) details of which can be seen in Table 3. Electrophoresis was carried out in Protean System II Apparatus (Biorad, Hertfordshire, UK) in electrophoresis buffer containing 25mM Tris + 250mM glycine pH 8.3 + 0.1%w/v SDS at 10V for 2-3 hours supplied by an APS powerpack (Holm-Nielson). Gels were stained with 90% Methanol (BDH): distilled H_2O. 1:1 v/v + 10%v/v Glacial Acetic Acid (Sigma)+ Coomassie Brilliant Blue R250 (Biorad) overnight on a lateral shaker and destained with two changes of destain solution (90% Methanol:distilled H_2O. 1:1 v/v + 10%v/v Glacial Acetic Acid) each after 30 minutes. Gels were placed in distilled water and kept at 4 °C for 1 hour. Gels were photographed using digital camera electrophoresis documentation and analysis system 120 (Kodak, Newhaven,USA). and analysed by ID image analysis software (Kodak).

Table 2: Details of Broad Range Molecular Weight Standards

Name of protein of marker	Molecular Weight of marker (Dalton)	Reference for marker
Myosin	200,000	Woods EF, Himmelfarb S and Harrington WF. *J.Biol. Chem.* 1963; **238**: 2374.
β-galactosidase	116,250	Fowler AV and Zabin I. *Proc. Natl. Acad. Sci. USA.* 1977; **74**: 1507.
phosphorylate b	97,400	Titani K *et al. Proc. Natl. Acad. Sci. USA.* 1977; **74**: 4762.
Bovine Serum Albumin	66,200	Brown JR. *Fed Proc.* 1975;**34**: 591.
Ovalbumin	45,000	Warner RC., "Egg Proteins," in: **The Proteins**, Vol. IIA, p.435 (Neurath H. and Bailey K., eds.), Academic Press, New York (1954).
Carbonic anhydrase	31,000	Davis R.P., "Carbonic Anhydrase," in: **The Enzymes**, Vol. V, p.545, (Boyer, P. D.,ed.) Academic Press, New York (1971).
Soybean trypsin inhibitor	21,500	Wu Y.V. and Scherage H.A.,*Biochemistry*. 1962; **1**:698.
Lysozyme	14,400	Jolles P., Angew. *Chem Intl. Edit.*1969; **8**: 227
Aprotinin	6,500	Kassell B. and Laskowski M. *Biochem. Biophys. Res. Com.* 1965; **20**:463.

2.19 Native PAGE analysis

Cell lysates were diluted with 1:5 in gel loading buffer 30%w/v glycerol (Sigma) + 0.25%w/v Bromophenol Blue (Sigma). Cell lysates were loaded onto Tris-HCL Ready gel containing a gradient of 4-15% polyacrylamide (Biorad, Hertfordshire, UK) with Broad Range Molecular Weight Markers (Biorad) details of which can be seen in Table 3. Electrophoresis was carried out in Protean System II Apparatus (Biorad, Hertfordshire, UK) in electrophoresis buffer containing 45mM Tris-borate + 1mM EDTA at 10V for 2-3 hours. Gels were stained with 90% Methanol (BDH): distilled H_2O. 1:1 v/v + 10%v/v Glacial

Acetic Acid (Sigma) + Coomassie Brilliant Blue R250 (Biorad) overnight on a lateral shaker and destained with two changes of destain solution (90% Methanol:distilled H_2O. 1:1 v/v + 10%v/v Glacial Acetic Acid) each after 30 minutes. Gels were placed in distilled water and kept at 4 °C for 1 hour. Gels were photographed using digital camera electrophoresis documentation and analysis system 120 (Kodak, Newhaven,USA) and analysed by ID image analysis software (Kodak).

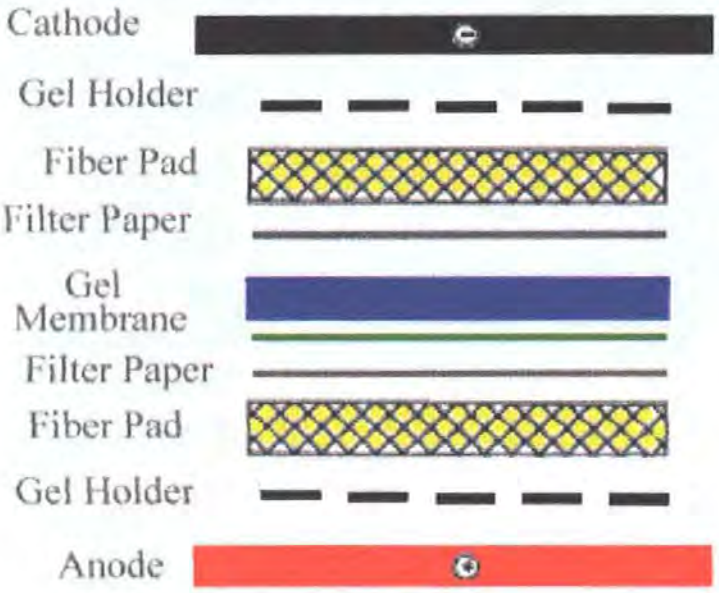
2.20 Electro-blotting of proteins onto membranes

Gels were not stained with Coomassie Blue if they were to be transferred onto either a nitro-cellulose membrane or PVDF.

2.20.1 Transfer to Nitro-cellulose for Western Blotting

Transfer buffer (Towbin Buffer[265]) containing 25mM Tris, pH 8.3 + 192mM glycine + 20% v/v Methanol was chilled. Filter paper (Biorad), Fibre pads (Biorad), 0.45μm nitro-cellulose membrane (Biorad) and gels were allowed to equilibrate in transfer buffer for at least 1 hour at 4°C. Gels were already marked for orientation and a corresponding mark placed on the nitro-cellulose membrane. The cassettes of Mini Trans-Blot Electropheretic Transfer Cell (Biorad) were assembled as shown in Figure 13. Air bubbles were carefully removed to ensure good transfer. Ice blocks were placed around the apparatus to reduce heat transfer. Transfer occurred overnight at 30V, 90mA using APS powerpack.

Figure 13: Arrangement of layers in blotting apparatus



2.20.2 Transfer to PolyVinylidene DiFluoride (PVDF) for protein sequencing

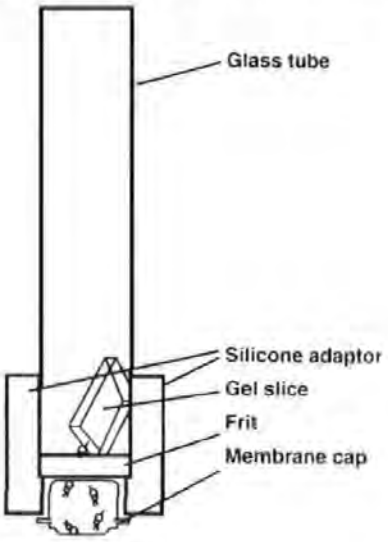
PVDF membrane is thought to be the best support for proteins as it has high protein binding capacity[266] and is resistant to acidic and organic solvents used in amino-terminal protein sequencing[267,268]. Transfer was carried out as previously stated in Section 2.20.1.

2.21 Electro-Elution of protein bands

B-CLL lysates were separated by reducing PAGE, as outlined in section 2.18. Protein bands were visualised by staining with Coomassie Blue. After equilibration in distilled water for 1 hour 65KDa, 42KDa, 31KDa and 25KDa bands were excised with a razor blade. Membrane caps containing dialysis membrane with 10,000 Dalton (Da) exclusion pore size were soaked at 60°C in elution buffer (25mM Tris, 192mM Glycine and 0.1% SDS) for 1 hour prior to use. The model 422 Electro-Eluter was assembled as shown in Figure 14. The gel slices were chopped and placed in individual labelled glass tubes on the Electro-Eluter. The glass tubes were filled with elution buffer. Elution took place

overnight with 8-10mA/glass tube. The eluted protein was found in the membrane cap. Approximately 400µl of protein solution was decanted. The membrane cap was rinsed with a further 200µl of fresh elution buffer. The protein solution was dialysed overnight at 4°C against PBS using 10,000Da Slid-A -Lyzer dialysis cassettes (Pierce, Perbio Science, Chester, UK). Protein content was estimated as outlined in section 2.9.

Figure 14: Arrangement of tubes for electro-elution.



2.22 Western blot analysis

Reducing SDS-PAGE was carried out on cell lysates from both B-CLL patients and normal healthy volunteers as stated in section 2.18. The gels were not stained with Coomassie Blue. The resulting gel was blotted onto nitro-cellulose as described in section 2.20.1. Protein bands were stained with 2% Ponceau S stain solution (Sigma) and destained in distilled water. All bands were marked in pencil at this stage. Non-specific binding was blocked in Bovine Lacto Transfer Technique Optimiser B (BLOTTO B) for 1 hour at room temperature with gentle shaking. BLOTTO B consisted of 10mM Tris HCL (Sigma) pH8 + 150mM Sodium Chloride (Sigma) + 1% skimmed milk powder (Safeway, UK) + 1% Bovine Serum albumin (BSA) (Sigma) + 0.05% Tween 20 (Sigma). Goat anti-Human

CD5, Goat anti-Human CD19, Goat anti-Human CD23, Rabbit anti-Human CD72, Mouse anti-Human CD38 monoclonal antibodies (Santa Cruz, USA) were incubated with the nitro-cellulose blot diluted at 1:500 in BLOTTO B for 1 hour at room temperature with gentle shaking. The blot was washed 3 times in 10mM Tris buffered 150mM saline pH8 (TBS) + 0.02% Tween 20. A secondary polyclonal Donkey anti-Goat Ig alkaline-phosphatase conjugate, polyclonal Rabbit anti-Mouse Ig alkaline-phosphatase conjugate or polyclonal Mouse anti-rabbit Ig alkaline-phosphatase conjugate (Santa Cruz) diluted 1:1000 in BLOTTO B respectively were incubated for 1 hour at room temperature with gentle shaking. The blot was washed 3 times in TBS + 0.02% Tween 20 and once in TBS alone. The Western blot was developed using an Alkaline-Phosphatase development kit (Biorad) for 2 –4 minutes, washed in distilled water and air-dried.

2.23 Protein Sequencing

Protein bands of interest were sequenced. B-CLL lysates were concentrated using a freeze drier and then separated using reducing SDS-PAGE as stated in section 2.18. Gels were blotted onto PVDF as stated in section 2.20.2. Bands were sent for automated N-terminal sequencing to 2 commercial services either Proseq (Boxford, USA) or PNACL (Leicester, UK).

2.24 Statistics.

Effects of treatment upon groups of 5 patients or more were analysed by one-way analysis of variance (ANOVA). Direct comparisons between treatment groups with smaller sample groups were analysed using the Student's *t*-test. Differences between median values were compared using the Kruskal-Wallis test. Statistics were generated using Statsgraphics Plus software.

3. RESULTS-DENDRITIC CELLS

3.1 Characterisation of dendritic cell immunophenotypes.

The results of immunophenotyping studies of DCs derived from normal volunteers and patients with B-CLL are summarised in Table 3.

Table 3: Dendritic cell surface markers

Cell surface marker	Normal DCs (Mean % ± sd)	BCLL patient DCs (Mean % ± sd)
HLA-DR	56.8 ± 17.1	54.0 ± 8.4
CD83	1.4 ± 0.9	1.2 ± 0.8
CD40	12.9 ± 5.9	26.7 ± 8.4 ***p=0.02***
CD86	27.8 ± 1.8	15.7 ± 3.8 ***P=0.003***
CD16	2.2 ± 1.2	19.2 ± 6.9
CD56	0.3 ± 0.3	0.2 ± 0.1
CD3	10.7 ± 5.2	9.2 ± 5.6
CD14	19.3 ± 18.2	11.2 ± 7.0
CD11c	4.2 ± 4.5	10.1 ± 6.0
CD20	4.1 + 2.7	4.7 + 3.0
CD1a	9.6 ± 7.3	9.6 ± 8.3
CD45	92.5 ± 3.5	89.0 ± 4.0

Table 3. Analysis of markers for CD19 depleted PBMC cultured in RPMI 1640 + 10% AB serum + IL-4 (1000U/ml) + GM-CSF (800 U/ml) for 6 days at 37°C, 5% CO_2 from 3 normal volunteers and 5 B-CLL patients. *p*-values were calculated using single-tailed unpaired Student's t-test.

There were no significant differences in mean cell surface marker expression of HLA-DR, CD1a, CD3, CD4, CD11c, CD14, CD16, CD20, CD45, CD56, and CD83 between patients and healthy volunteers. However, CD40 was found to be significantly increased in patients compared to healthy volunteers *(p=0.02)*, and CD86 was found to be significantly decreased in B-CLL patients compared to healthy volunteers *(p=0.003)*.

3.2 Photographs of monocyte DCs

Dendritic cells from 1 B-CLL patient were isolated according to the method in

section 2.5 and photographed using a Diavert microscope (Leitz) using x20 objective and automatic MPS 45 camera (Wild). It can be observed from Figure 15 that monocyte derived DCs change from rounded monocyte like cells to cells with dendrite protrusions over the 6 day incubation. Numbers of monocyte cells with in the culture did not increase. Dendritic cells were thus a consequence of cell differentiation from the common granulocyte-macrophage precursor monocyte cells observed after 1 day of culture. The DCs on day 7 had been incubated at 37°C, 5% CO_2 overnight with IL-12 (100ng/ml) and then subsequently pulsed with autologous B-CLL lysate for 4 hours at room temperature. Larger numbers of dead cells were seen after the overnight incubation with IL-12 than without.

Figure 15: Monocyte derived dendritic cell cultures

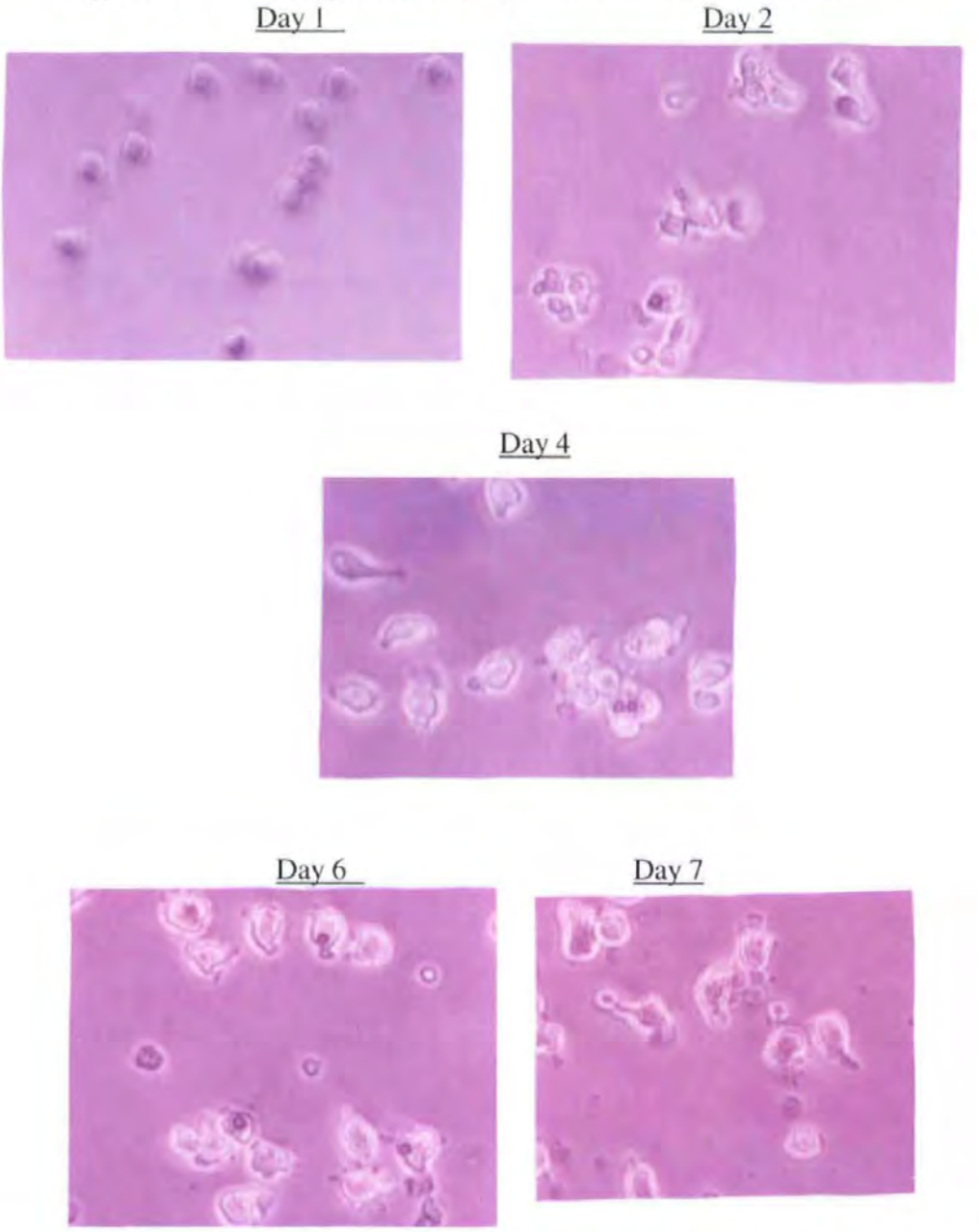


Figure 15: Dendritic cells from Patient 25 were isolated as stated previously and cultured for the indicated period at 37°C, 5% CO_2 in RPMI 1640 + Penicillin + Streptomycin + Glutamine + 10% AB serum. Cells in 24 well plates were photographed using Diavert microscope (Leitz) with x20 objective and automatic MPS 45 camera (Wild).

3.3 Validation of system using autologous DCs pulsed with Tetanus Toxin and Tuberculin PPD.

T cells were co-cultured with commonly used antigens to assess the efficacy of DCs to stimulate T cell activation (IL-2 receptor expression). IL-2 production is initiated by antigenic stimulation and the IL-2 receptor is constitutively expressed after approximately 48 hours [1269,2701]. T cell activation was observed when autologous DCs, pulsed with either Tetanus Toxin or Tuberculin PPD, were cultured with autologous T cells from a single healthy volunteer (Figure 16a). However, when the Tetanus Toxin and Tuberculin PPD antigens were added to T cells without DCs there was an increase in T cell after 6 and 7 days co-culture. The T-cells were responding to the Tetanus Toxin and Tuberculin PPD as a recall antigen after *in vivo* vaccination. The activation of T-cells by autologous DCs pulsed with tetanus toxin occurred much quicker after 3 days of co-culture. The activation of T cells by DCs pulsed with Tuberculin PPD occurred after 5 days co-culture.

Figure 16a: T cell proliferation to autologous DCs pulsed with Tetanus Toxin and Tuberculin PPD in a normal healthy volunteer.

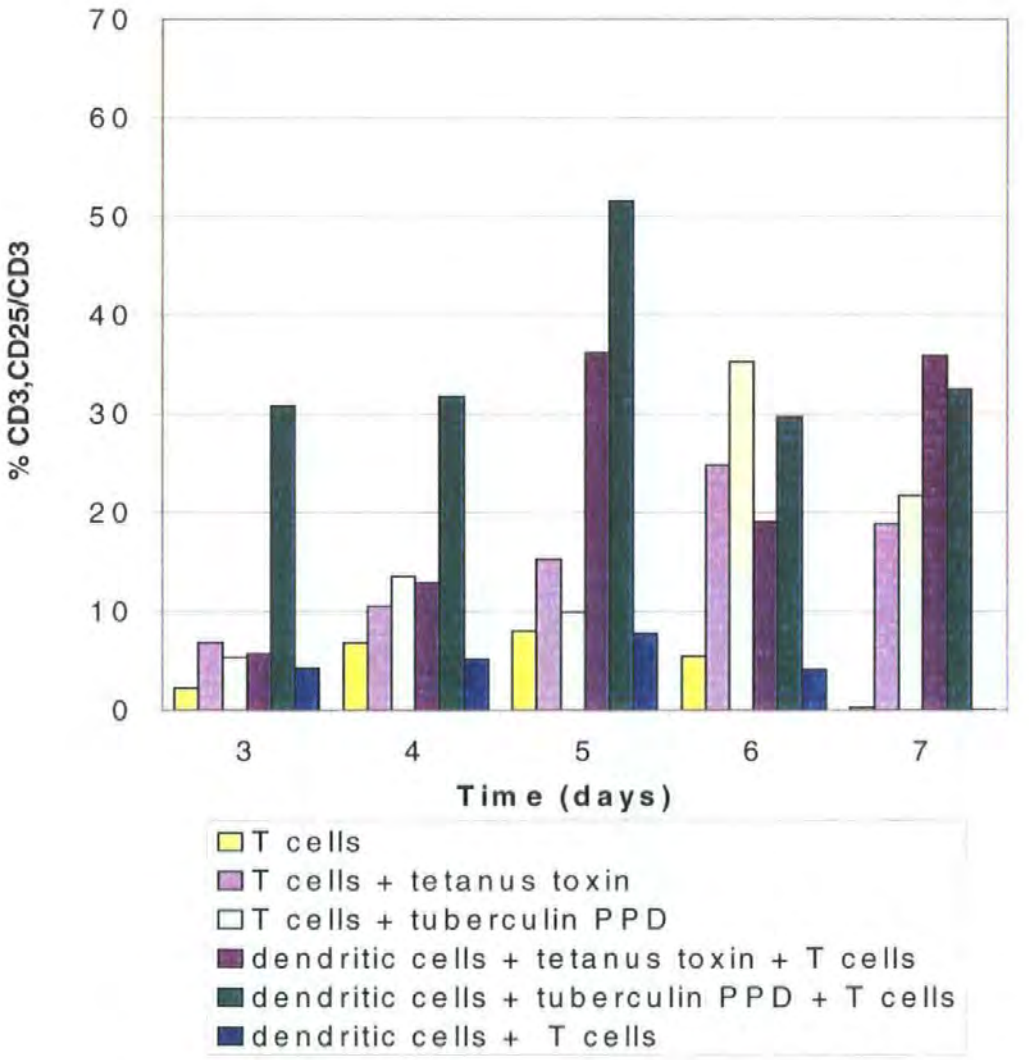


Figure 16a: T-cells from 1 normal healthy volunteer were cultured alone, with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) or with DCs pulsed with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) for 4 hours at room temperature. Cells were harvested on day 3-7 of culture and assessed by flow cytometry for CD3/CD25 co-expression.

T cells from B-CLL Patient 26 showed an increase in T cell activation when stimulated by autologous DCs pulsed with Tetanus Toxin or Tuberculin PPD (Figure 16b). However, when Tetanus toxin and Tuberculin PPD were added to T-cells without DCs increased T-cell activation was observed after 5 days. The response to antigen alone without being pulsed onto DCs was greater in the B-CLL patient. This may be because the individuals recall antigen response is stronger due to recent vaccination or increased exposure to the antigens. However, B-CLL patients have been shown to express increased levels of CD25 as the disease progresses [271]. Pulsing Tuberculin PPD onto DCs stimulated more T cells to express the IL-2 receptor than administration of the Tuberculin antigen directly to T-cells. These experiments validated that specific antigens pulsed onto DCs could stimulate T cell activation, using markers such as the IL-2 receptor (CD25).

Figure 16b: T cell proliferation to autologous DCs pulsed with stimulated by Tetanus Toxin and Tuberculin PPD in a B-CLL patient.

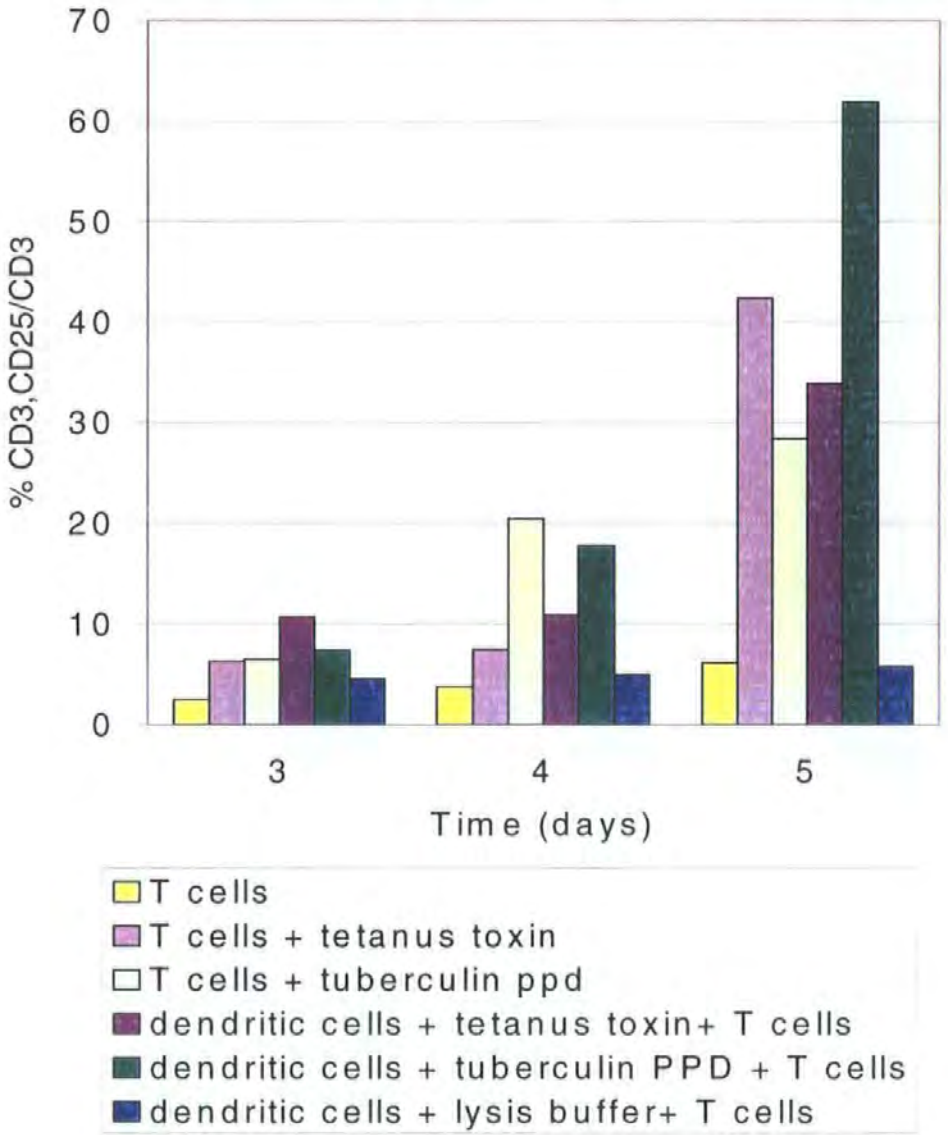


Figure 16b: T-cells from 1 B-CLL Patient 26 were cultured alone, with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) or with DCs pulsed with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) for 4 hours at room temperature. Cells were harvested on day 3-7 of culture and assessed by flow cytometry for CD3/CD25 co-expression.

Cytokine production by T cells was also assessed by ELISA to show T helper cell activity. IFN-γ was measured from the same patients and healthy volunteer that had shown an increase in IL-2 receptor. T-cells from the healthy volunteer secreted increased amounts of IFN-γ when cultured with autologous DCs pulsed with either Tetanus Toxin or Tuberculin PPD (Figure 17a). IFN-γ secretion in response to the administration of Tuberculin PPD to T-cells without DCs after 5 days was the same as that when using pulsed DCs. However, IFN-γ secretion followed the same pattern as IL-2 receptor expression.

T-cells from the B-CLL Patient 26 secreted increased amounts of IFN-γ when cultured with autologous DCs pulsed with either Tetanus Toxin or Tuberculin PPD (Figure 17b). When comparing the T-cell responses from the normal healthy volunteer (Figure 16a and 17a) and B-CLL Patient 26 (Figure 16b and 17b) it was observed that although the peak response was weaker in the B-CLL patient the responses followed similar overall patterns.

Figure 17a: IFN-γ secretion by T cells to autologous DCs pulsed with Tetanus Toxin and Tuberculin PPD in a normal healthy volunteer.

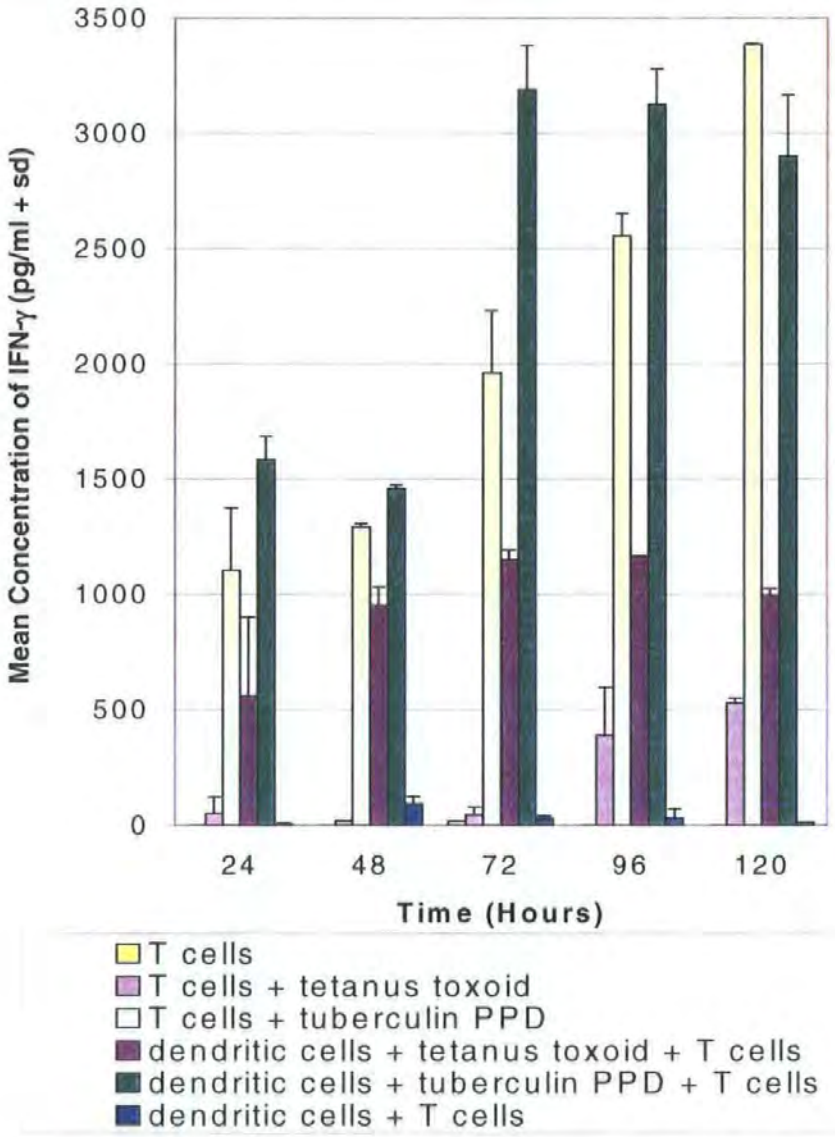


Figure 17a: T-cells from 1 normal healthy volunteer were cultured with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) alone or with DCs pulsed with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) for 4 hours at room temperature. Tissue culture supernatant was assessed by ELISA. Each treatment group was measured in duplicate per time point.

Figure 17b: IFN-γ secretion by T cells to autologous DCs pulsed with Tetanus Toxin and Tuberculin PPD in a B-CLL patient

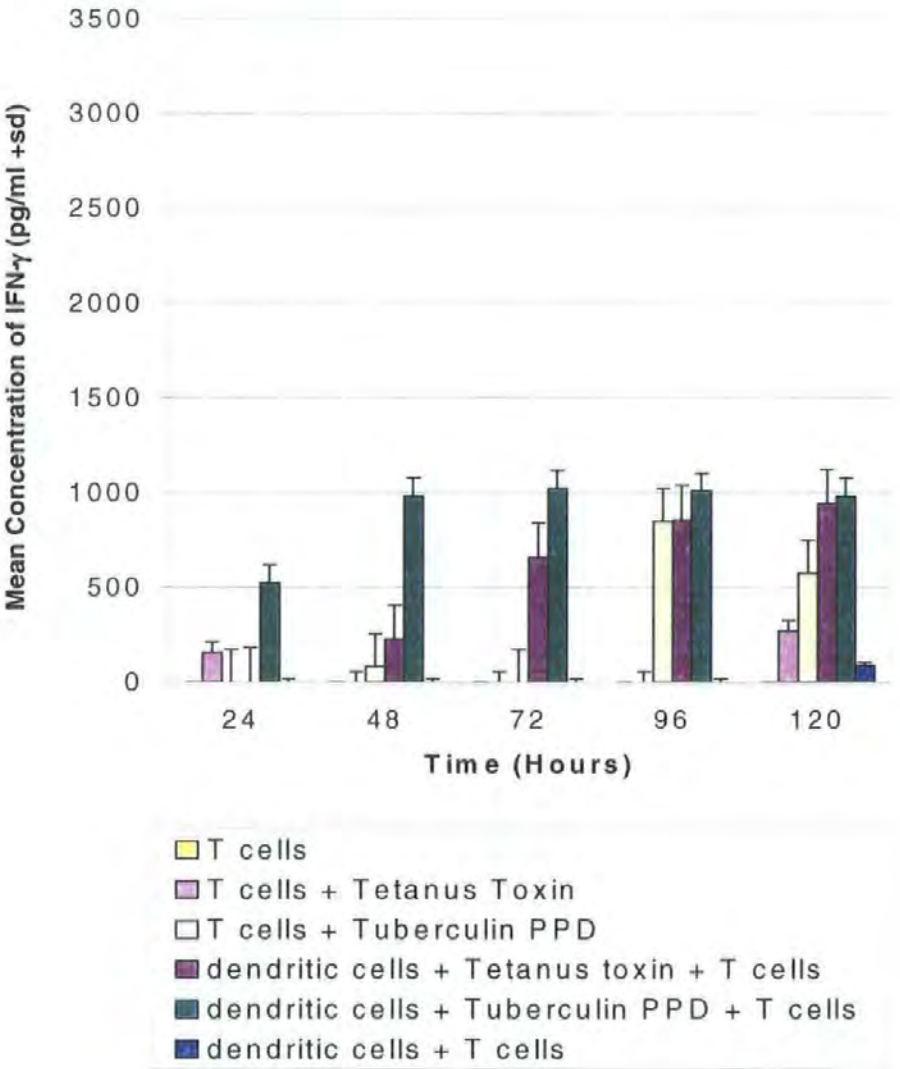
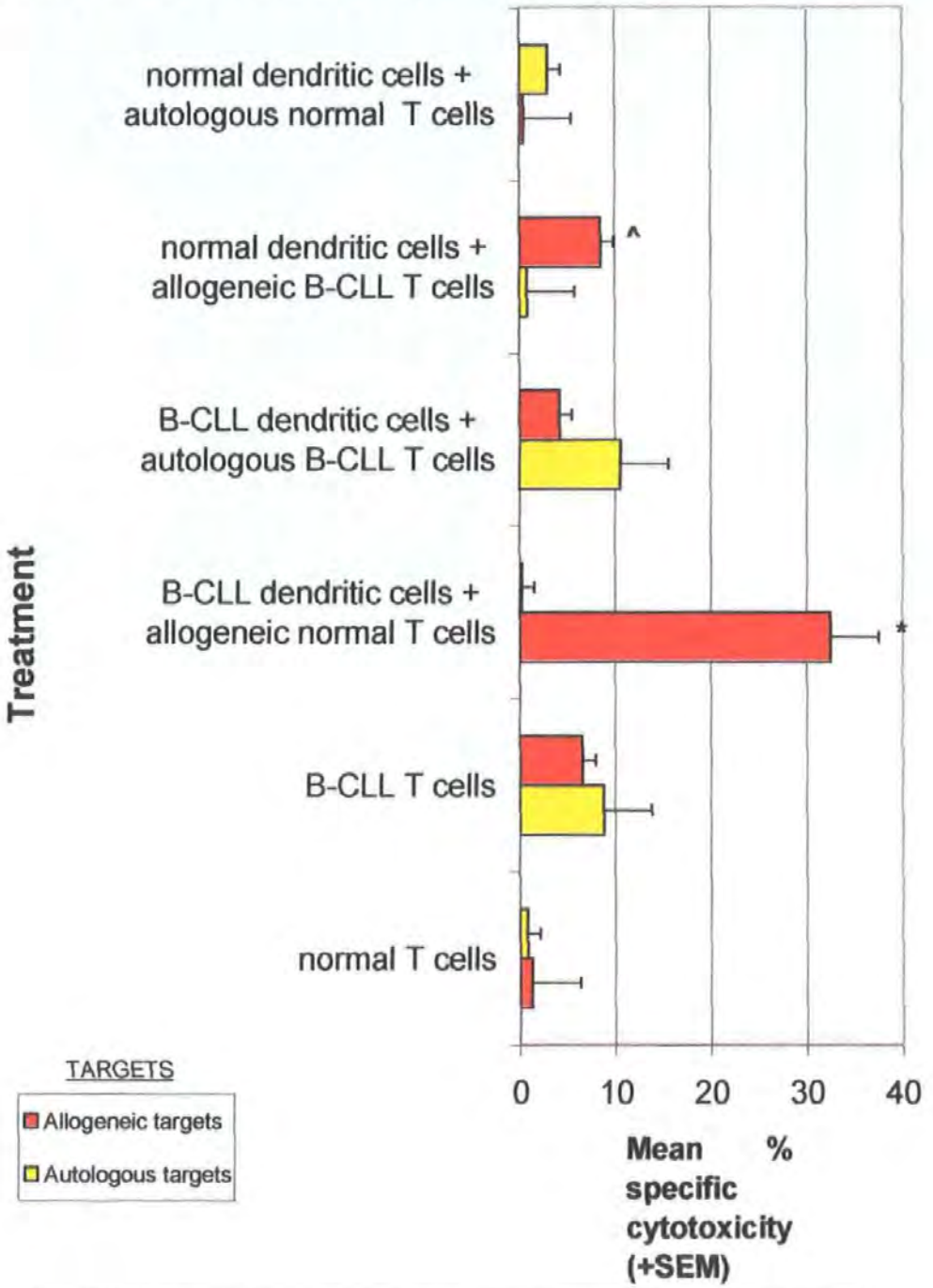


Figure 17b: T-cells from 1 B-CLL Patient 26 were cultured with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) alone or with DCs pulsed with Tetanus Toxin (0.4U/ml) and Tuberculin PPD (900U/ml) for 4 hours at room temperature. Tissue culture supernatant was assessed by ELISA. Each treatment group was measured in duplicate per time point.

3.3 Allogeneic dendritic cell: T cell interactions

In order to test the ability of DCs to stimulate cytotoxicity cell-cell allogeneic reactions between DCs and T cells were investigated by mixed cultures. Figure 18 shows the results from 3 B-CLL patients and 3 healthy normal volunteers. It should be noted that DCs from B-CLL patients cultured with allogeneic T cells from healthy volunteers stimulated cytotoxicity towards allogeneic B-CLL B cell targets (*). The converse was true in that DCs from normal healthy volunteers stimulated allogeneic B-CLL T cells to kill allogeneic normal healthy B cells (^). However, the cytotoxicity generated by B-CLL T cells was of a lower percentage than normal T-cells. T-cells from B-CLL patients showed higher background cytotoxicity when not stimulated. These two phenomenon were probably due to the fact the T-cells from B-CLL patients have shown dysfunctional characteristics. Collectively the antigen presentation data suggests that DCs can be used as efficient antigen presenting cells.

Figure 18: Cytotoxicity generated in mixed lymphocyte culture between T cells and DCs from B-CLL patients and healthy normal volunteers.



Dendritic cells from Patients 2, 7 and 8 and 3 healthy volunteers were cultured for 21 days in RPMI 1640 + 5% AB serum + Glutamine + Penicillin + Streptomycin + IL-2 (5U/ml) at 37°C, 5% CO_2 with autologous T cells or allogeneic normal T cells and tested for cytotoxicity against allogeneic or autologous targets.

4. RESULTS- T CELL RESPONSES

4.1 Measurement of T cell proliferation (IL-2R expression) in B-CLL patients.

T-cells derived from patients with B-CLL were cultured alone or with autologous B-CLL lysate-pulsed or unpulsed DCs. T-cells were assessed for co-expression of CD3 and CD25 (IL-2R). Figure 19 illustrates typical flow cytometric profiles generated from co-cultures of B-CLL Patient 22. It can clearly be seen that the proportion of T cells co-expressing CD3 cells and CD25 increased when T-cells were stimulated by B-CLL B-cell lysate pulsed DCs.

Figure 19: Typical example of flow cytometry profiles assessing T cell proliferation (IL-2R expression) in a B-CLL patient

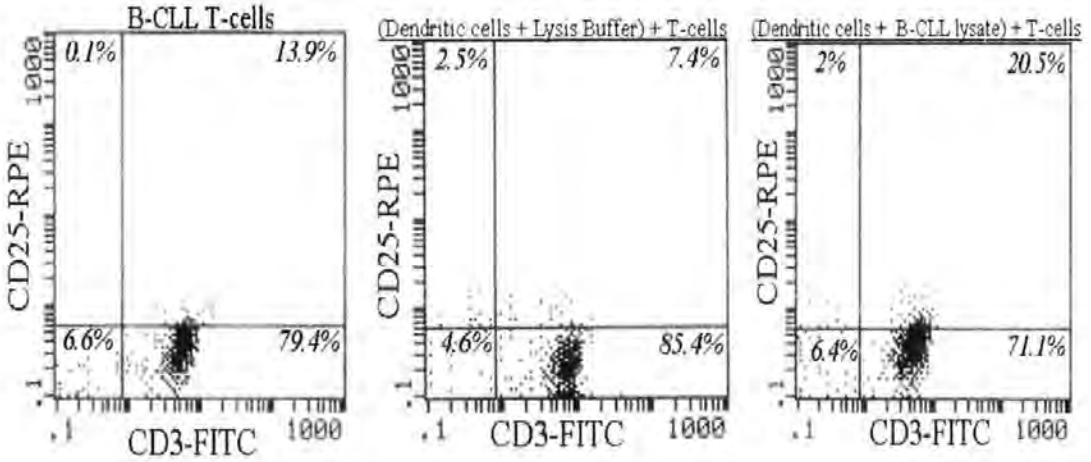


Figure 19: T-cells from B-CLL Patient 22 were co-cultured alone, with lysis buffer pulsed DCs or B-CLL lysate pulsed DCs for 7 days in RPMI+5% AB Serum + Penicillin + Streptomycin + Glutamine at 37°C, 5%CO_2. Cells were harvested and assessed for CD3/CD25 co-expression by flow cytometry. Gates were set at 2% of FITC-Ig and RPE-Ig negative controls.

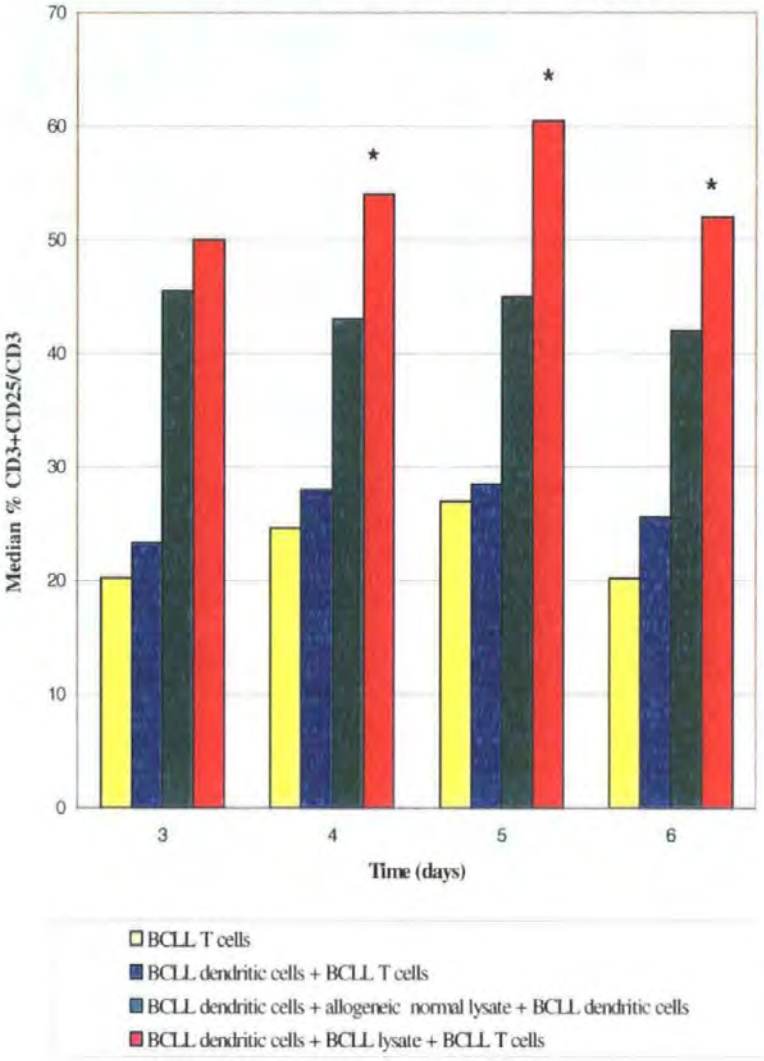
Activation markers were assessed in the co-cultures of 5 patients and analysed as a sample group. A significant increase in T-cell activation was found after 4 days of culture by T-cells cultured with autologous lysate-pulsed DCs compared to T-cells cultured with DCs pulsed with lysis buffer *($p=0.03$)* (*) (Figure 20a).

A lysate from a normal healthy volunteer was used as a control and pulsed onto DCs from B-CLL patients. It was important to see whether an allogeneic lysate from a normal healthy volunteer could also stimulate T-cells from B-CLL patients. Although there was an increase in the percentage of activated B-CLL T cells after co-culture with autologous B-CLL DCs pulsed with an allogeneic B-cell lysate from a healthy volunteer, this was not significant (Figure 20a).

4.2 Measurement of T-cell proliferation (IL-2R expression) in healthy volunteers

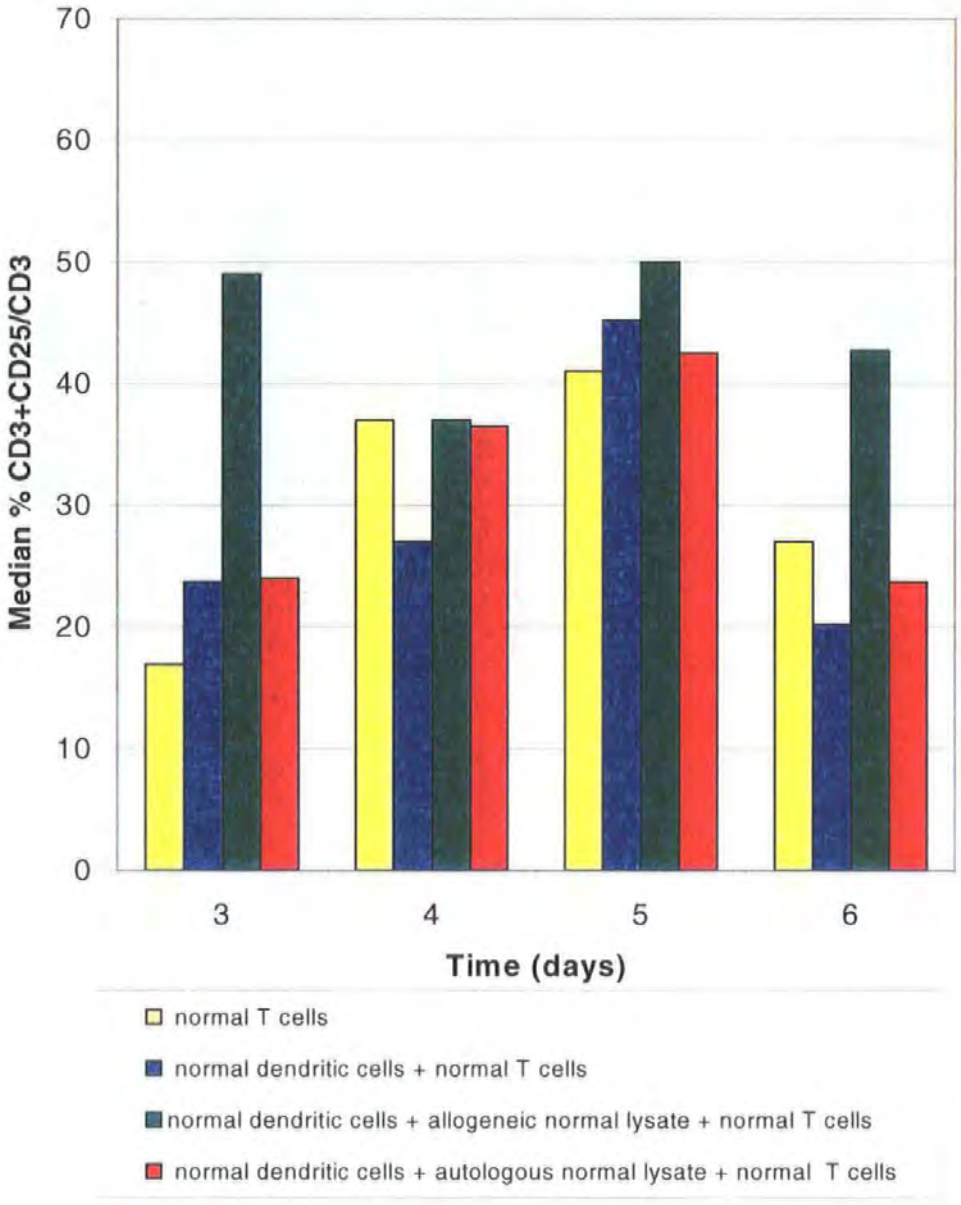
Dendritic cells from 5 normal healthy volunteers were pulsed with autologous normal lysate and then co-cultured with autologous normal T-cells. This allowed a direct comparison between the autologous system in normal healthy volunteers and B-CLL patients. When an autologous non-B-CLL B-cell lysate from healthy volunteers was pulsed onto autologous DCs, there was no increase in T cell activation (Figure 20b). Interestingly, when an allogeneic non-B-CLL B-cell lysate from healthy volunteers was pulsed onto autologous DCs, the percentage of activated autologous T cells was increased at day 3 and 6 but not significantly (Figure 20b). A strong allogeneic response would have been expected when using an allogeneic B cell lysate from normal healthy volunteers.

Figure 20a: T cell proliferation (IL-2R expression) to autologous lysate pulsed DCs from B-CLL patients



Numbers of CD3/CD25 positive T-cells from 5 B-CLL were measured (Figure 20a). Median values expressed were tested using the Kruskal-Wallis test. (*) Indicates a significant increase in double positive cells with $p=0.03$ when co-cultures of pulsed DCs were compared with unpulsed DCs.

Figure 20b: T cell proliferation (IL-2R expression) to autologous lysate pulsed DCs from normal healthy volunteers



Numbers of CD3/CD25 positive T-cells from 5 healthy volunteers' (Figure 20b) were measured. Median values expressed were tested using the Kruskal-Wallis test. No significant increases were found.

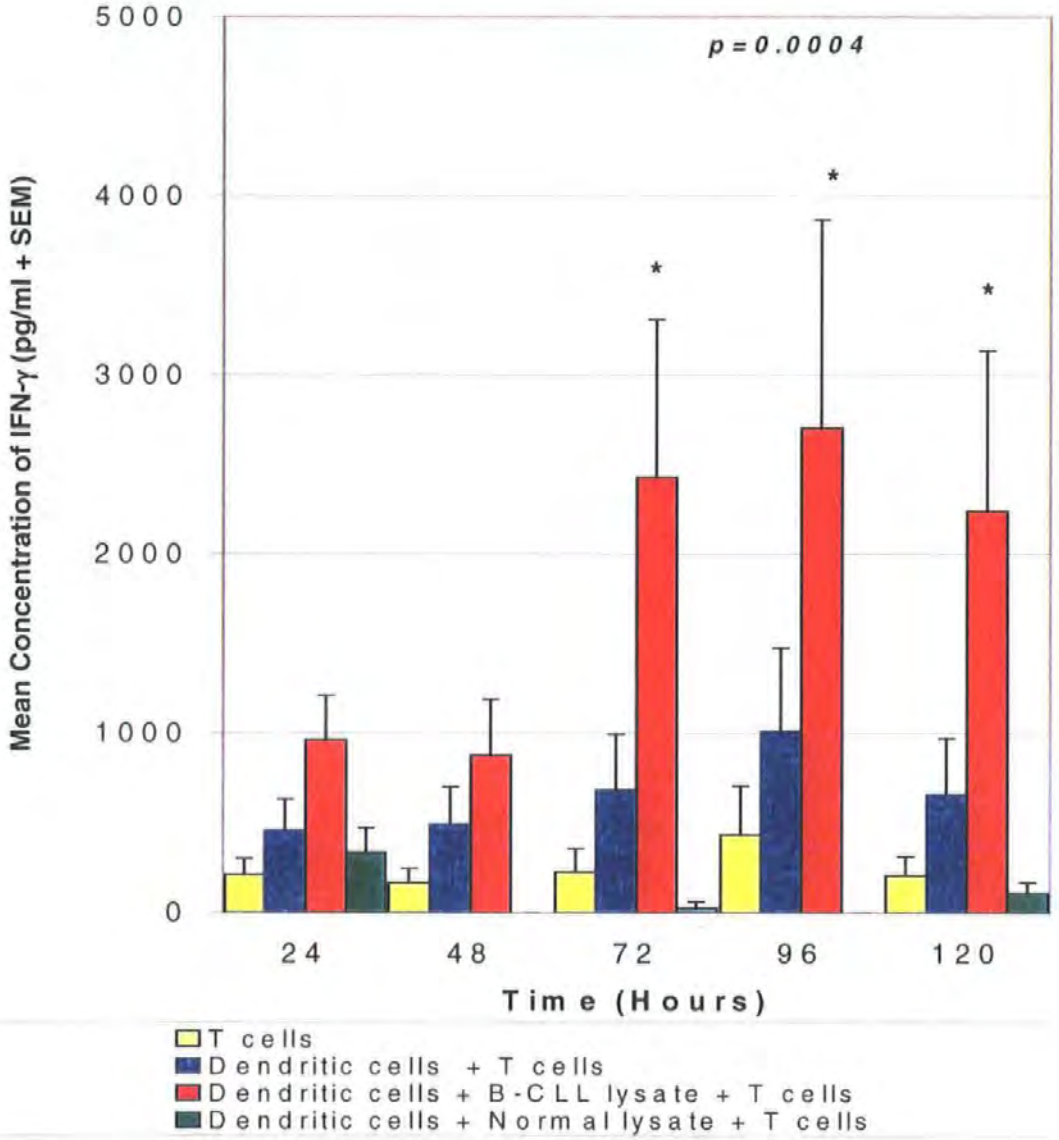
4.3 Quantitation of cytokine secretion in B-CLL patients.

T-cells derived from patients with B-CLL were cultured alone or with lysate-pulsed or unpulsed (lysis buffer added in 5 patients) DCs. The supernatant from these cultures was harvested and tested for IFN-γ protein levels by ELISA. IFN-γ was chosen as it is an important T helper cell type 1 (Th1) cytokine. A significant increase of IFN-γ secretion in culture supernatant was found after 72 hours by T-cells cultured with B-CLL lysate-pulsed autologous DCs compared to both T-cells cultured with unpulsed DCs and T-cells cultured alone *($p=0.0004$)* (Figure 21a). In addition there was no secretion of IFN-γ by T-cells cultured with autologous DCs pulsed with soluble allogeneic B-cell lysate derived from healthy volunteers (non-B-CLL) (Figure 21a).

4.4 Quantitation of cytokine secretion in healthy volunteers

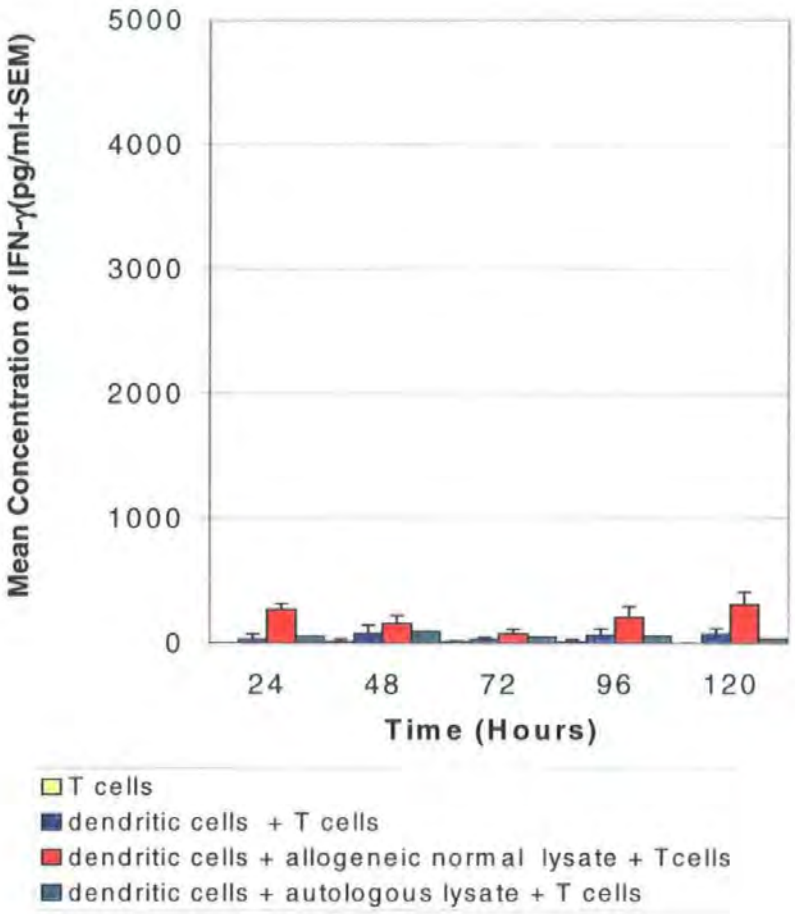
Secretion of IFN-γ by T-cells derived from healthy volunteers and cultured with autologous DCs pulsed with autologous B-cell lysate from healthy volunteers (non-B-CLL) was measured. Normal T-cells, stimulated using the same system as that for B-CLL patients, did not secrete significant amounts of IFN-γ (Figure 21b). Dendritic cells from a normal healthy volunteer that had been pulsed with allogeneic B-CLL lysate could not stimulate autologous normal healthy T-cells to secrete IFN-γ. Even background levels, of T cells alone or T-cells with DCs pulsed with lysis buffer, of IFN-γ secretion in the group of 5 healthy volunteers was lower than the 10 B-CLL patients (Figure 21b compared with 21a).

Figure 21a: IFN-γ secretion by T-cells cultured with autologous lysate pulsed DCs from B-CLL patients.



Tissue culture supernatants from cultures of 10 B-CLL patients' (Figure 21a) were measured for IFN-γ by ELISA. Data was analysed by one-way ANOVA. Where (*) indicates an overall significant effect of treatment with *p=0.0004*. Each treatment group per patient was measured in duplicate.

Figure 21b: IFN-γ secretion by T-cells cultured with autologous lysate pulsed DCs from normal healthy volunteers



Tissue culture supernatants from cultures of 5 normal healthy volunteers (Figure 21b) were measured for IFN-γ by ELISA. Each treatment group per patient was measured in duplicate.

4.5 Secretion of IL-4

To establish the nature of the cytokine profile generated by the stimulated T-cell cultures a typical T Helper cell type 2 (Th 2) cytokine was measured. Concentrations of IL-4 in tissue culture supernatants were measured in 2 patients with B-CLL and found to be less than 50 pg/ml.

4.6 Measurement of cytotoxic T-cell activity in B-CLL patients.

T-cells were cultured with DCs for 21 days with IL-2 fed on days 3,7,10,14, and 17. Soluble lysate was added to the pulsed DCs on day 7 and 14. T-cell effectors were then tested in a flow cytometric cytotoxicity assay. A typical example of the flow cytometric profiles at a 40:1 effector:target ratio from Patient 13 are shown below in Figure 22. It can be seen that cytotoxicity to B-CLL B cell targets is greater by T-cells cultured with autologous DCs pulsed with B-cell lysate from B-CLL patient than that seen by T cell effectors stimulated by DCs pulsed with lysis buffer or T-cells cultured alone.

Figure 22: Typical Flow cytometric profiles of cytotoxicity to B-CLL B cells

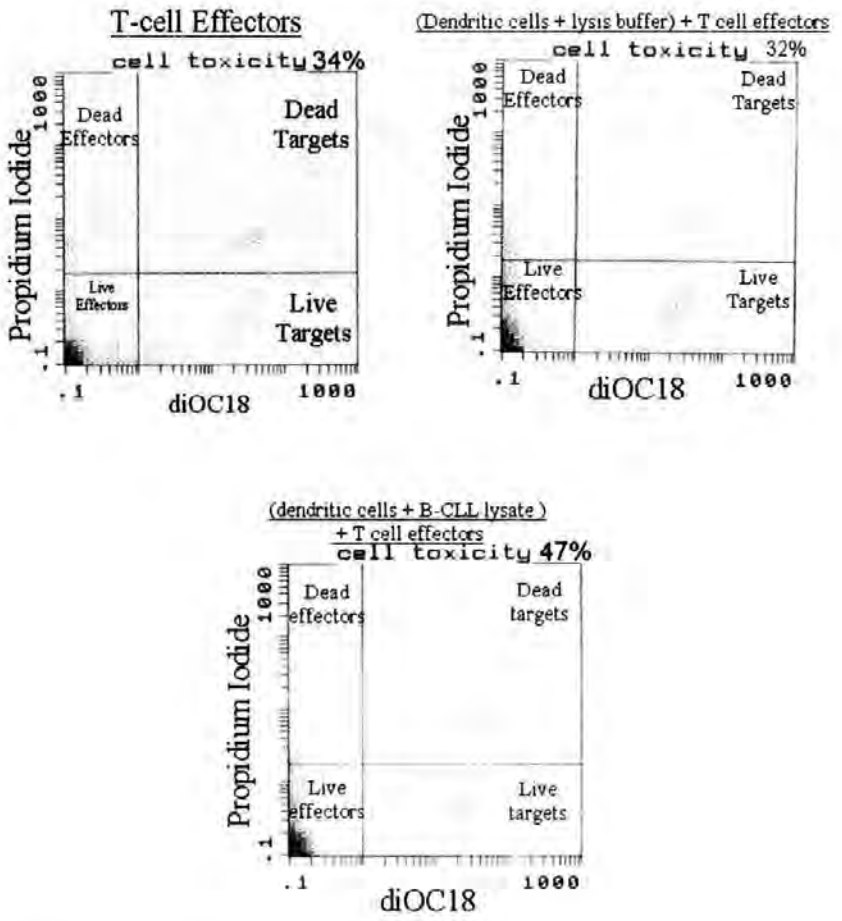
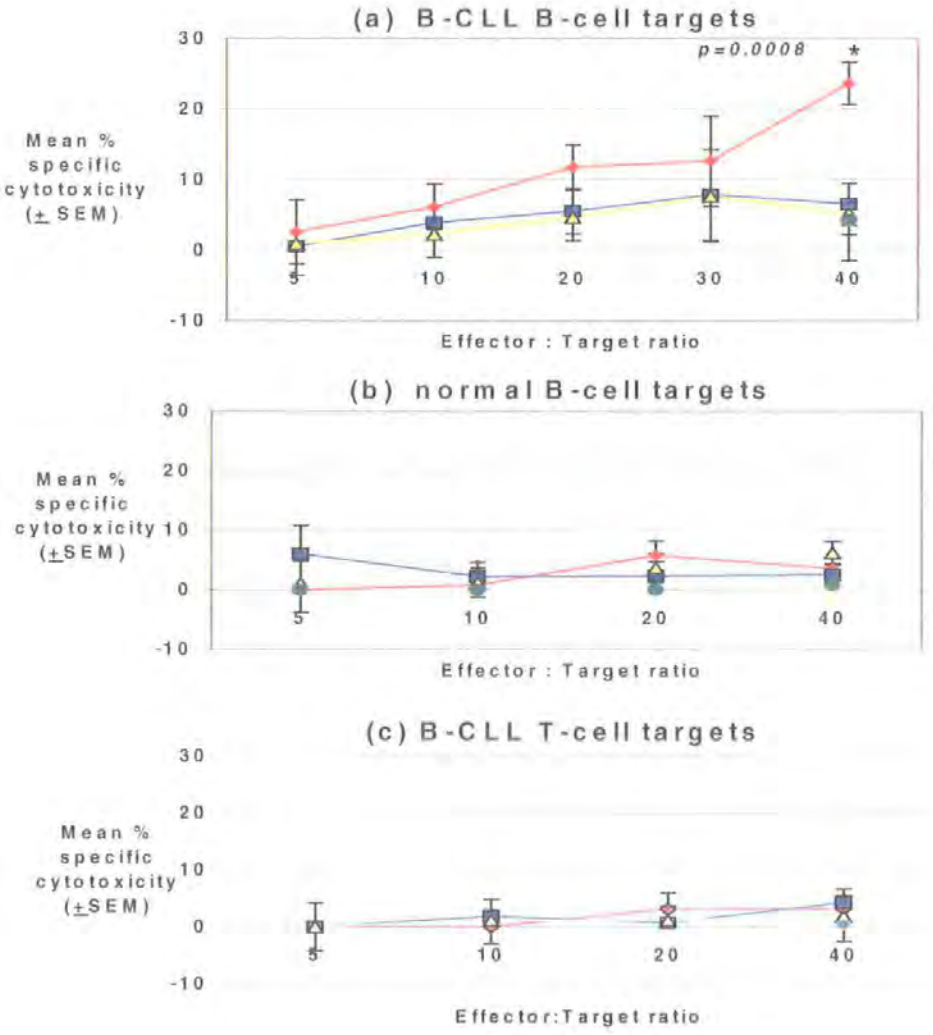


Figure 22: T-cells from Patient 13 were cultured and are shown here after incubation at 40:1 effector ratio in RPMI 1640 + 10% AB Serum + Penicillin + Streptomycin + Glutamine for 4 hours at 37oC, 5%CO2. Gates were set as illustrated in Figure 11. Non-specific cytotoxicity to B-CLL B-cell targets was measured at 29%.

T-cells derived from 10 patients with B-CLL were cultured alone or with lysate-pulsed or unpulsed (lysis buffer added in 8 patients) DCs and then tested for cytotoxicity against B-CLL B-cell targets. At the 40:1 effector: target ratio, a significant increase in cytotoxicity against B-CLL targets was generated by T-cells cultured with B-CLL lysate-pulsed autologous DCs compared with both T-cells cultured with unpulsed DCs and with T-cells cultured alone *($p=0.0008$)* (Figure 23a). In order to check the specificity of this cell mediated cytotoxicity other control targets were tested. To test whether the cell-mediated cytotoxicity was directed towards a Pan B-cell antigen, such as CD19, control B cell targets from normal healthy volunteers were used. Significant cytotoxicity was not demonstrated against allogeneic B-cell targets from healthy volunteers (non B-CLL targets) (Figure 23b). Secondly to test for any autoimmune reactivity that may have a detrimental effect upon the immune system of the patient, autologous T-cells from B-CLL patients were used as targets. Significant cytotoxicity against autologous T-cells derived from B-CLL patients (non-B-cell targets) was not stimulated by co-culture T cell effectors with a B-CLL B cell lysate pulsed DCs (Figure 23c). T-cells derived from patients with B-CLL cultured with soluble B-CLL lysate in the absence of DCs did not show significant cytotoxicity against B-CLL or B-cell targets from healthy volunteers. Presentation of the B-CLL B cell lysate by DCs was required in order to stimulate T-cell responses.

Figure 23a, 23b and 23c : Cytotoxicity of T cell effectors from B-CLL patients



Effectors from 10 B-CLL patients were cultured for 21 days (see Chapter 2) and cytotoxicity against autologous B-CLL B-cell targets (Figure 23a), allogeneic B-cell targets from healthy volunteers (non-B-CLL) (Figure 23b) and autologous B-CLL T-cell targets (Figure 23c) was measured. T-cells had been cultured alone △ or with autologous DCs pulsed with autologous B-CLL lysate◆ , allogeneic lysate from healthy volunteers (non B-CLL) ● or lysis buffer■ (except in patient 7 and 13 no lysate). Data was analysed by one-way ANOVA. Where (*) indicates an overall significant effect of treatment with *p=0.0008*.

Figure 24: Cytotoxicity against HLA matched normal B-cells by T-cell effectors from B-CLL patient stimulated by lysate pulsed DCs

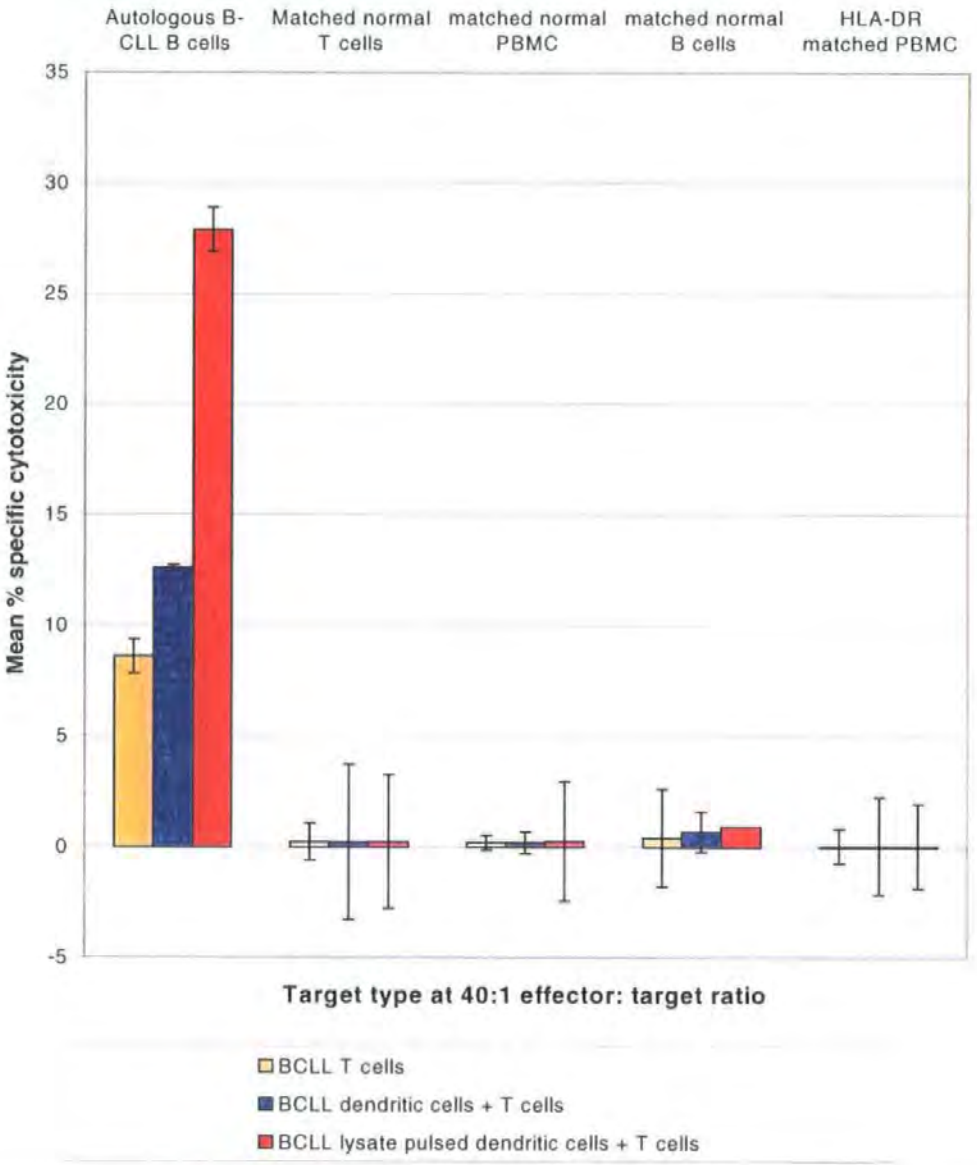


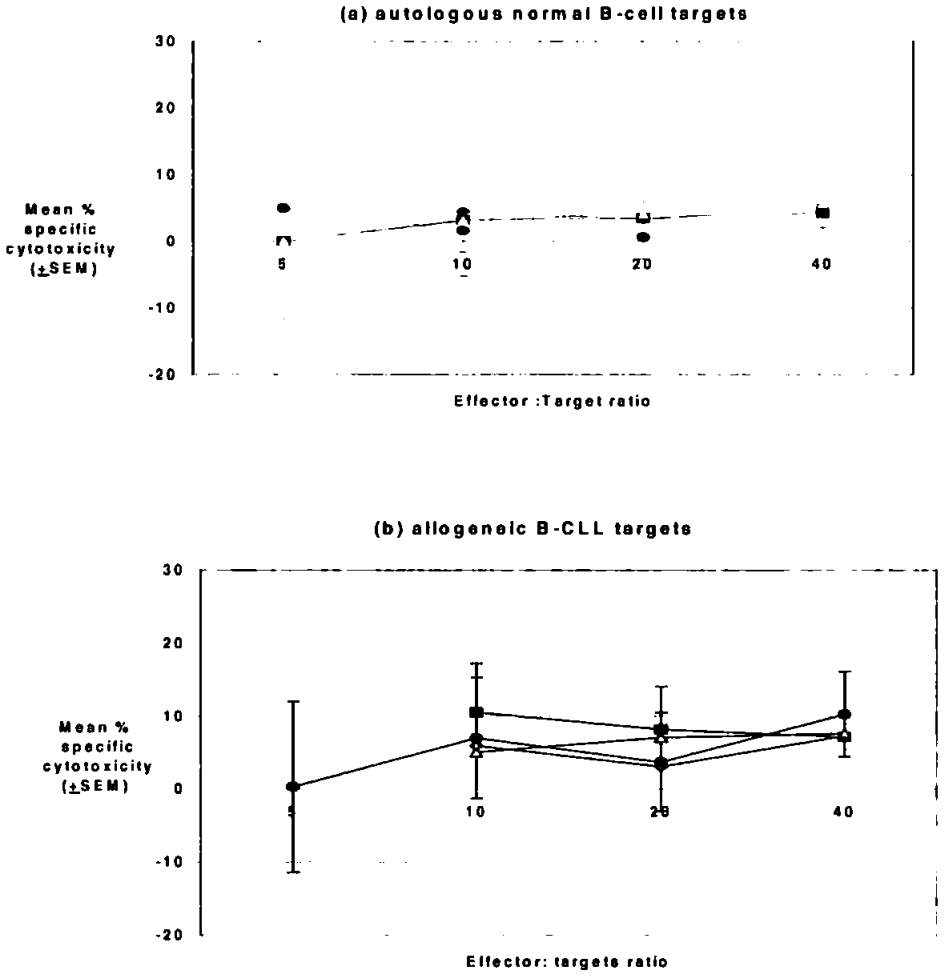
Figure 24: T-cells from B-CLL Patient 26 were co-cultured alone, with lysis buffer pulsed autologous DCs or lysate pulsed autologous DCs for 28 days at 37°C, 5%CO_2. Cultures were fed IL-2 and restimulated as stated in Section 2.12. Cytotoxicity was measured against HLA-matched PBMC's, T cells and B cells. Duplicates per treatment per target were measured.

Many anti-tumour responses have been shown to be HLA restricted. Therefore, it was important to test B-CLL targets of the same HLA type as control targets. In Patient 26, effectors were tested against B cells, PBMC's and T cells from 2 HLA class I and II matched healthy volunteers and 1 HLA class II matched healthy volunteer. No cytotoxicity to these targets was demonstrated as shown in Figure 24.

4.7 Measurement of cytotoxic T-cell responses in healthy volunteers

As a direct comparison with the B-CLL autologous system, T-cells from normal healthy volunteers were stimulated by autologous DCs pulsed with B-cell lysate from normal healthy volunteers. T-cells derived from healthy volunteers showed no specific cytotoxicity to autologous B-cell targets from healthy volunteers (non-B-CLL targets) (Figure 25a). T-cells were also cultured with autologous DCs pulsed with allogeneic B-CLL lysate. Effector from normal healthy volunteers after stimulation by normal DCs pulsed with an allogeneic B-CLL B-cell lysate did not demonstrate significant cytotoxicity against autologous B-cell targets from B-CLL patients (Figure 25b).

Figure 25. Cytotoxicity of T cell effectors from normal individuals.



Effectors from 5 normal healthy volunteers were cultured for 21 days (see Chapter 2) and cytotoxicity against autologous B-cell targets from healthy volunteers (non-B-CLL) (Figure 25a) and allogeneic B-CLL B-cell targets (Figure 25b) was measured. T-cells had been cultured alone△ or with autologous DCs pulsed with autologous normal B-cell lysate ● , allogeneic lysate from B-CLL patient◆ or lysis buffer■ . Data was analysed by one-way ANOVA.

4.8 Stimulation of cytotoxicity by Granulocyte lysate from B-CLL patient

In addition, it was thought necessary to demonstrate that a lysate from a cell type other than B-cells could not stimulate similar anti-B-CLL responses. A cell not shown to be involved in the disease of B-CLL was chosen. Granulocytes have shown limited involvement in the pathology of B-CLL [272]. Granulocytes were isolated as described in Section 2.7. Lysate generation, pulsing of DCs and culture conditions for the granulocyte lysate were the same as those as B-cells. T-cells cultured with autologous DCs pulsed with an autologous granulocyte lysate from B-CLL Patient 1 showed no significant increase in cytotoxicity against autologous granulocytes or autologous B-CLL cells as shown in Figure 26. Although the cytotoxicity of T-cell effectors cultured alone or with DCs pulsed with lysis buffer was higher against granulocyte targets than B-CLL cells this was thought to be due to the fragility of the granulocyte cells.

Figure 26: Cytotoxicity stimulated by granulocyte lysate from a B-CLL patient

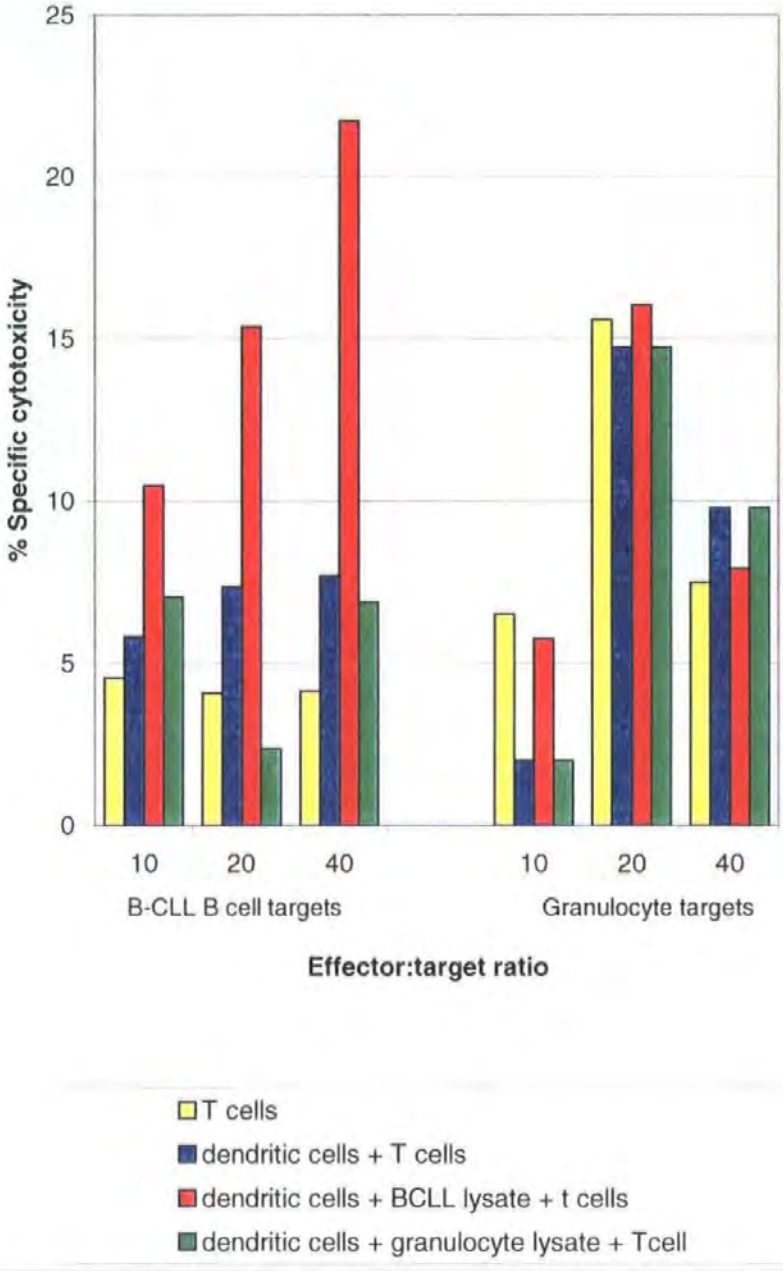
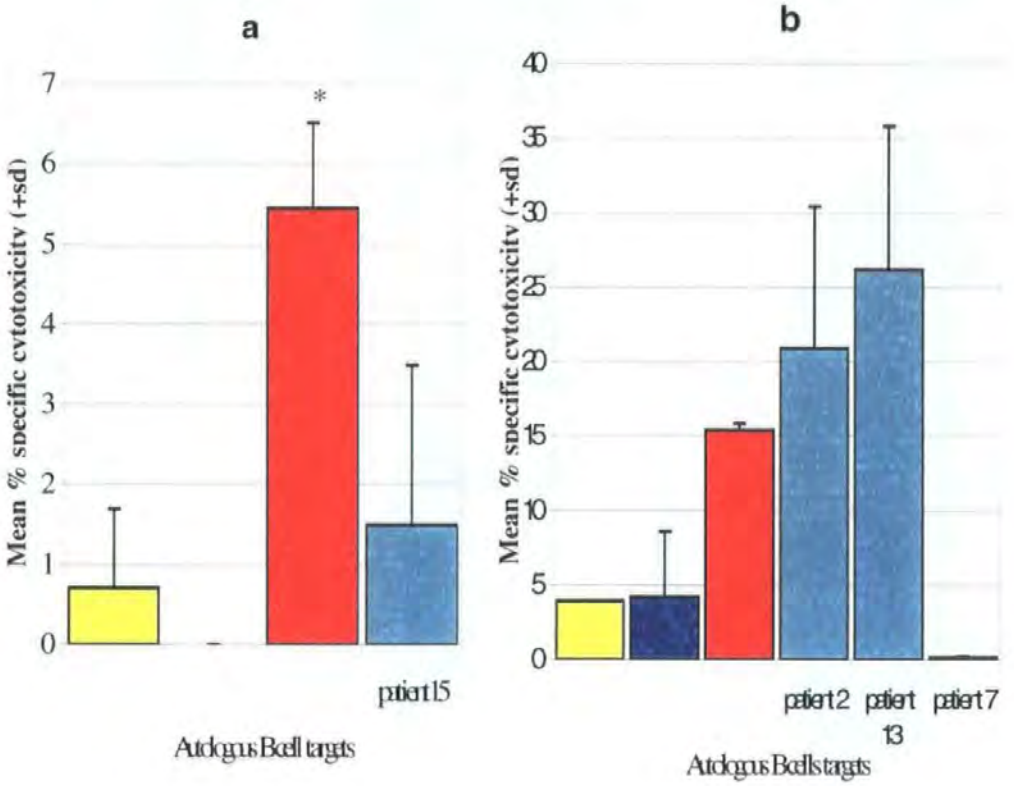


Figure 26: Granulocytes and B-CLL B cells were separated from B-CLL Patient 1 and cell lysates were prepared by identical methods according to Chapter 2. A single batch of DCs was pulsed with the 2 different lysates under the same conditions. T-cell effectors were cultured without or with pulsed DCs under the same conditions for 21 days as stated in Chapter 2. All targets were autologous.

4.9 Stimulation of cytotoxicity using allogeneic B-cell lysates from B-CLL patients

In order to further characterise the specificity of the stimulatory ability of the B-CLL B-cell lysates, allogeneic B-cell lysates from B-CLL patients were pulsed onto DCs from another B-CLL patient and cultured with T-cells from that B-CLL patient. Mixed results were obtained. T-cells derived from Patient 14 and cultured with autologous DCs pulsed with an allogeneic B-CLL lysate from Patient 15 did not demonstrate cytotoxicity to B-CLL targets from Patient 14 (Figure 27a). However, T-cells derived from Patient 6 and cultured with autologous DCs pulsed with allogeneic B-CLL lysates from Patients 2 and 13 demonstrated significant cytotoxicity to B-CLL targets from Patient 6 *($p=0.003$* and *$p=0.009$)*. Allogeneic B-CLL lysates from B-CLL Patient 7 did not stimulate cytotoxicity to B-CLL targets from Patient 6 (Figure 27b). The lysates from Patient 7 and 15 had previously generated specific cytotoxicity when used as autologous lysate, pulsed onto autologous DCs, cultured with autologous effectors and tested against autologous targets.

Figure 27: Cytotoxicity generated by allogeneic-B-CLL-lysate pulsed DCs



Effectors from B-CLL patients were cultured alone □, with autologous DCs pulsed with autologous B-CLL lysate ■, B-CLL allogeneic lysates from patients 15, 2, 13 and 7 ■ and lysis buff ■ for 21 days. Cytotoxicity shown was performed at a target: effector ratio of 40:1. Where (*) indicated a significant increase in mean specific cytotoxicity compared with autologous DCs with lysis buffer with $p<0.01$ when analysed by Students t-test. Each treatment group was measured in duplicate per patient.

4.10 Characterisation of effector cell immunophenotypes.

Effector cells from B-CLL patients 14 and 16 where immunophenotyped after 21 days in culture. The mean cell surface expression was found to be 47% $CD4^+$ cells and 13% $CD8^+$ cells, the remaining cells being $CD3^-$. CD16 and CD56 expression was absent.

4. 11 Antibody blocking studies.

Although Natural Killer cells were not found by flow cytometry in the effector cell population, they could still be responsible for the killing of B-CLL cells. In order to characterise the mechanism of cytotoxicity, antibodies were used to inhibit the cytotoxic T-cells. Antibody blocking experiments were performed at the effector stage of the cytotoxicity assay on 3 patients chosen at random. Significant inhibition of cytotoxicity was demonstrated with anti-class II but not with anti-class I monoclonal antibodies in Patient 2 (Figure 28a) $p=0.006$ and 12 $p=0.031$ (Figure 28b). Significant inhibition of cytotoxicity was demonstrated in Patient 4 with anti-pan TCR $\alpha\beta$ and anti-CD4 but not with anti-CD8 monoclonal antibodies ($p=0.03$ and $p=0.046$) (Figure 28c). This confirmed that the cytotoxicity induced by B-CLL lysate pulsed DCs was reliant upon a HLA class-II restricted mechanism.

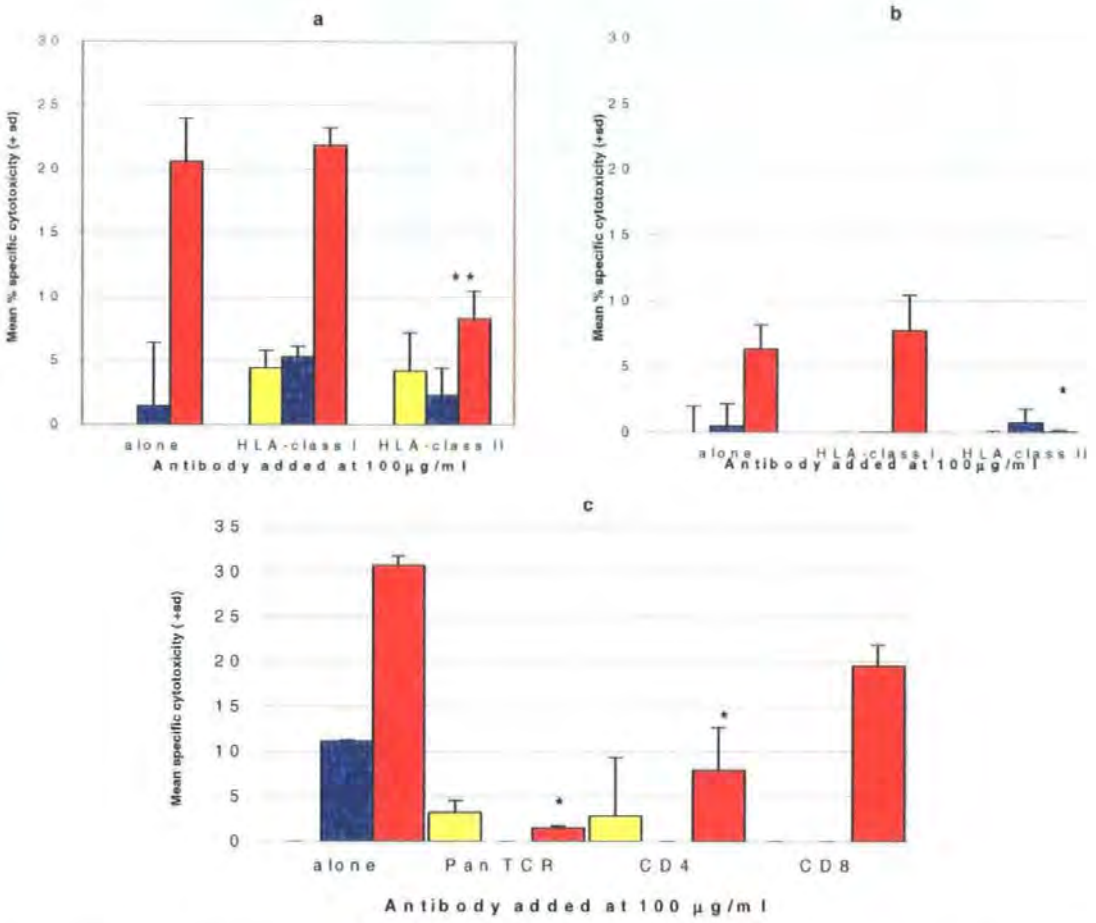


Figure 28: Antibody blocking of cytotoxicity generated from B-CLL patient T-cell effectors.

Figure 20: Antibody Blocking. T-cell effectors from B-CLL patient 2 (Figure 20a) and B-CLL patient 12 (Figure 20b) and patient 4 (Figure 20c) were cultured alone □ , with autologous B-CLL DCs pulsed with B-CLL lysate ■ or lysis buffer■ for 21 days. Anti-human HLA-class I and anti-human HLA-class II antibodies were present whilst effectors were incubated at a 40:1 effector :target ratio with autologous B-CLL B-cell targets (Figure 20a and Figure 20b). Anti-Pan TCR αβ , Anti-CD8 and Anti-CD4 antibodies were incubated with autologous B-CLL B-cell targets at a target: effector ratio of 40:1 (Figure 20c). Where (*) and (**) indicate a significant inhibition of specific cytotoxicity in the presence of antibody compared with none with $p<0.05$ and $p<0.01$ when analysed by Student's t-test. Each treatment group was measured in duplicate per patient.

5. RESULTS- FURTHER OPTIMISATION

5.1 Maturation of DCs with IFN-α

The low levels of cytotoxicity may be due to the relatively immature state of the monocyte derived DCs ($CD83^-$) used to present lysate antigens to the T-cells in our experimental system. After loading of antigen onto the dendritic cell surface a further 'danger signal' is required to achieve maximal presentation of that antigen to the T-cell [273]. Type I Interferon's (IFN) have shown maturation effects upon $CD34^+$ derived DCs [274]. IFN-α (2μg/ml) (Cambridge Bioscience) was added to monocyte derived DCs after pre-treatment with IL-12 and pulsing with either B-CLL lysate or lysis buffer on 7 days of culture. After 24 hours incubation the DCs were washed by centrifugation and added to T-cells as stated in Section 2.11. Cytotoxicity against B-CLL B-cell targets was assessed after a further 21 days of T-cell effectors in co-culture. In Patient 17, autologous T-cells cultured with lysate pulsed DCs matured by IFN-α showed decreased specific cytotoxicity to B-CLL targets (Figure 29). The addition of IFN-α to lysate pulsed monocyte derived DCs from Patient 17 or 27 did not stimulate cell surface expression of CD83. In Patient 27, a decrease in the number of cells positive for HLA-DR antibody staining was observed when the maturation agent was added to DCs after lysate pulsing. However, when IFN-α was added before lysate pulsing during the same experiment there was an increase in HLA-DR expression (Figure 30).

Figure 29: Effect of maturation by IFN-α on dendritic cell maturation as measured by cytotoxicity by T cells to autologous B-CLL targets.

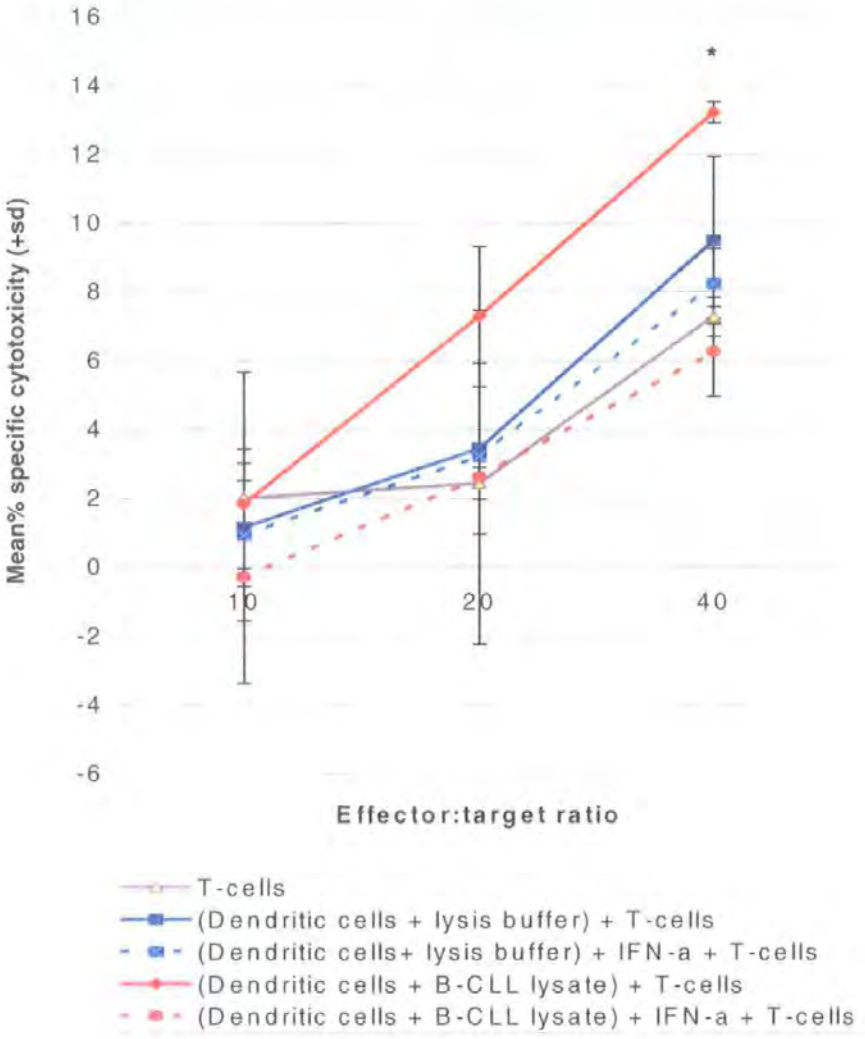


Figure 29: T-cell effectors generated from DCs treated or untreated with IFN-α (2μg/ml) for 24 hours were tested against autologous B-CLL B-cells from Patient 17. Mean specific cytotoxicity was generated from duplicates for each treatment point. (*) Indicates a significant decrease in cytotoxicity ($p=0.018$) when IFN-α was added to lysate pulsed DCs compared with no IFN-α.

Figure 30: Cell surface expression of HLA-DR on DCs with maturation agent IFN-α

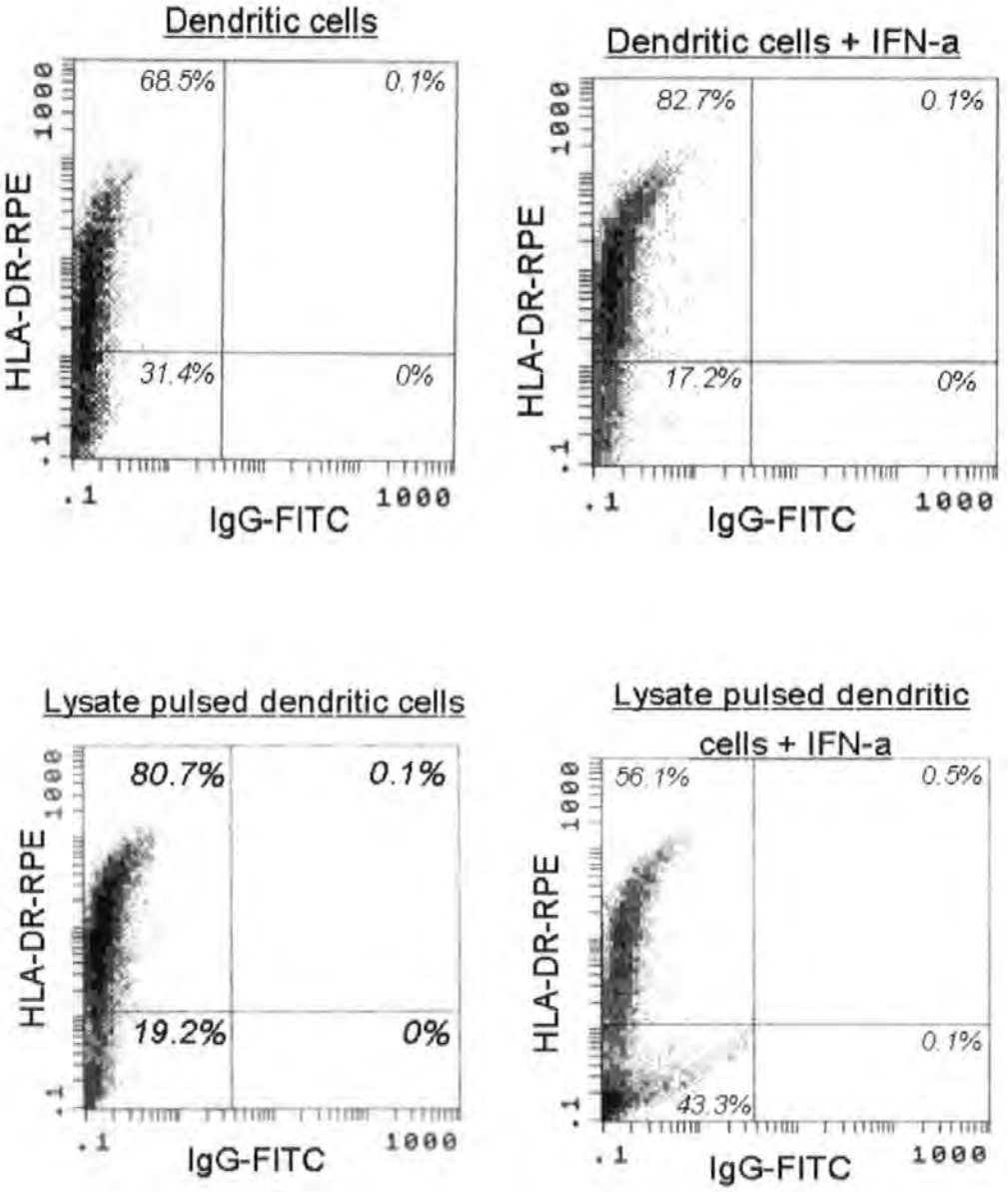


Figure 30: Dendritic cells from B-CLL Patient 27 were treated with IFN-α (2μg/ml) with and without previous exposure to autologous lysate and were stained with anti- Human HLA-DR-RPE conjugated antibodies as stated in Section 2.2.

5.2 Maturation of DCs with Poly (I:C)

Polyriboinosinic Polyribocytidylic Acid (Poly(I:C)) induces stable mature DCs capable of stimulation in a primary allogeneic MLR [275]. Therefore, it was another possible dendritic cell maturation agent that should be investigated. Poly (I:C) at 50μg/ml was added for 3 days after pulsing with B-CLL B-cell lysate to monocyte DCs. Although 50μg/ml was not the optimal concentration to achieve maximal effect, it did increase the cell surface expression of CD83 on immature monocyte DCs (Figure 31). T-cell effectors, from Patient 18, stimulated by Poly(I:C) matured and immature autologous DCs were tested for cytotoxicity against autologous B-CLL B-cell targets. At the 40:1 effector target ratio, autologous effector T-cells cultured with Poly (I:C) treated DCs exhibited decreased specific cytotoxicity to B-CLL B-cells. However, at the target: effector ratio of 20:1 specific anti-B-CLL B-cell cytotoxicity was increased when T-cell effectors had been cultured with autologous lysate pulsed DCs matured with Poly (I:C) (Figure 31).

Figure 31: The effect of maturation agents on CD83 expression by dendritic cells.

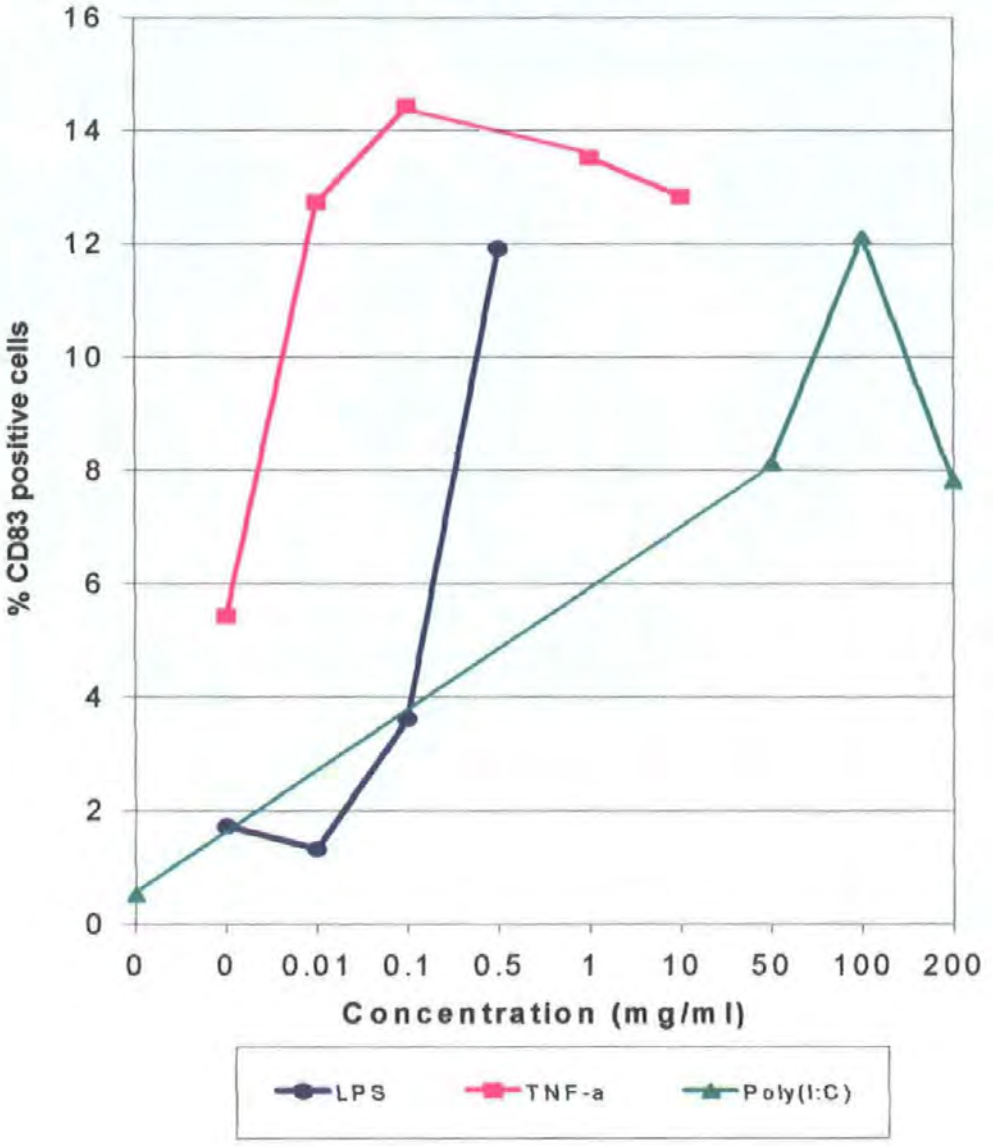


Figure 31: Dendritic cells from Patient 21 were isolated and cultured for 6 days as stated in Section 2.5. IL-12 pre-treatment and lysate were administered as previously stated in Section 2.10. Maturation agents were added to duplicate wells of 24 well plate at various concentrations. After 3 days incubation at 37°C, 5%CO_2 cells were removed using EDTA and stained for CD83-FITC staining as stated in Section 2.2.

Figure 32: Effect of Poly (I:C) on dendritic cell maturation as measured by cytotoxicity by T cells to autologous B-CLL B-cells

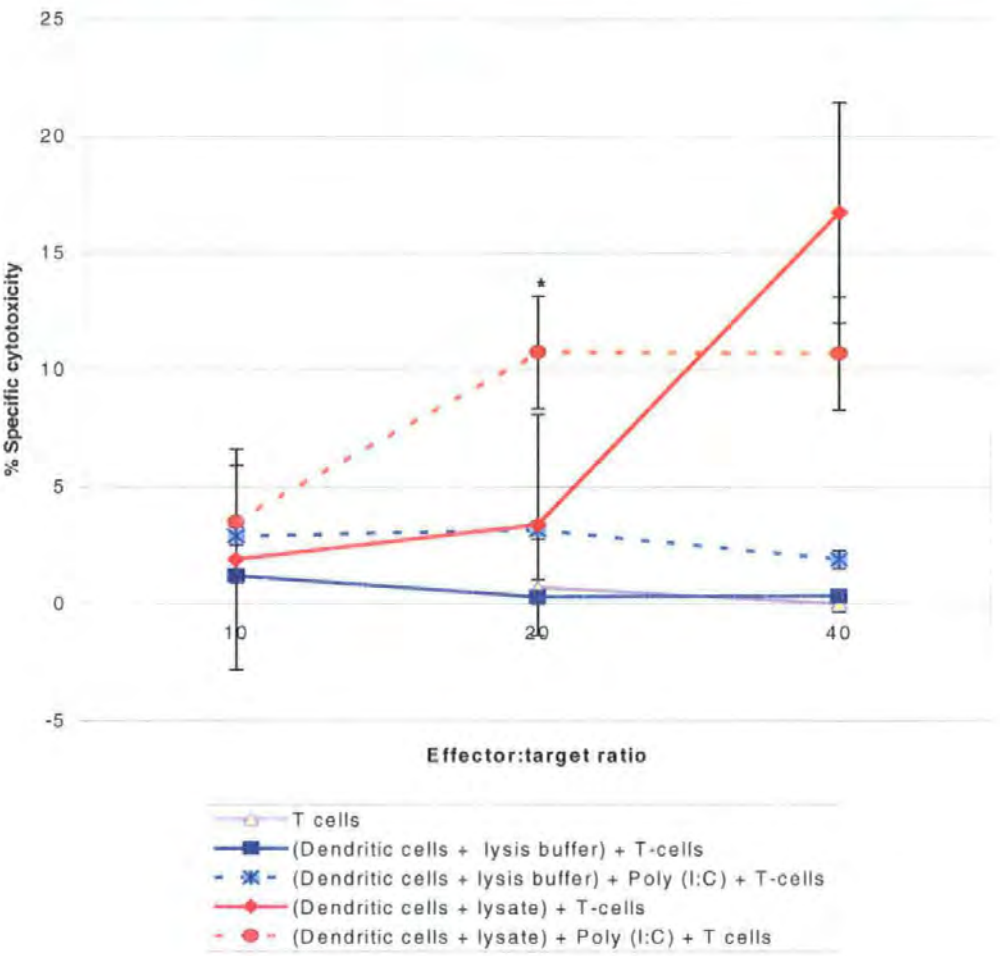


Figure 32: T-cell effectors generated from DCs treated or untreated with Poly (I:C) (50µg/ml) for 3 days were tested against autologous B-CLL B-cells from Patient 18 after co-culture for 21 days at 37°C, 5%CO_2. Mean specific cytotoxicity was generated from duplicates for each treatment point. (*) Indicates an increase due to use of Poly (I:C).

5.3 Maturation of DCs with LPS

Lipopolysaccharide (LPS) has been shown to induce maturation of DCs *in vivo* [109] and *in vitro* [276,277]. Therefore, LPS was another good candidate to increase the maturation of monocyte derived DCs from B-CLL patients. LPS was added to autologous DCs after pulsing with a B-CLL B cell lysate at 100ng/ml for 24 hours before co-culture with autologous T cells. Although this was not optimal for Patient 21 and so did not show peak expression in the concentration curve (Figure 31), the addition of LPS as a maturation agent resulted in 12% CD83 cell surface expression in Patient 19. A decrease in the numbers of double positive CD86/CD40 cells was also observed. There was no significant difference in IFN-γ secreted by T cells co-cultured with DCs matured with LPS after pulsing with B-CLL B cell lysate. There was no increase in specific cytotoxicity to B-CLL B-cell targets by effectors that had been cultured with lysate pulsed DCs matured with LPS. However, the autologous T cells co-cultured with DCs pulsed with lysis buffer and then treated with LPS showed increased levels of non-specific cytotoxicity (Figure 33).

Figure 33: Effect of LPS on dendritic cell maturation as measured by cytotoxicity by T cells to autologous B-CLL B-cell targets

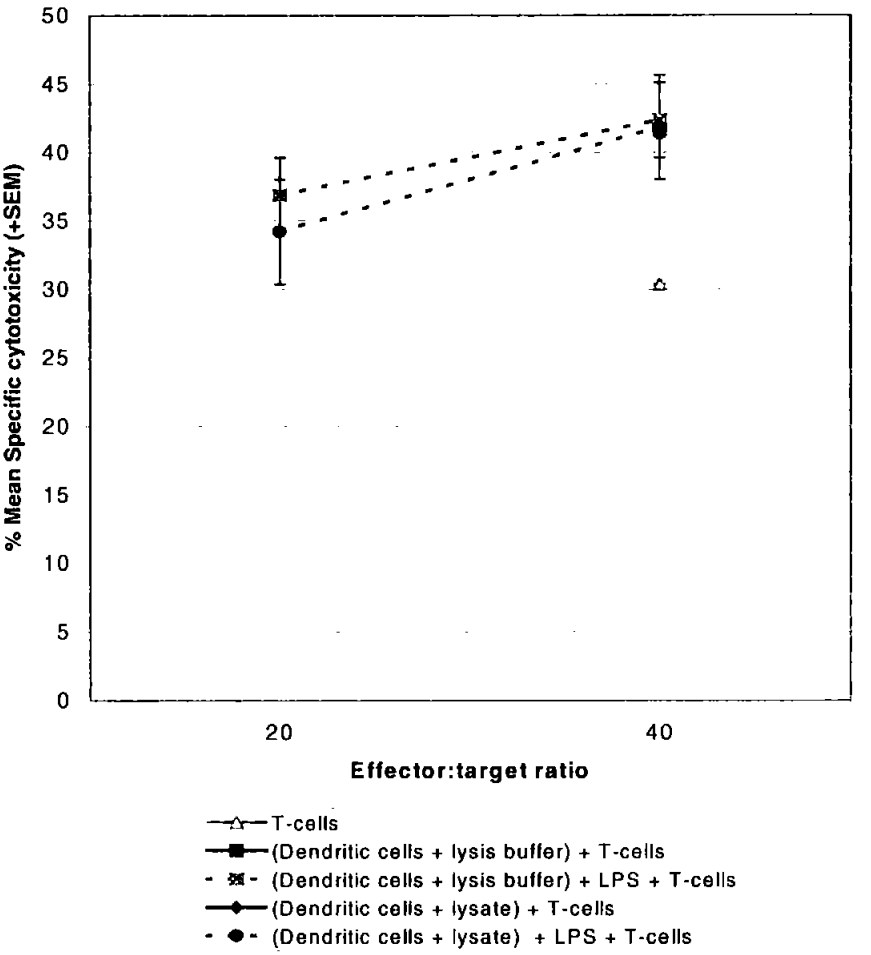


Figure 33: T-cell effectors from Patient 19 were generated from DCs treated or untreated with LPS (100ng/ml) for 24 hours and tested against autologous B-CLL B-cells after co-culture for 21 days at 37°C, 5%CO_2.

5.4 Maturation of DCs with TNF-α

Tumour Necrosis Factor-alpha (TNF-α) administration to monocyte derived DCs produced $CD1a^+$, $CD83^+$ DCs capable of maximal stimulation in an allogeneic Mixed Lymphocyte Reaction (MLR) [110]. Therefore TNF-α was used as a potential maturation agent. The addition of TNF-α as a maturation agent resulted in an increase in CD83 cell surface expression (Figure 31). TNF-α was added to DCs, from Patient 20, at 10ng/ml for 48 hours after pulsing with autologous B-CLL B-cell lysate and then co-cultured with autologous T cells. There was no difference in the numbers of double positive CD86/CD40 cells. At effector: target ratios of 10:1 and 20:1 there was increased levels of specific cytotoxicity to B-CLL B-cells by effector T-cells which had been stimulated by TNF-α treated autologous lysate pulsed DCs. However, at the higher effector: ratio of 40:1 there was no significant difference in cytotoxicity against B-CLL B cells by T-cells cultured with TNF-α matured lysate pulsed DCs (Figure 34).

Figure 34: Effect of TNF-α on dendritic cell maturation as measured by cytotoxicity by T cells to autologous B-CLL B-cell targets

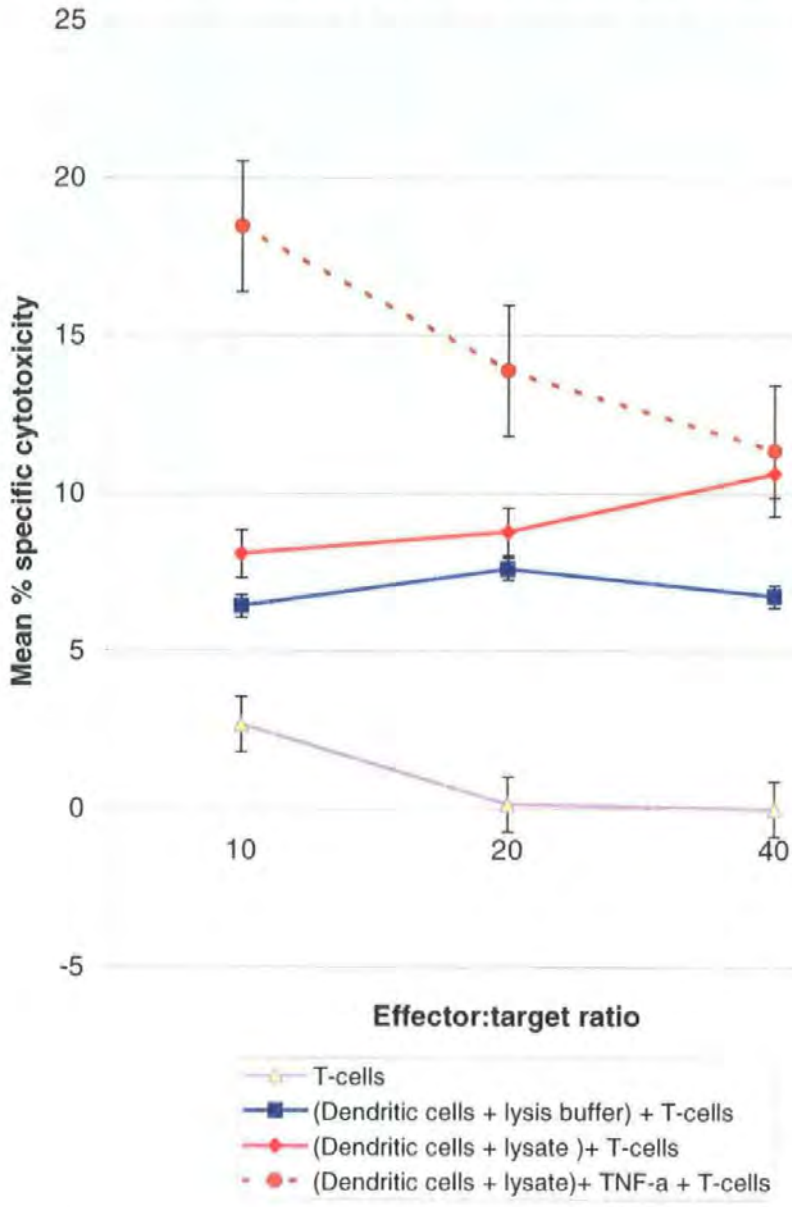


Figure 34: T-cell effectors from Patient 20 were generated from DCs treated or untreated with TNF-α (10ng/ml) for 48 hours and tested against autologous B-CLL B-cells after co-culture for 21 days at 37°C, 5%CO_2.

5.5 Use of Heat Shock to improve antigen presentation

Antigenic peptides have been coupled with tumour cell derived proteins to enhance macrophage responses against a wide range of tumours [278,279]. Therefore, it was thought that if the B-cells from B-CLL patients' experienced heat shock and that when they were used to prepare the lysate, these lysates would prove more antigenic. After isolation as outlined in Sections 2.4 and 2.6, the B-CLL B-cells were placed at a 45°C water bath for 90 minutes. This treatment has shown to induce heat-shock protein-70 (HSP-70) [280], heat-shock protein-72 (HSP-72) [281] and used in many studies involving heat shock protein [282, 283, 284]. B-cell lysate generated from heat-shocked B-CLL B-cells was compared with B-cell lysate from untreated B-CLL cells by pulsing onto autologous DCs and co-culture with autologous T cells from Patient 11. T cell effectors generated were then tested for cytotoxicity against autologous B-CLL B-cell targets. Heat-shock treatment of the B-cells used to prepare B-CLL lysate when pulsed onto DCs, increased observed cytotoxicity by autologous T-cell effectors at 40:1 and 20:1 effector: target ratios (Figure 35a). However, the increase in cytotoxicity was not significant when compared with that observed from T-cell effectors stimulated by DCs pulsed with lysate from B-cells not receiving heat-shock treatment. Interestingly, T-cell effectors cultured without DCs but with soluble heat shock lysate showed increased cytotoxicity to autologous B-cell targets. When the lysate was analysed by SDS-PAGE it was observed that the lysate from B-CLL B-cells were different to the lysate from heat-shock treated B-CLL B-cells in the same patient (Figure 35b).

Figure 35a: Effect of heat-shock treatment of B-CLL B-cells before lysate preparation on cytotoxicity against autologous B-CLL B-cell targets.

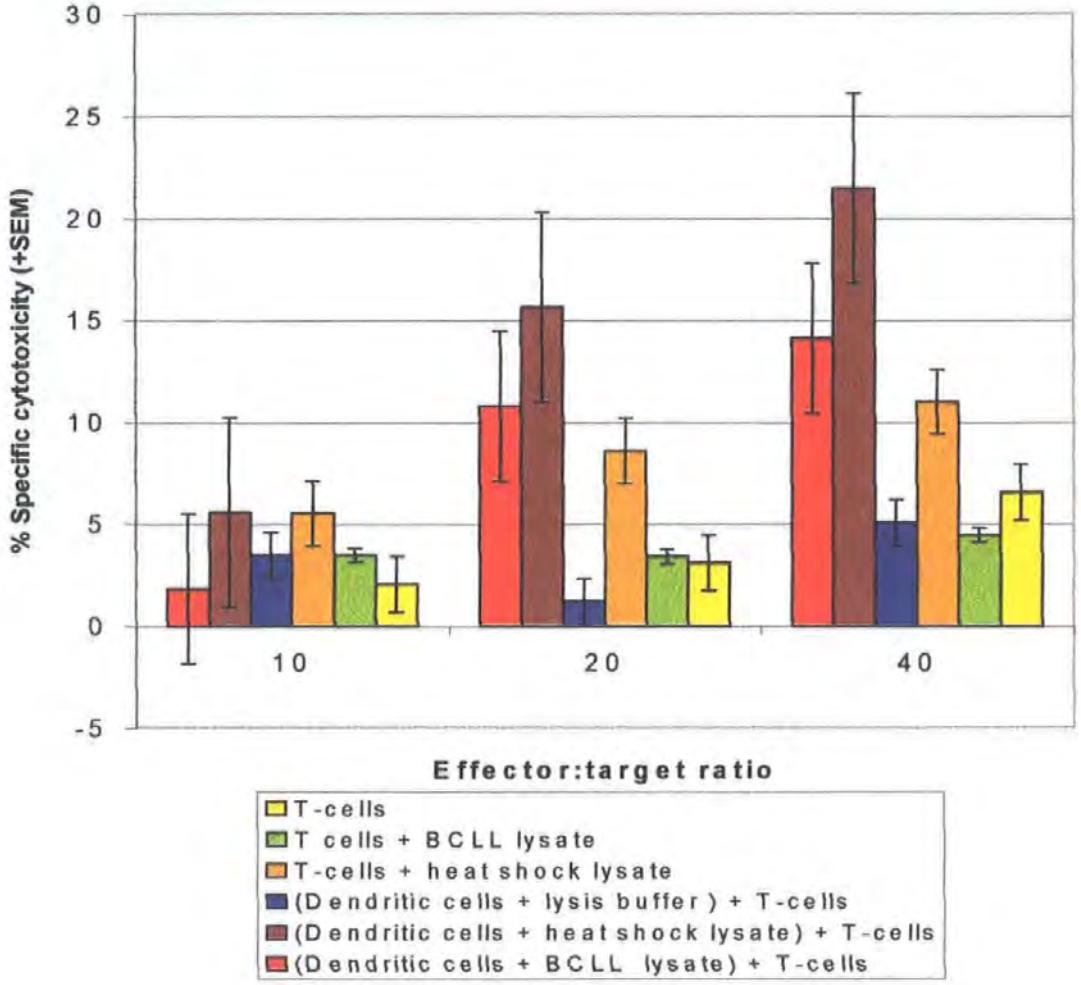
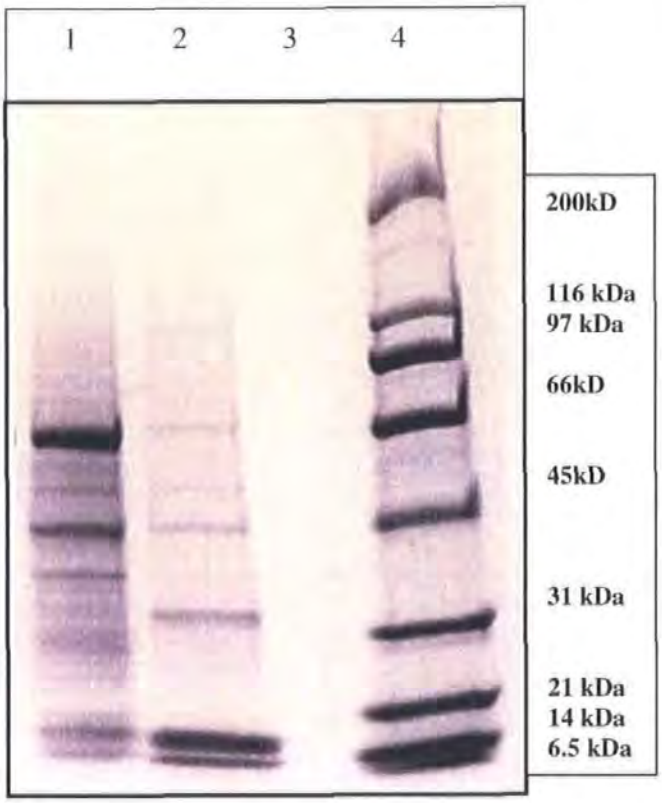


Figure 35a: B-cells from Patient 11 were heat-shocked at 45°C for 90 minutes prior to lysate preparation. Heat-shocked and untreated lysates were pulsed onto DCs which were co-cultured with autologous T-cells for 21 days at 37oC, 5%CO2. Cytotoxicity was measured against autologous B-cells.

Figure 35b: SDS-PAGE of heat shock treated B-cell lysate from B-CLL patient



1- 50 µg B-CLL B-cell lysate from Patient 11 *(626362.21)*

2- 50µg B-CLL lysate from heat shock treated B-cells from Patient 11*(254647.75)*

3- Lysis buffer

4- 100µg Broad Spectrum Molecular Weight markers.

Figure 35b: B cells from Patient 11 were isolated. Lysate was prepared from half and the remaining half was heat shock treated in a 45°C water bath for 90 minutes. Lysate was prepared from the heat shock treated cells. Both lysates were assayed for total protein content and analysed by SDS-PAGE. Italic numbers in brackets indicate total net intensity of each lane.

5.6 Pre-treatment of T cells with IL-15

T-cells from patients with B-CLL may have been exposed *in vivo* to possible immunosuppressive factors secreted by the B-CLL cells. T-cells of B-CLL patients show dysfunctional cell surface molecule expression[255]. Treatment with cytokines in conjunction with B-CLL B-cell lysates presented by monocyte derived DCs may be necessary to stimulate dysfunctional T cells. $CD4^+$ T-cells treated with IL-15 have shown enhanced antigen specific proliferation *in vitro* [285]. Since IL-15 can maintain T-cell activation normally suppressed by multiple myeloma cells [286], another late B lineage tumour, this effect was tested on T-cells from B-CLL patients. T-cells were plated at 8 x 10^5/ml in 24 well plates (Life Technologies, Invitrogen) in culture medium + 10% AB Serum + 10ng/ml IL-15 (Cambridge Bioscience). Cells were incubated for 16 hours at 37°C in 5% CO_2. T cells were washed in culture medium twice to remove any residual IL-15 before co-culture with DCs as stated in Section 2.12.

5.6.1 Proliferation (IL-2R expression) by IL-15 treated T-cells

A typical flow cytometric profile from Patient 21 after 7 days incubation can be seen in Figure 36. T-cells were stimulated by DCs pulsed with autologous B-CLL B-cell lysate as previously observed in Section 4.1. However, the percentage of activated T-cells was further increased by the T-cells being pre-treated with IL-15 before stimulation by lysate pulsed DCs (Figure 36). The experiment was repeated in B-CLL patient 22. There was an observable increase in the numbers of activated T-cells after 5 days (in patient 22) and 7 days (in patient 21) co-culture with autologous B-CLL lysate pulsed DCs than T cells co-cultured with autologous DCs pulsed with lysis buffer (Figure 37). T-cells treated with IL-15 prior to culture with autologous DCs pulsed with B-cell lysate showed an observable increase in activation after 7 days than IL-15 pre-treated T-cells co-cultured with autologous DCs pulsed with lysis buffer in both Patients 21 and 22 (Figure 37). T-cells that had received prior treatment with IL-15 showed greater activation levels after 7 days of stimulation by B-CLL lysate pulsed DCs than those not pre-treated with IL-15 in both patients (Figure 37).

Figure 36: Comparison of T cell proliferation (IL-2R expression) after IL-15 treatment from a B-CLL patient

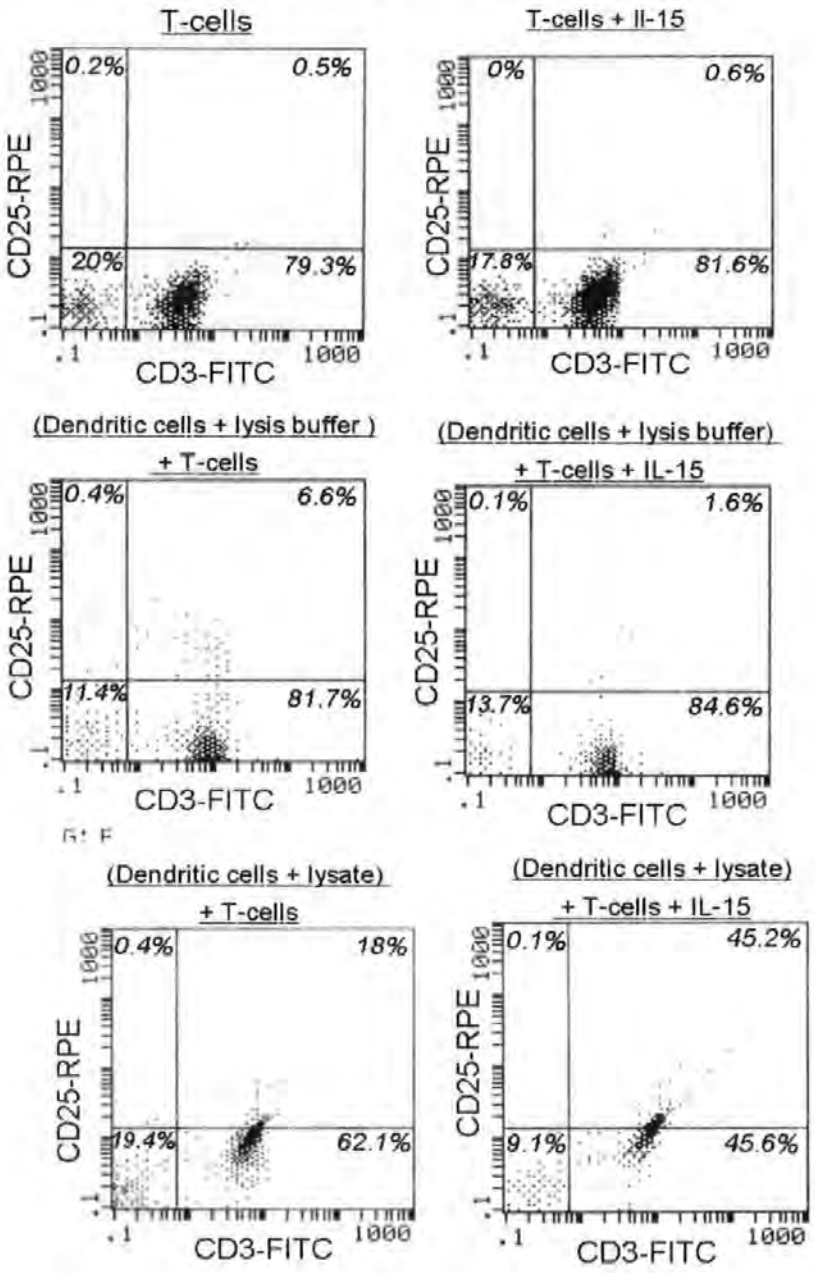


Figure 36: Pulsed or unpulsed DCs from Patient 21 were co-cultured with T-cells alone or T-cells treated with IL-15 (10ng/ml) for 16 hours. Typical examples shown were incubated for 7 days at 37°C, 5% CO_2 in RPMI 1640+ 5%AB serum + Penicillin + Glutamine + Streptomycin. Cells were harvested and stained for activation markers as stated in Section 2.14.

Figure 37a: Effect of IL-15 pre-treatment upon T cell proliferation (IL-2R expression) after stimulation by B-CLL lysate pulsed DC

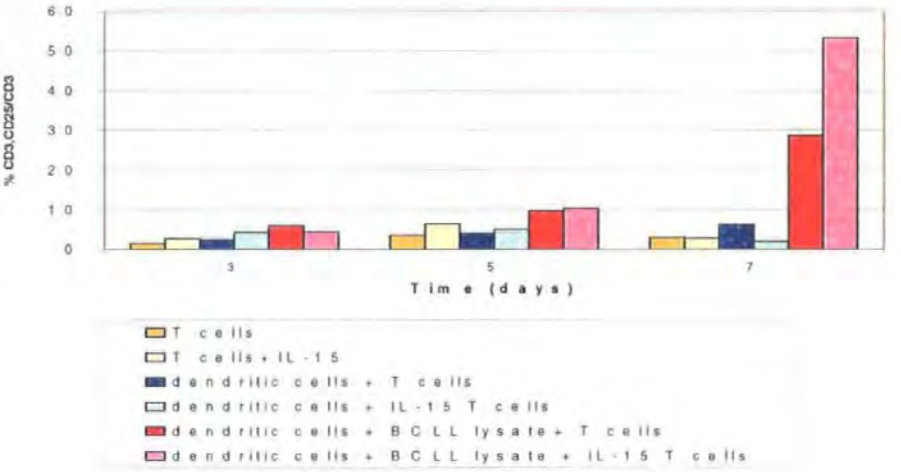


Figure 37b: Effect of IL-15 pre-treatment upon T-cell proliferation (IL-2R expression) after stimulation by B-CLL lysate pulsed DC

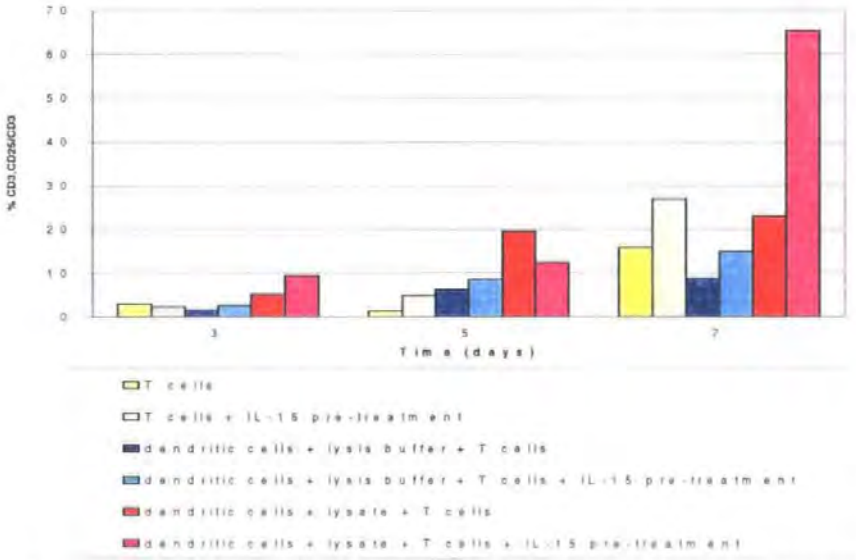


Figure 37a and b: Numbers of CD3/CD25 positive T-cells were measured from Patient 21 (Figure 37a) and Patient 22 (Figure 37b). T-cells were cultured alone or pre-incubated with IL-15 (10ng/ml) for 16 hours and then cultured alone or with autologous B-CLL DCs pulsed with a soluble B-CLL lysate or lysis buffer.

5.6.2 IFN-γ secretion by IL-15 pre-treated T cells

A significant increase of IFN-γ secretion in culture supernatant was found after 4 days by T-cells cultured with B-CLL lysate-pulsed autologous DCs compared to both T-cells cultured with lysis buffer-pulsed DCs and T-cells cultured alone *(p=0.038)* (Figure 38). T-cells derived, from the same B-CLL patients, treated with IL-15 prior to culture with autologous DCs pulsed with B-cell lysate showed a significant increase in IFN-γ secretion after 1 day *(p=0.018)*(Figure 38). More IFN-γ was secreted by T-cells which had been IL-15 treated and then cultured with B-CLL lysate-pulsed autologous DCs *(p=0.030)*(Figure 38).

5.6.3 Cytotoxicity to B-CLL targets generated by T-cell effectors pre-treated with IL-15

T-cells co-cultured with B-CLL lysate pulsed DCs showed significantly higher levels of specific cytotoxicity to autologous B-CLL B cell targets when compared with DCs pulsed with lysis buffer *(p=0.019)*(Figure 39). T-cells, from the same B-CLL patients, pre-treated with IL-15 prior to culture with autologous DCs pulsed with B-cell lysate also showed significantly higher levels of specific cytotoxicity to B-CLL B-cell targets *(p=0.002)*(Figure 39). T cells co-cultured with autologous B-CLL lysate pulsed DCs showed significantly greater specific cytotoxicity against B-CLL B-cell targets when pre-treated with IL-15 *(p=0.006)* (Figure 39).

Figure 38: Effect of IL-15 pre-treatment on IFN-γ secretion by T-cell effectors stimulated by autologous B-CLL B-cell lysate pulsed DCs.

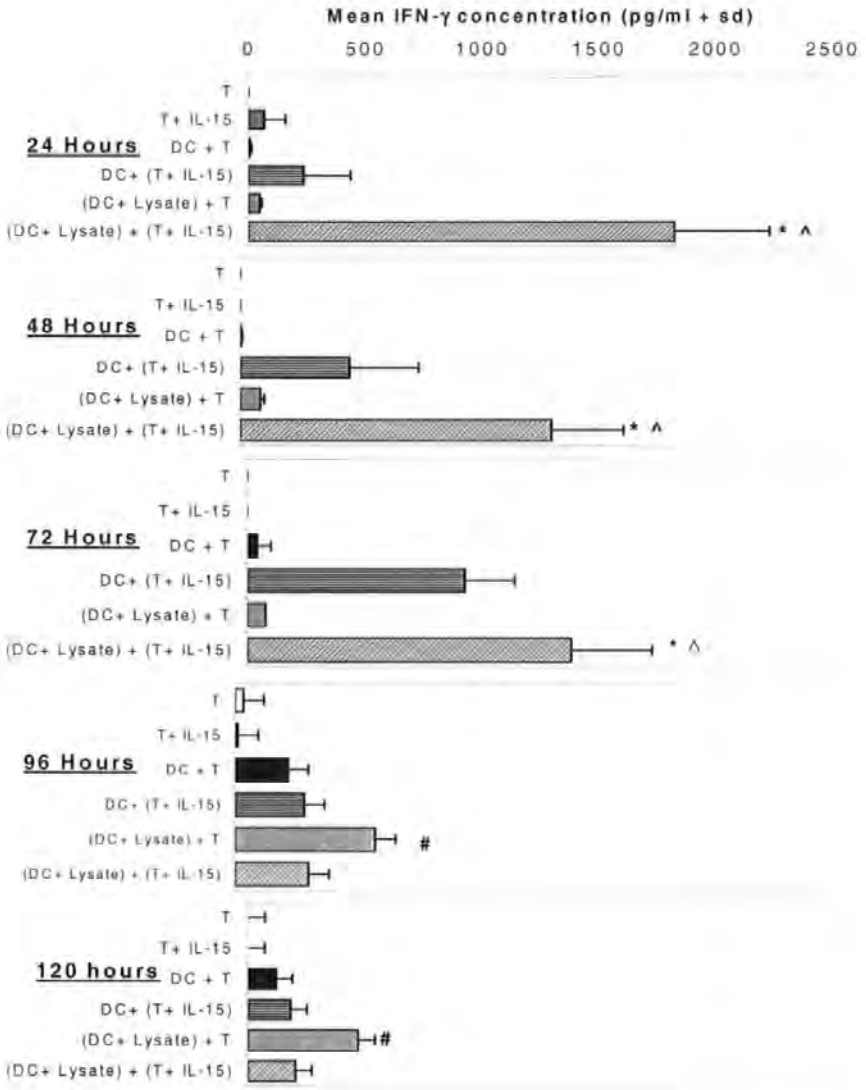


Figure 38: Tissue culture supernatants from patients 21 and 23 were measured for secreted IFN-γ by ELISA. T-cells were cultured alone or pre-incubated with IL-15 (10ng/ml) for 16 hours and then co-cultured for 21 days. Mean values were tested using unpaired Students' t-test. # indicates a significant *(p=0.038)* difference between {(DC+Lysate)+T} and {DC+T}. * indicates a significant *(p=0.018)* difference between {(DC+Lysate)+(T+IL-15)} and {DC+(T+IL-15)}. ^ indicates a significant *(p=0.030)* difference between {(DC+Lysate)+(T+IL-15)} and {(DC+Lysate)+T }. Each treatment group per patient was measured in duplicate.

Figure 39: Effect of IL-15 pre-treatment on cytotoxicity of T-cell effectors from B-CLL patients

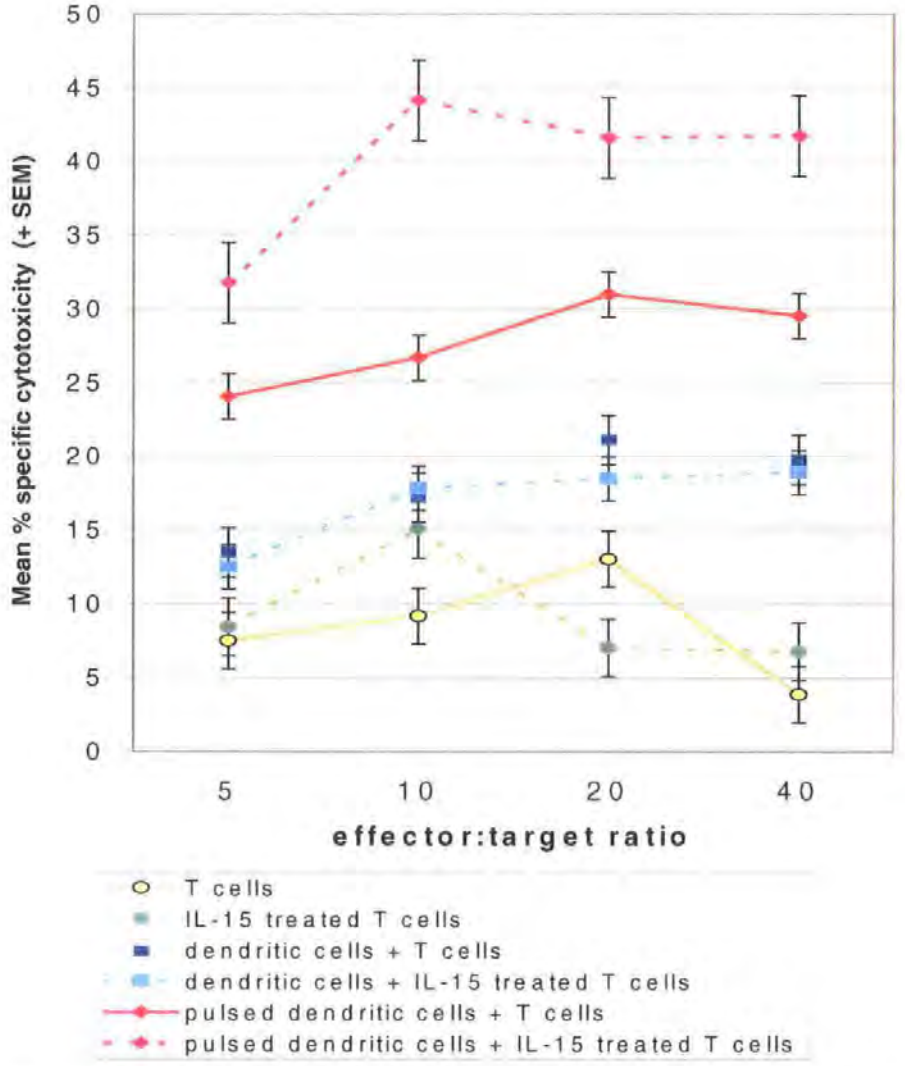


Figure 39: Effectors from patients 5,6,and 7 were pre-incubated with IL-15 (10ng/ml) for 16 hours and then co-cultured for 28 days and tested for cytotoxicity against autologous B-CLL B-cell targets. One measurement was made per patient per treatment. Data was analysed by one-way ANOVA. Overall significant effect of stimulation by lysate pulsed DCs *($p=0.019$)*. Overall significant effect of stimulation of IL-15 pre-incubated T-cells by lysate pulsed DCs *($p=0.002$)*. Overall significant effect of pre-incubation with IL-15 *($p=0.006$)*.

5.6.4. Effect of IL-15 pre-treatment on cytotoxicity to K562 cells

IL-15 can induce Natural Killer (NK) cell development [287] and increase cytotoxic activity of NK cells [288]. K562, an erythroleukemia cell line, are sensitive to NK cell mediated cytotoxic activity [289]. It was possible that the increase in cytotoxicity observed in Figure 39 was due to NK activity and not T-cell effectors. Therefore, pre-treated T-cell effectors were also tested for cytotoxicity against K562 cells. K562 T cells co-cultured with B-CLL lysate pulsed DCs showed no specific cytotoxicity to K562 cell targets (Fig 40). IL-15 pre-treated T cells co-cultured with autologous B-CLL lysate pulsed DCs did not show significant cytotoxicity to K562 cell targets (Figure 40). T cells co-cultured with autologous DCs pulsed with B-CLL B-cell lysate showed similar levels of cytotoxicity to K562 cell targets whether pre-treated with IL-15 or without cytokine (Figure 40).

Figure 40: Effect of IL-15 pre-treatment on cytotoxicity of T-cell effectors from B-CLL patients against K562 cells.

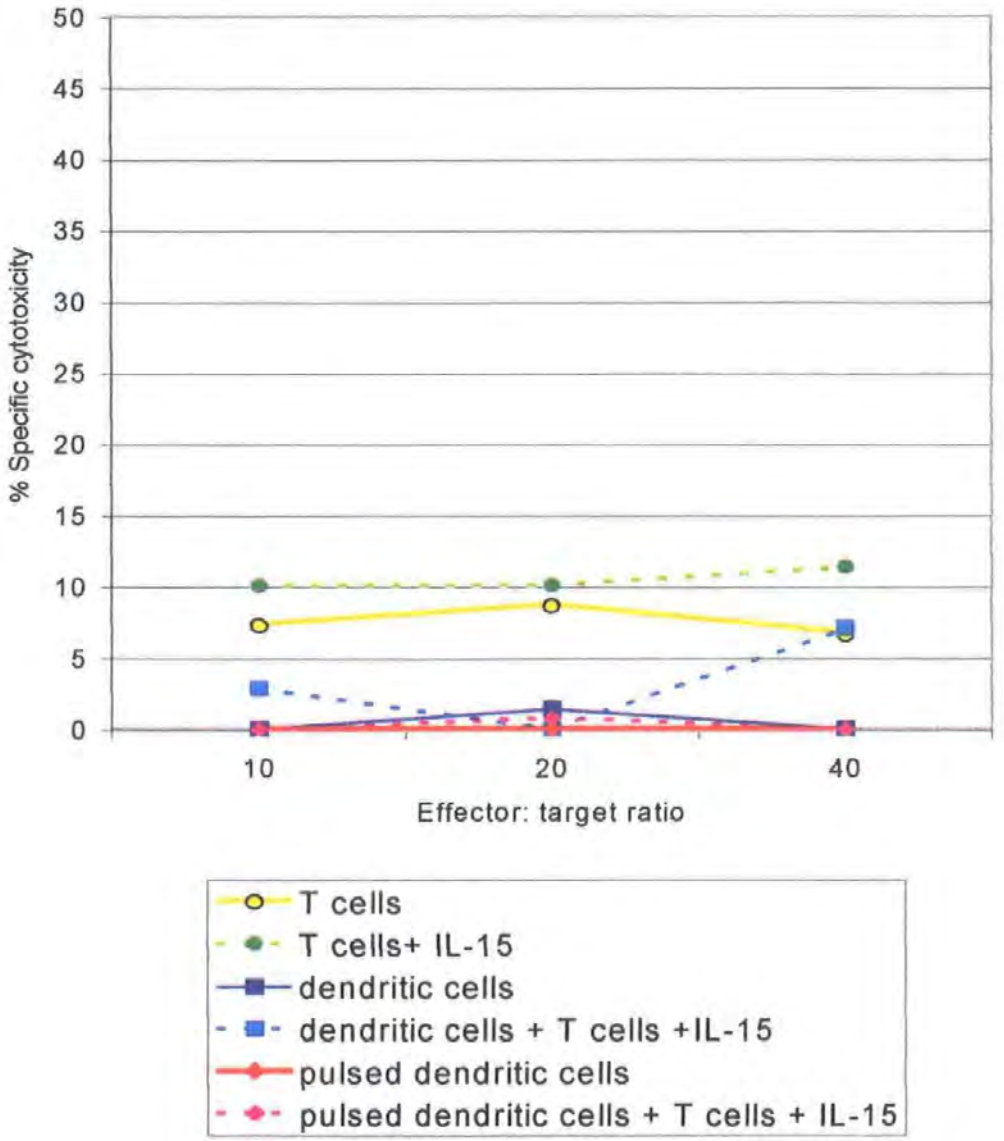


Figure 40: Effectors from patient 7 were pre-incubated with IL-15 (10ng/ml) for 16 hours and then co-cultured for 28 days and tested for cytotoxicity against K562 cell targets. One measurement was made per treatment group

5.7 Fusion of DCs and B-CLL B cells

5.7.1 Introduction

The majority of effective anti-tumour responses generated are MHC class-I-restricted, but data presented in this thesis suggests a predominant MHC class-II-restricted anti-tumour response in B-CLL. The generation of $CD8^+$ cytolytic T-lymphocytes (CTL) as well as a $CD4^+$ cytokine and cytotoxic anti-tumour response would be necessary for effective vaccination in patients with B-CLL. Priming of CTL by native antigens requires introduction into the cytoplasm [290, 291]. A strategy that assures that antigens will be delivered and processed efficiently in a MHC class-I –restricted manner is to fuse APCs with tumour cells. Whole cell vaccines have been produced in this manner for several different tumours [292, 293, 294]. Therefore fusion of monocyte derived DCs with autologous B-CLL B cells was attempted by electroporation in an effort to generate $CD8^+$ CTL.

Table 4: Summary of electrofusion efficiencies and yields

Fusion method	Number of viable cells	Percentage fused cells	Total fused cell yield
Mixed not fused (control)	4.3×10^5	6.6%	2.8×10^4
250 V non-separated	3×10^5	10.4	3.1×10^4
250V separated on 10% Dextran	1×10^4	50.5	5×10^3
500V non-separated	1×10^5	45.0	4.5×10^4
500V separated on 10% Dextran	5×10^4	83.1	4.1×10^4

Table 4: B-CLL B-cells from Patient 8 were electrofused using Gene Pulser Transfection Apparatus (Biorad). Viability counts were estimated by counting cells excluding Trypan Blue dye using haemocytometer 16 hours after voltage application. Percentage of cells fused were calculated from flow cytometry labelling with CD20-RPE and CD86-FITC antibodies under stated conditions 16 hours after voltage application.

Figure 41: Flow cytometric analysis of fused B-CLL and DCs

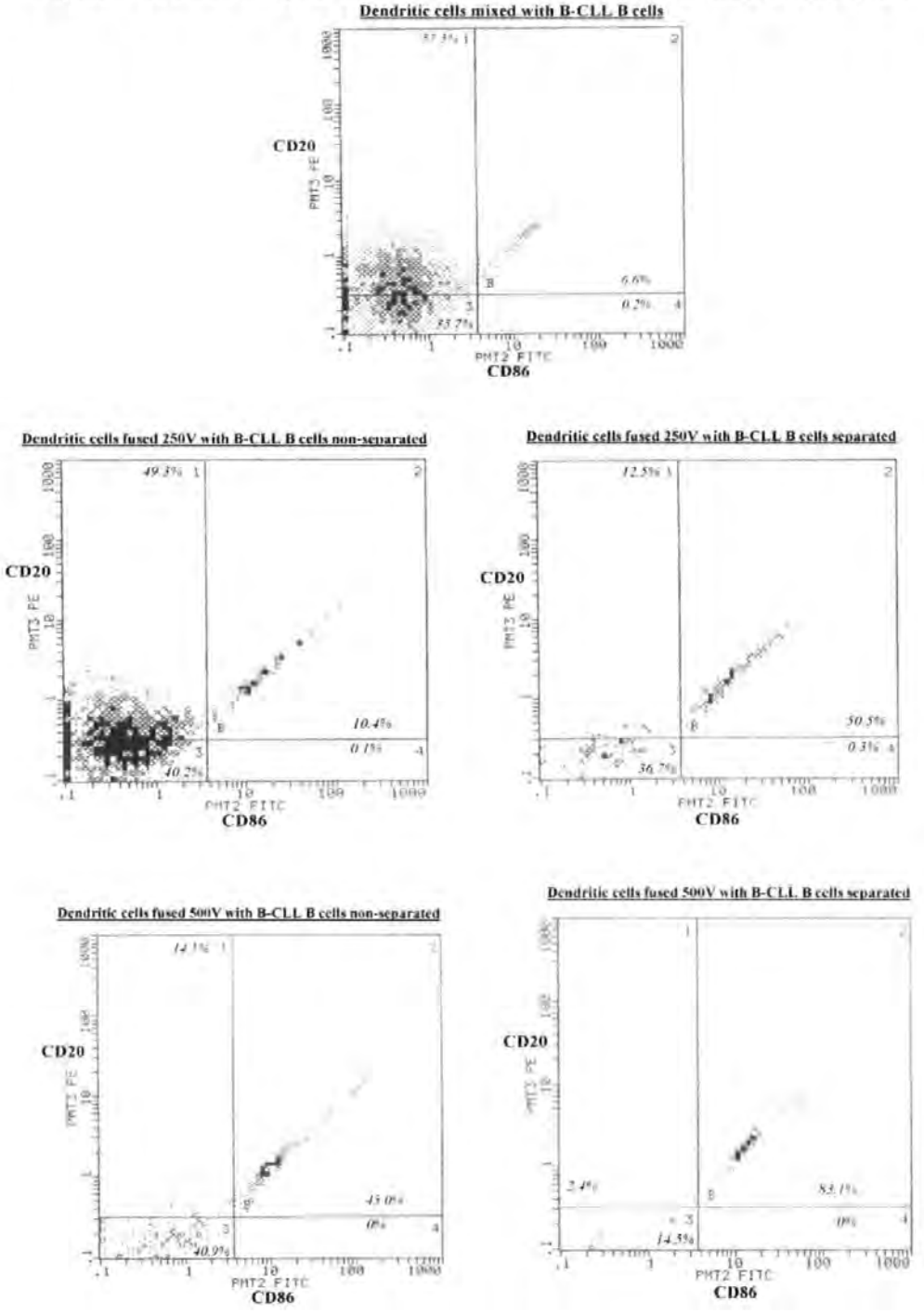


Figure 24: Fused cells were labelled with CD20- RPE and CD86-FITC antibodies, as stated in Section 2.2, 16 hours after voltage application.

5.7.2. Results

T-cell effectors from the same B-CLL patient were generated via two different approaches. Some were generated by co-culture of T-cells with B-CLL lysate pulsed DCs and others were generated by co-culture of T-cells with B-CLL dendritic cell-B-cell fusion cells. T-cell effectors generated in these two different ways were then tested for cytotoxicity against autologous B-CLL B-cell targets as a direct comparison. As previously observed (Section 4.6), T-cell effectors cultured with lysate pulsed autologous DCs showed a significantly higher percentage specific cytotoxicity against B-CLL B-cell targets than T-cell effectors cultured with autologous DCs pulsed with lysis buffer *($p=0.037$)* (Figure 42). T-cell effectors cultured with autologous fused dendritic-cell-B-cell hybrids showed a significantly higher percentage specific cytotoxicity against B-CLL B-cells than other T-cell effectors *($p=0.016$)*(Figure 42). A direct comparison in the same B-CLL patient showed that autologous fused dendritic-cell-B-cell hybrids were more effective at stimulating T-cell effectors with specific cytotoxicity against B-CLL B-cells than autologous DCs pulsed with B-CLL B-cell lysate *($p=0.013$)* (Figure 42).

Figure 42: Cytotoxicity to B-CLL B-cells by T-cells generated by co-culture with fusion hybrids

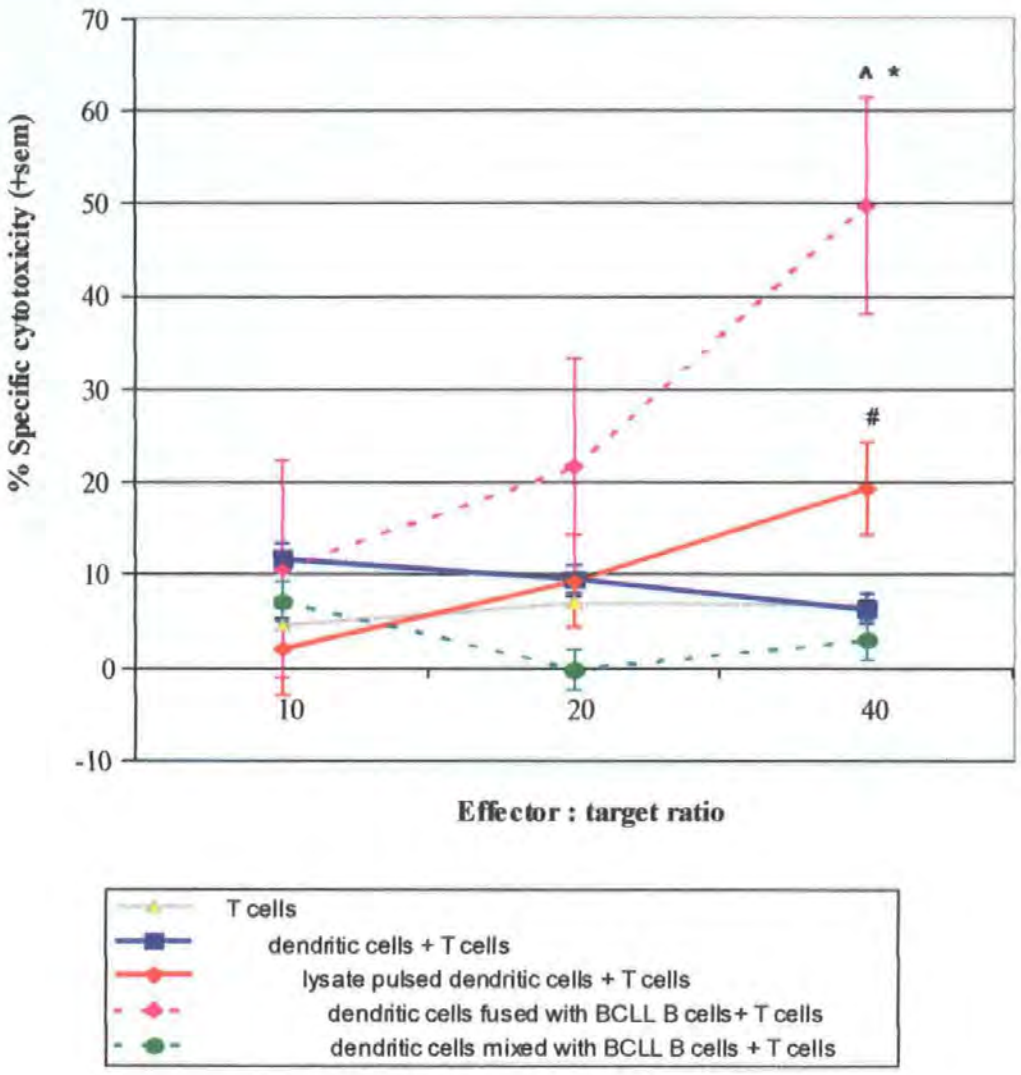


Figure 42: Effectors from Patient 8 were cultured for 28 days and cytotoxicity against autologous B-CLL B cell targets at a 40:1 ratio was measured. Data were analysed by unpaired Students' t-test. Where # indicates significant *(p=0.037)* increase in cytotoxicity due to pulsing DCs with lysate. Where ^ indicates significant *(p=0.016)* increase in cytotoxicity due to electrofusion of DCs with B-CLL B-cells rather than mixing the 2 cell types. Where * indicates significant *(p=0.013)* increase in cytotoxicity due to electrofusion of DCs with B-CLL cells rather than pulsing with lysate. Each treatment group was measured in duplicate.

Figure 43: Effect of monoclonal antibodies on cytotoxicity of T-cell effectors stimulated by co-culture with fusion hybrids.

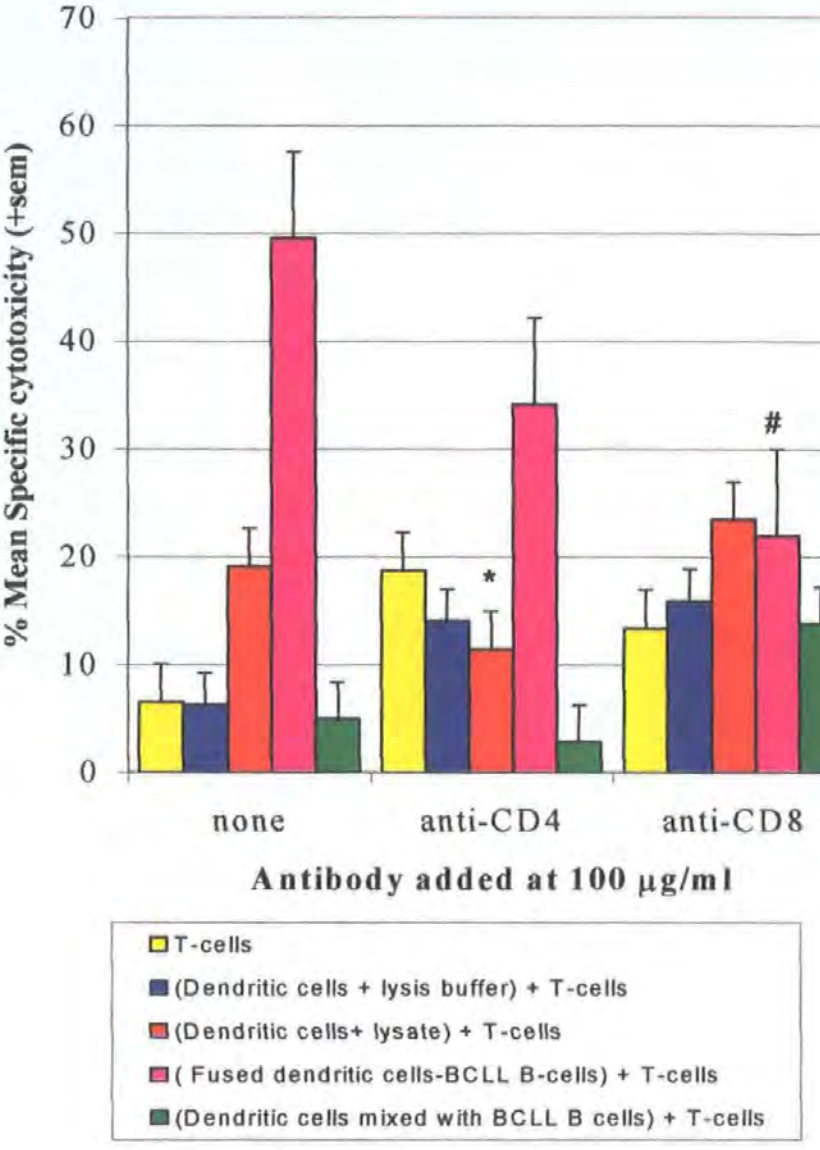


Figure 43: Effectors from Patient 8 were cultured for 28 days and cytotoxicity against autologous B-CLL B-cell targets at a 40:1 ratio was measured. Anti-human CD4 and anti-human CD8 antibodies (100mg/ml) were added to effectors for 4 hours when incubated with targets. Data were analysed by unpaired Students' t-test, where * indicates significant *($p=0.039$)* inhibition with anti-human CD4 antibodies of cytotoxicity stimulated by lysate pulsed DCs and # indicates significant *($p=0.018$)* inhibition with anti-human CD8 antibodies of cytotoxicity stimulated by electrofused DCs. Each treatment group was measured in duplicate.

Monoclonal antibodies against Human CD4 and CD8 were incubated with all the T cell effectors during the cytotoxicity assay incubation period. T-cell effectors stimulated by lysate pulsed autologous DCs showed a significant inhibition of specific cytotoxicity against B-CLL B-cell targets by human anti-CD4 antibodies *($p=0.039$)* (Figure 43). T-cell effectors stimulated by autologous dendritic-cell-B-cell hybrids showed a decrease in cytotoxicity against B-CLL B-cells of 14% when incubated with anti-human CD4 antibodies *($p=0.062$)*(Figure 43). Most importantly, T-cell effectors stimulated by autologous dendritic-cell-B-cell hybrids showed a significant inhibition of cytotoxicity against B-CLL B-cells by anti-human CD8 antibodies *($p=0.018$)* (Figure 43).

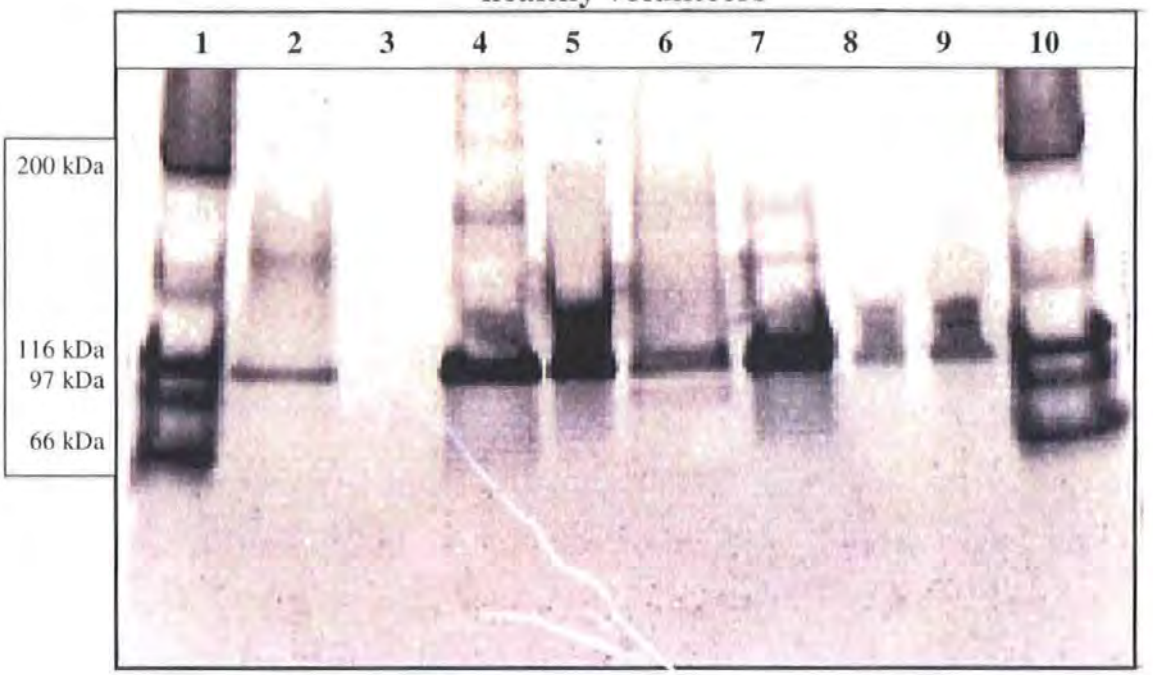
6. Results-Properties of Lysates

At present there are no documented peptide sequences that could be responsible for the anti-tumour responses demonstrated. However, differences between B-cell lysates of normal healthy volunteers and B cells from B-CLL patients have previously been detected by 2-D electrophoresis [295]. However, no functional tests have ever been performed to assess the importance of these B-CLL specific proteins. A quick method for identification of possible proteins within the lysate that could act as target anti-tumour antigens was required in order to perform functional tests using the *in vitro* dendritic cell system.

6.1 Native PAGE

A typical example of the native gel containing B-cell lysates from both B-CLL patients and normal healthy volunteers is illustrated in Figure 44. A single dominant band at between 116 kDa and 97 kDa were observed in all the lysates. Additional fainter bands were seen in both lysates from B-CLL patients and normal healthy volunteers. There was not a clearly visible difference between lysates generated from B-CLL patients and normal healthy volunteers. Any differences observed using non-reduced gels was dependant upon the amount of protein contained within the lysate loaded onto each gel.

Figure 44: Native gel of B-cell lysates from B-CLL patients and normal healthy volunteers



1- 100µg Broad spectrum molecular weight standards
2- 50µg B-cell lysate B-CLL patient 13
3- Lysis buffer
4-100µg B-cell lysate B-CLL patient 10
5-100µg B-cell lysate normal healthy volunteer
6-50µg B-cell lysate B-CLL patient 28
7-100µg B-cell lysate normal healthy volunteer
8-50µg B-cell lysate B-CLL patient 15
9-50µg B-cell lysate B-CLL patient 6
10-100µg Broad spectrum molecular weight standards

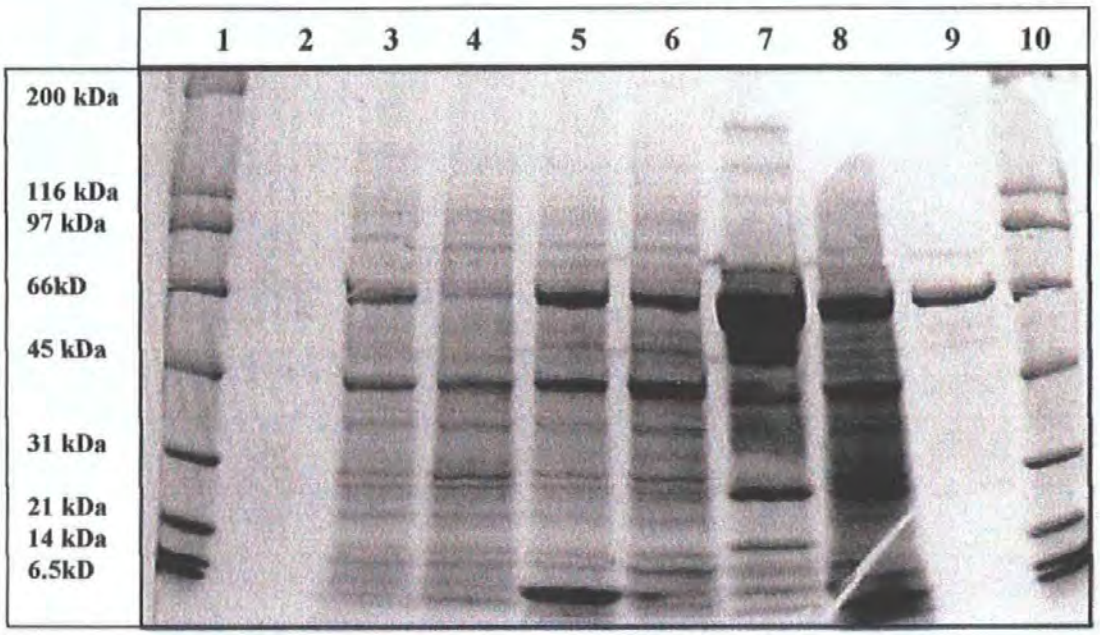
Figure 44: Lysates were assayed for protein content as stated in section 2.9. Samples were loaded onto a native gel and stained according to section 2.19.

6.2 SDS-PAGE

It should be noted from Figure 45a that all the B cell lysates had an obvious protein visible at the 65 kDa molecular weight. Many more protein bands were observed in the protein lysates from B-CLL patients where the same total protein had been loaded onto the

reducing gel as the B cell lysate from a control healthy volunteer (Lanes 3, 4, 13, 14 vs. Lane 9, 16, 18). Proteins were detected in some patients that were not observed in all of them (Lanes 5 and 7). We have not correlated these patient specific bands with stage or cyto-genetics.

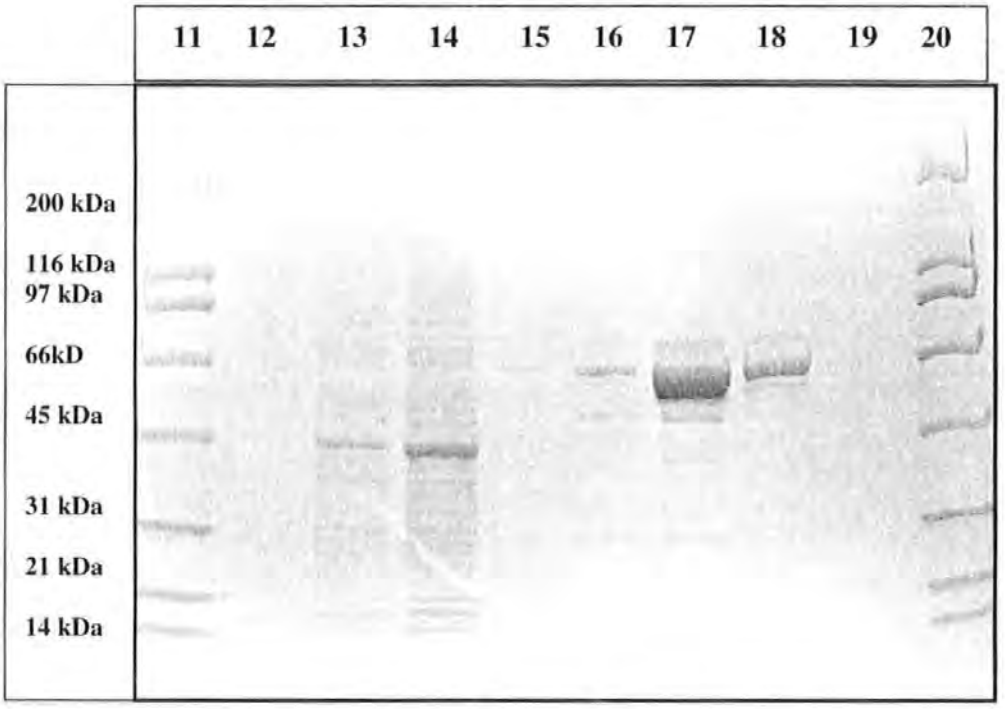
Figure 45a: SDS-PAGE of B-cell lysates from B-CLL patients and normal healthy volunteers



1- 100µg Molecular weight standards
2- Lysis buffer
3- 50µg B-cell lysate B-CLL patient 28
4- 50µg B-cell lysate B-CLL patient 9
5- 100µg B-cell lysate B-CLL patient 13
6- 100µg B-cell lysate B-CLL patient 2
7- 100µg B-cell lysate B-CLL patient 10
8- 100µg B-cell lysate B-CLL patient 16
9- 50µg B-cell lysate healthy volunteer 1
10- 100µg Molecular weight standards

Figure 45a: Lysates were assayed for protein content as stated in section 2.9. Samples were loaded onto a reducing gel and stained according to section 2.18.

Figure 45b: SDS-PAGE of B-cell lysates from B-CLL patients and normal healthy volunteers



11- 100µg Molecular weight standards

12- Lysis buffer

13- 50µg B-cell lysate B-CLL patient 17

14- 50µg B-cell lysate B-CLL patient 29

15- 10µg B-cell lysate healthy volunteer 2

16- 50µg B-cell lysate healthy volunteer 3

17- 100µg B-cell lysate healthy volunteer 4

18- 50µg B-cell lysate healthy volunteer 5

19- lysis buffer

20- 100µg Molecular weight standards

Figure 45b: Lysates were assayed for protein content as stated in section 2.9. Samples were loaded onto a non-reducing gel and stained according to section 2.18.

When 50 μg of B-cell lysate from B-CLL patients was loaded onto the gel a 42 kDa band not seen in the B cell lysate from a healthy volunteer was observed. This observation was made in 10 B-CLL patients and 5 normal controls as summarised in Figure 46a. However, when 100ug was loaded onto the gels a faint 42 kDa protein could be observed in the lysate from 1 healthy volunteer's B-cells (Figure 45b). Therefore the lysates loaded at 100μg were analysed separately as summarised in Figure 46b. In order to draw a direct comparison between these lysates a series of dilutions of both lysates from B-CLL patients and normal healthy controls was performed (Figure 47). This was because the protein estimates may have been incorrect. The difference in expression of this B-CLL specific band may have been due to less total protein being added in the B-cell lysates from healthy volunteers. Figure 47 illustrates that at comparable total protein concentrations the 42 kDa protein band is clearly more highly expressed within the B-cell lysate from a B-CLL patient than a healthy volunteer. A band of less intensity was also observed at 25 kDa in most B-CLL patients (Figure 46a and 46b). We chose to test the functional capability of the 65 kDa, 42kDa and 25kDa bands present in all the B-CLL lysates by pulsing these separated bands onto DCs.

Figure 46a: Comparison of digital optical densimetry profiles of B-cell lysates from B-CLL patients and healthy volunteers

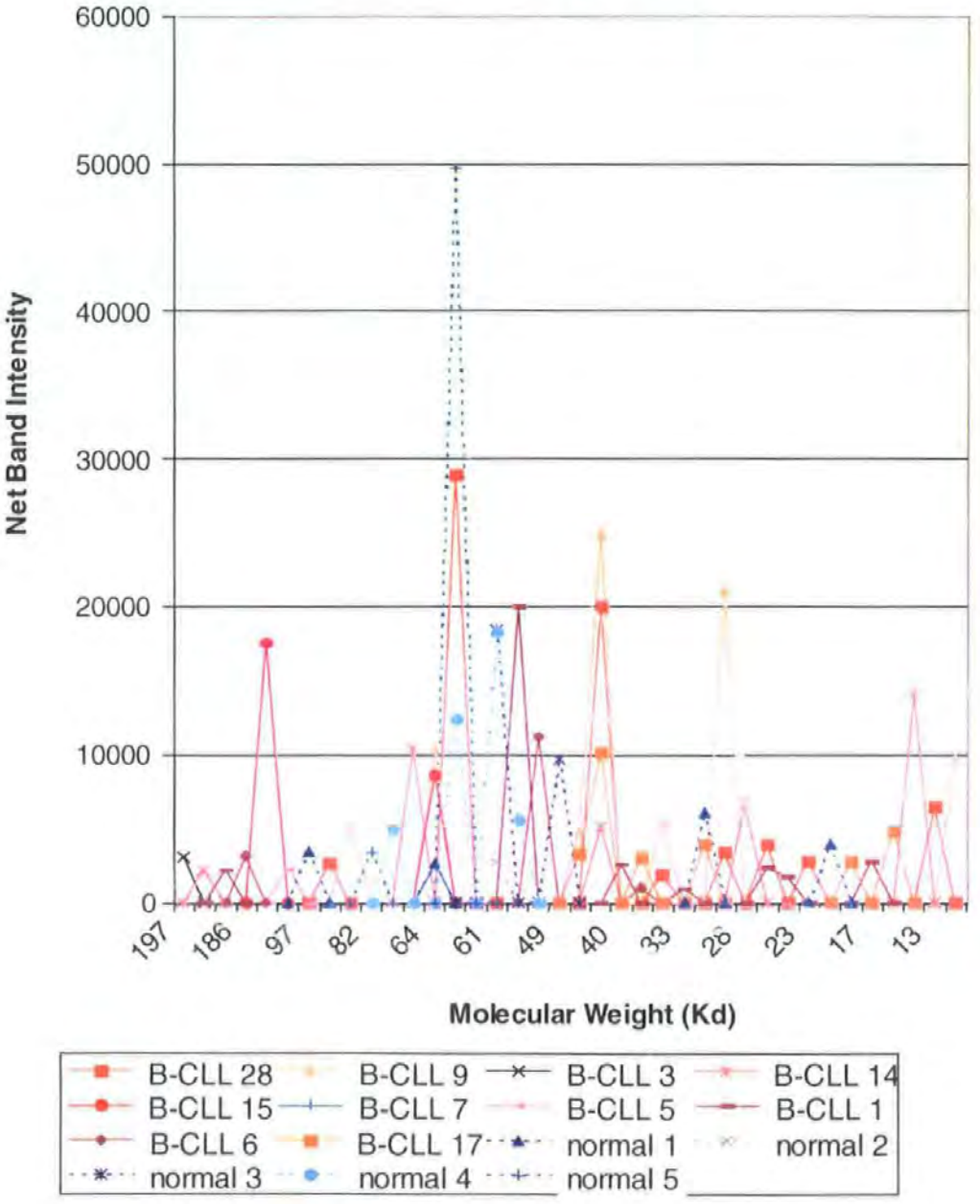


Figure 46a: Cell lysates from 10 B-CLL patients and 5 normal healthy volunteers were prepared as stated in Section 2.7. 50µg was loaded into each well and analysed by SDS-PAGE as described in Section 2.18. Gels were scanned using Kodak image processing software and net band intensity was measured.

Figure 46b: Comparison of digital optical densimetry profiles of B-cell lysates from B-CLL patients and healthy volunteers

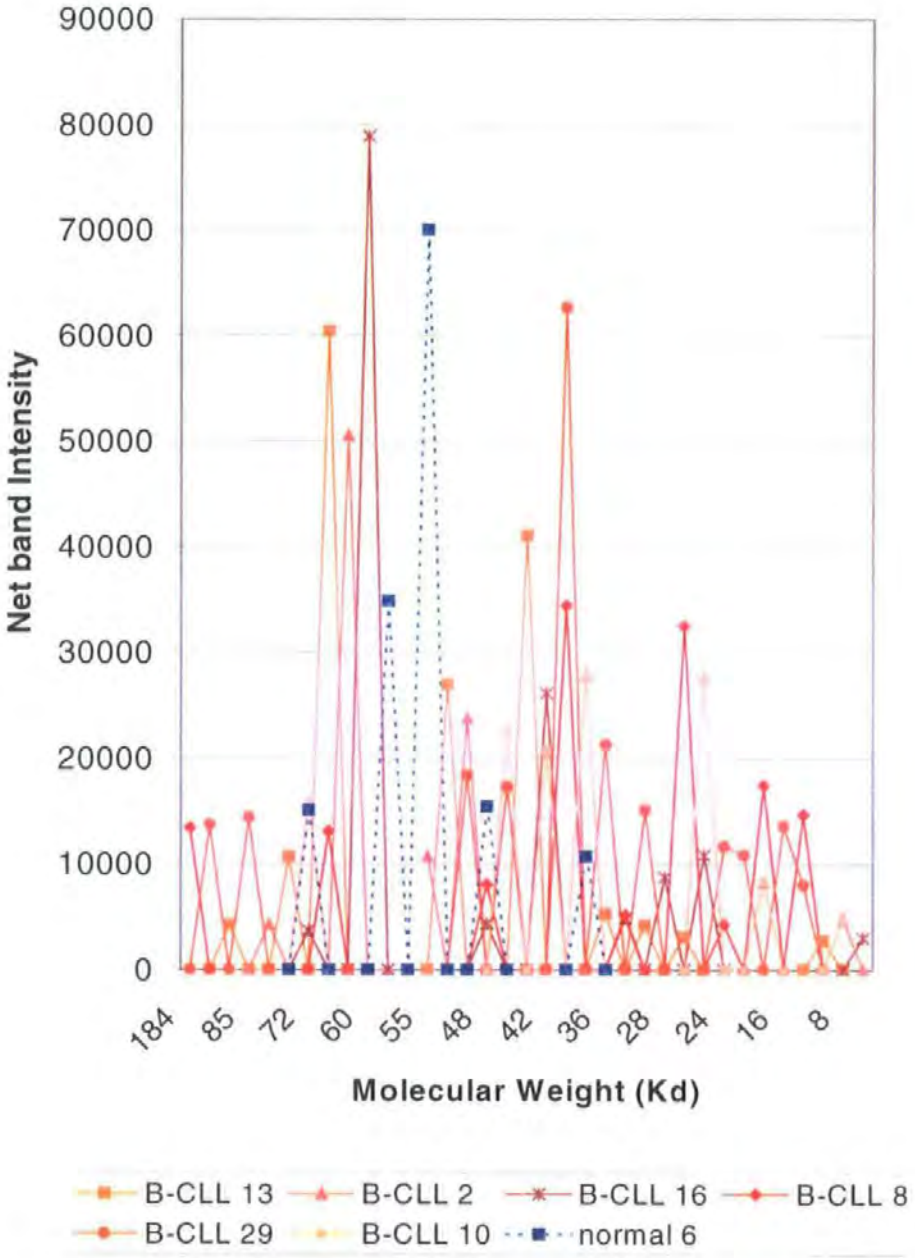
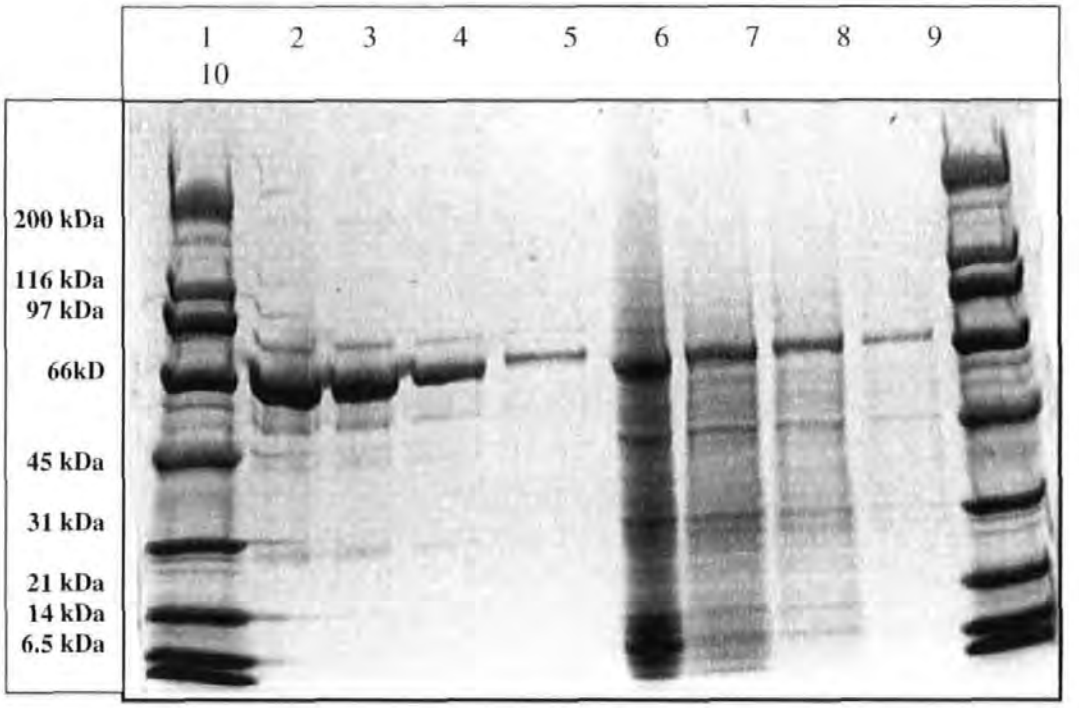


Figure 46a: Cell lysates from 6 B-CLL patients and 1 normal healthy volunteers were prepared as stated in Section 2.7. 100µg was loaded into each well and analysed by SDS-PAGE as described in Section 2.18. Gels were scanned using Kodak image processing software and net band intensity was measured.

Figure 47: SDS-PAGE of various dilutions of B-cell lysate from healthy volunteer and B-CLL patient



LANES

1-100µg Molecular weight standards

2-75µg normal B-cell lysate *(317,250)*

3-50µg normal B-cell lysate *(240,589)*

4-25µg normal B-cell lysate *(122,833)*

5-5µg normal B-cell lysate *(27,593)*

6-75µg B-CLL lysate *(604,148)*

7-50µg B-CLL lysate *(295,728)*

8-25µg B-CLL lysate *(88,881)*

9-5µg B-CLL lysate *(21,978)*

10-100µg Molecular weight standards

Figure 47: Lysates were assayed for protein content as stated in section 2.9. Samples were loaded onto a non-reducing gel and stained according to section 2.18. Italic numbers in brackets depict total net intensity readings for each lane.

6.3 Immunogenicity of individual protein bands.

In 5 B-CLL patients, B-cell lysates were isolated as stated in Section 2.8 whilst DCs were cultured according to Section 2.5. Individual protein bands were extracted from the polyacrylamide gel by electro-elution as stated in Section 2.21. Proteins were pulsed onto DCs and then co-cultured with T-cells as the lysates had done previously. IFN-γ secretion and cytotoxicity against autologous B-cell targets were compared for the 65kD, 42kD, 25kD and whole lysate.

As previously illustrated in Figure 21a, whole B-cell lysate from B-CLL patients when pulsed onto autologous DCs after 3 days co-culture with autologous T-cells produced a significant increase in IFN-γ secretion *(p=0.0198)* (Figure 48). Both 65kD *(p=0.0025)* and 42kD *(p=0.0125)* band, when pulsed onto autologous DCs, stimulated autologous T-cells to produce increased levels of IFN-γ. The 65 kDa protein band was more effective at stimulating the secretion of IFN-γ than the 42kD band or whole B-cell lysate.

Figure 48: Effect of individual protein bands upon IFN-γ secretion by T-cells from B-CLL patients.

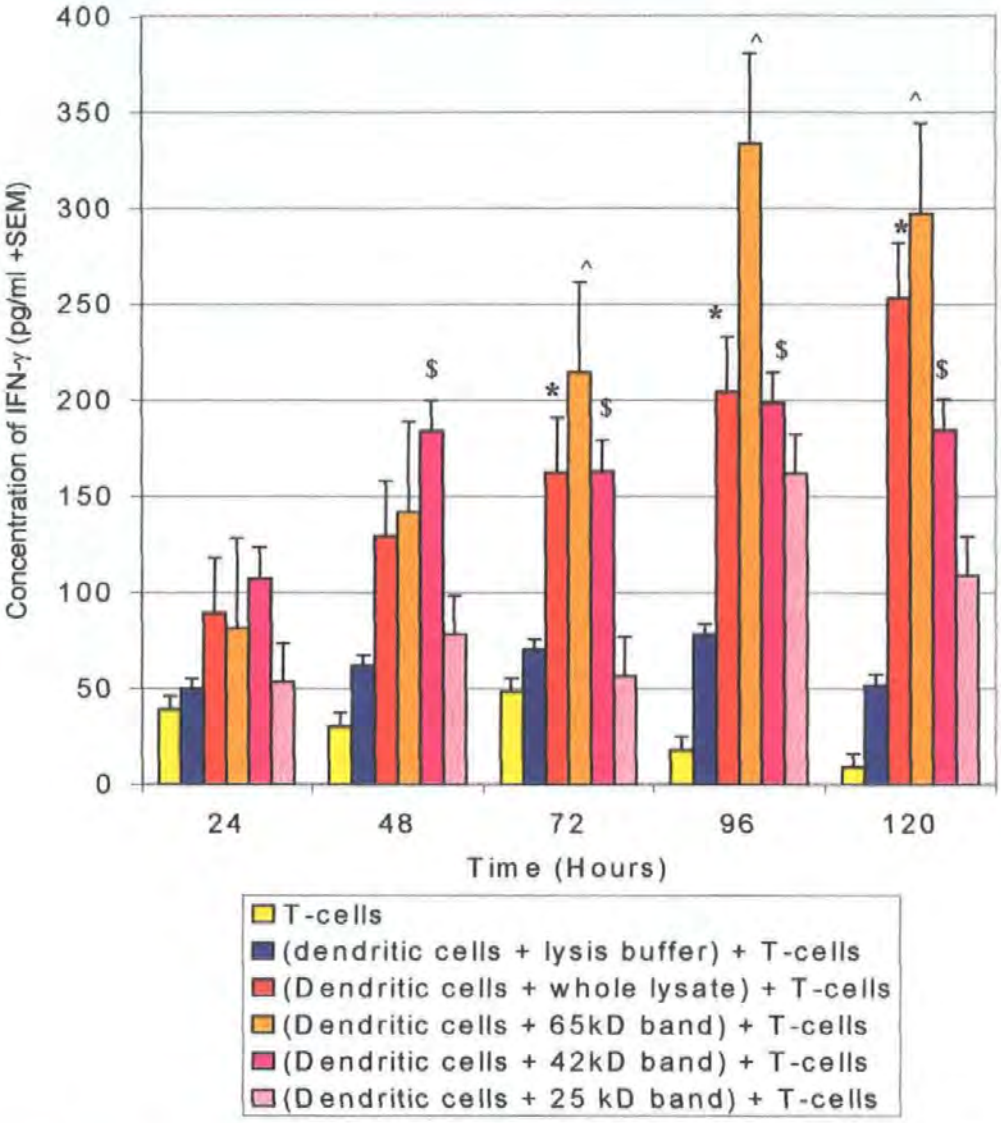


Figure 48: IFN-γ was measured by ELISA from Patients' 25,29,30,31,32. Overall effect of treatment was measured by one-way ANOVA. (*) indicates significant increase *(p=0.0198)* in IFN-γ secretion by T-cells cultured with DCs pulsed with whole lysate compared with those cultured with DCs pulsed with lysis buffer. (^) indicates significant *(p=0.0025)* increase in IFN-γ secretion by T-cells cultured with DCs pulsed with 65kD band compared with those cultured with DCs pulsed with lysis buffer. ($)indicates significant *(p=0.0125)* increase in IFN-γ secretion by T-cells cultured with DCs pulsed with 42kD band compared with those cultured with DCs pulsed with lysis buffer.

As previously illustrated in Figure 23a, whole B-cell lysate from B-CLL patients when pulsed onto autologous DCs and co-cultured with autologous T-cells generated T-cell effectors with increased cytotoxicity, at the effector: target ratio of 40:1, against autologous B-cells *(p=0.008)*. Dendritic cells pulsed with the purified 65kDa *(p=0.014)* or 42 kDa *(p=0.005)* protein band generated T-cell effectors with significantly increased cytotoxicity at the 40:1 effector: target ratio against autologous B-cells (Figure 49). At the lower effector: target ratio's of 10:1 and 20:1 the 42kDa band pulsed onto autologous DCs generated T-cell effectors with greater cytotoxicity against autologous B-CLL B-cell targets. Therefore, it was possible that the 42 kDa band contained the antigen responsible for stimulating a cytotoxic T-cell response. The purified protein band of molecular weight 25 kDa did not stimulate significant increases in either IFN-γ secretion or cytotoxicity against autologous B-CLL B-cell targets. The 25 kDa protein band was not sent to be sequenced in order to save valuable resources.

Figure 49: Stimulation of cytotoxicity against B-cells by T-cell effectors from B-CLL patients by different protein bands pulsed onto DCs

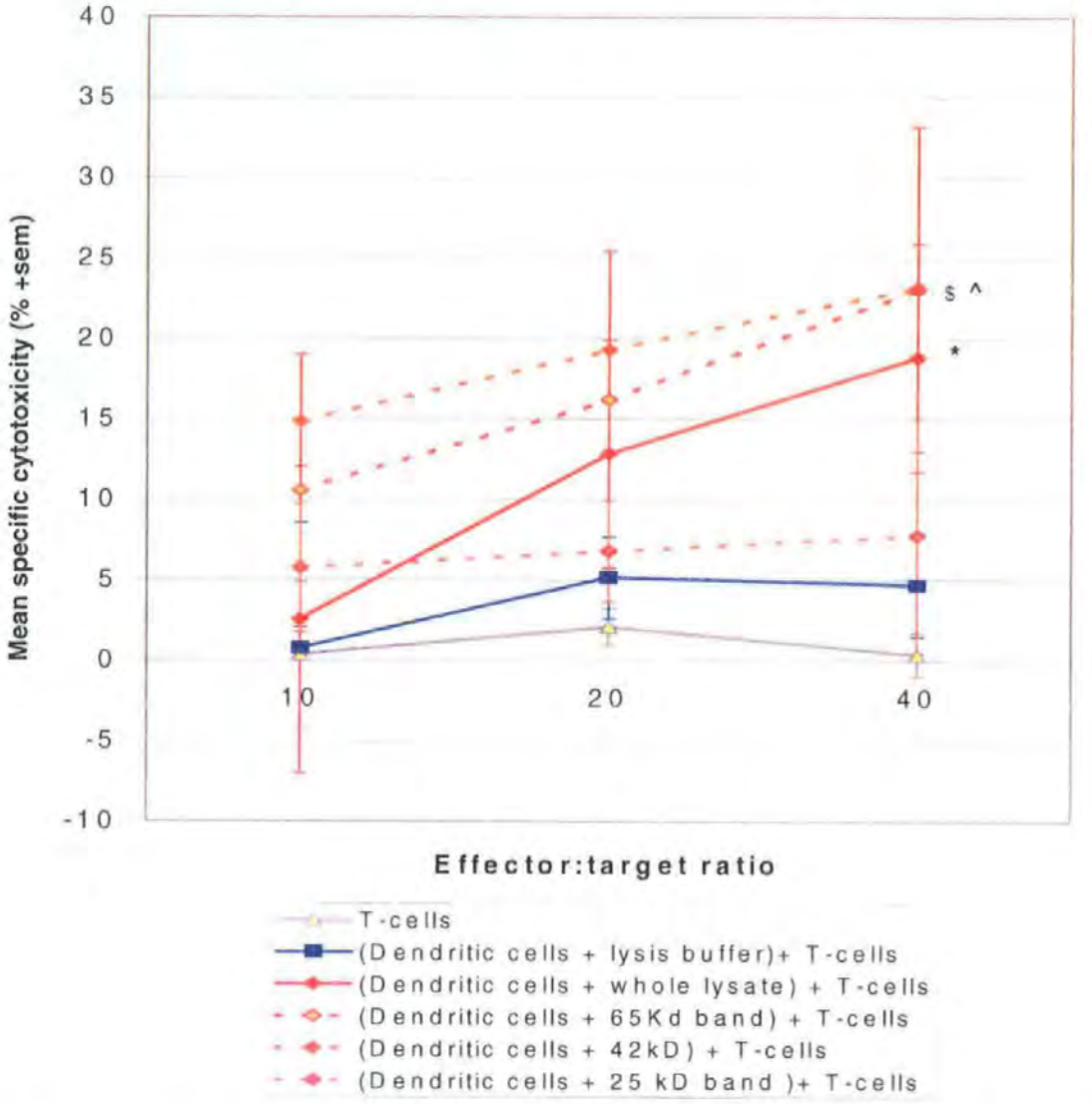


Figure 49: T-cell effectors were generated by 28 days culture from Patients' 25,29,30,31,32. Overall effect of treatment was measured by one-way ANOVA. (*) indicates significant *(p=0.008)* increase in cytotoxicity by T-cells cultured with DCs pulsed with whole lysate compared with those cultured with DCs pulsed with lysis buffer. (^) indicates significant *(p=0.014)*increase in cytotoxicity by T-cells cultured with DCs pulsed with 65kD band compared with those cultured with DCs pulsed with lysis buffer. ($)indicates significant *(p=0.005)* increase in cytotoxicity by T-cells cultured with DCs pulsed with 42kD band compared with those cultured with DCs pulsed with lysis buffer.

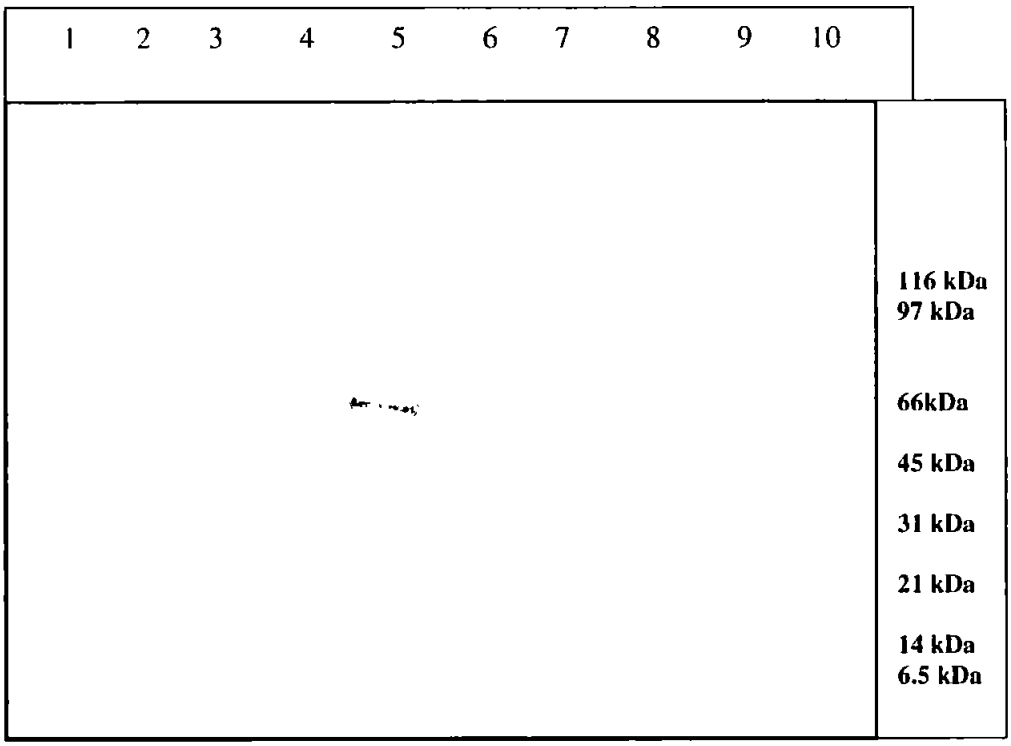
6.4 Western blot analysis of SDS-PAGE

There were two protein bands of particular interest that needed identification. One approach was to choose proteins with corresponding molecular weights of 65kDa and 42 kDa that were known to be over-expressed in B-CLL patients. Western blot analysis would then confirm whether these proteins were a possible target antigen.

6.4.1 CD19

CD19 is a 95 kDa transmembrane glycoprotein that contains two extracellular immunoglobulin domains [296, 297]. CD19 is selectively expressed on the cell surface of B-lymphocytes, where it activates intracellular signalling cascades [298]. Expression of CD19 is continuous throughout B-cell development and through terminal differentiation of B-cells into plasma cells [299]. CD19 forms functional complexes with B-lymphocytes surface proteins, including Integrin β1, CD21 and CD81. As such it would provide a good control marker to differentiate B-lymphocytes from T-lymphocytes. CD19-dynabeads had been used in all the purification steps and so it was a good idea to investigate whether either of the two bands were merely an artefact due to the method of B-cell separation. Decreased CD19 expression on the cell surface of B-cells from B-CLL patients has been reported [300]. Figure 50 shows that in Lanes 5 and 8 two protein bands reactive to anti-human CD19 at approximately 64kDa and 31kDa were detected. No reactive bands were detected in Lane 7 (containing T-cell lysate) despite plenty of protein bands being detected by Ponceau S staining. There was a decreased detection signal from B-CLL B cells than those from healthy volunteers.

Figure 50: Western blot of various cell lysate using affinity-purified polyclonal goat anti-human CD19



LANES

1- Broad Spectrum Molecular Weight markers

2- 50µgT-cell lysate from healthy volunteer 3

3- 50µgB-cell lysate from healthy volunteer 3

4- 50µg B-cell lysate from healthy volunteer 2

5- 100µg B-cell lysate from healthy volunteer 1

6- 50 µg B-cell lysate from B-CLL Patient 2

7- 100µg T-cell lysate from B-CLL Patient 26

8- 100 µg B-cell lysate from B-CLL Patient 26

9- 50 µg B-cell lysate from B-CLL Patient 10

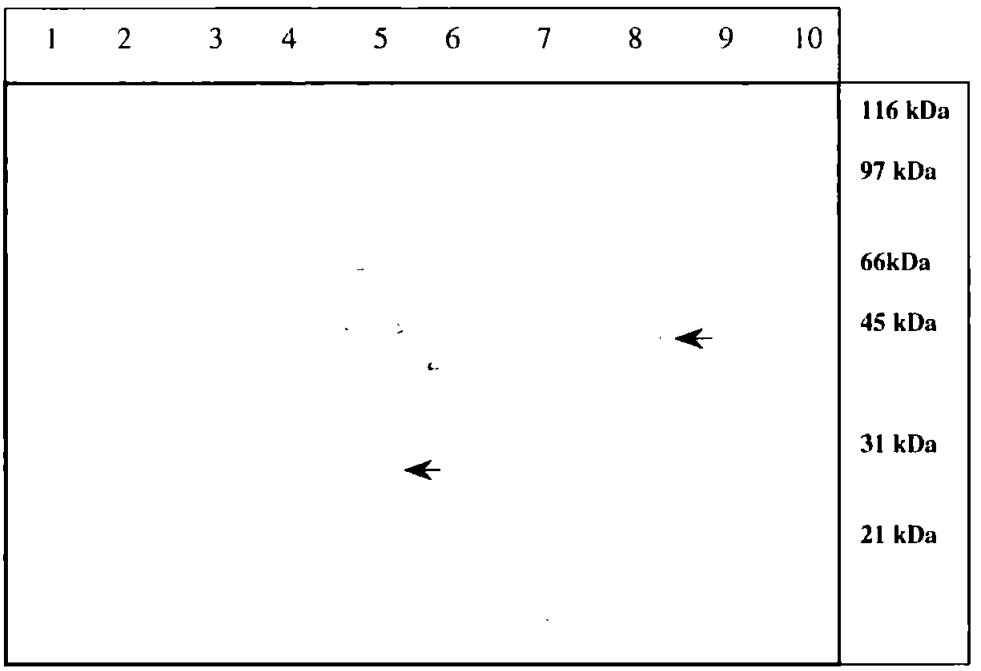
10- Broad Spectrum Molecular Weight markers

Figure 50: Lysates were run on a 4-15% Tris-HCL polyacrylamide gel under reducing conditions. Gel was left unstained and electro-blotted onto nitro-cellulose. Protein bands were stained with Ponceau S and marked in pencil. Primary antibody goat-anti-human CD19 (1:500) was added for 1 hour. Secondary antibody donkey anti-goat alkaline phosphatase conjugate (1:1000) was added for 1 hour.

6.4.2 CD5

CD5 is a 67kDa transmembrane glycoprotein expressed on virtually all T-lymphocytes, some thymocytes and B-cells [11]. It resembles CD6, as both share a common structure of 3 scavenger receptor cysteine-rich domains and similar tissue distribution [301]. CD5 is associated with T-cell signalling through the TCR/CD3 complex [302]. A B-cell antigen known as CD72 is the ligand for CD5 [303]. CD5 expression has been found upon B-cells from B-CLL patients [304]. Cross-linking of CD5 with antibodies has induced apoptosis of B-cells from B-CLL patients [305]. Therefore, it was considered a prime candidate as a protein not expressed in normal B-cells but that may have attributed to the immunogenicity of the 65kDa and in the B-cell lysate. Samples that showed the clearest detection of CD5 were those loaded with 100µg of total protein. CD5 was detected as 2 bands of approximately 50kDa and 30kDa in lysates from B-cells from both B-CLL patients and normal healthy volunteers (Figure 51, Lanes 5 and 8). In T-cell lysates from both normal healthy volunteers and B-CLL patients (Figure 51, Lanes 2 and 7) the 31kDa band was detected alone. This may well be a consequence of different glycosylation or different disulphide bonds of the protein subunits.

Figure 51: Western blot of various cell lysate using affinity-purified polyclonal goat anti-human CD5



LANES

1- Broad Spectrum Molecular Weight markers

2- 50µgT-cell lysate from healthy volunteer 6

3- 50µgT-cell lysate from healthy volunteer 3

4- 50µg B-cell lysate from healthy volunteer 2

5- 100µg B-cell lysate from healthy volunteer 1

6- 50 µg B-cell lysate from B-CLL Patient 2

7- 100µg T-cell lysate from B-CLL Patient 26

8- 100 µg B-cell lysate from B-CLL Patient 26

9- 50 µg B-cell lysate from B-CLL Patient 10

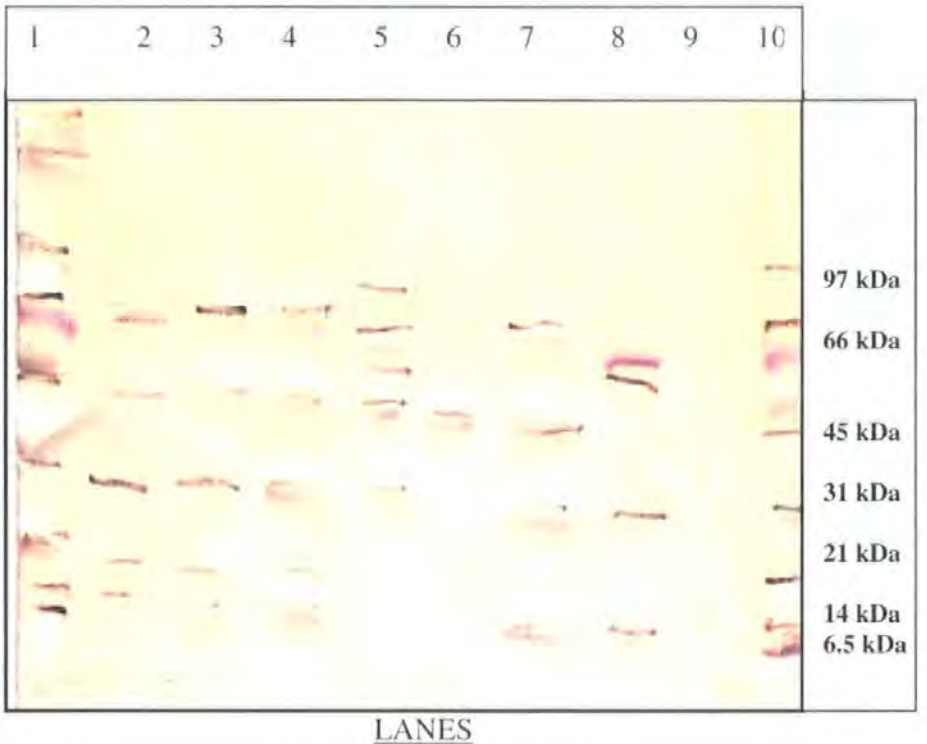
10- Broad Spectrum Molecular Weight markers

Figure 51: Lysates were run on a 4-15% Tris-HCL polyacrylamide gel under reducing conditions. Gel was left unstained and electro-blotted onto nitro-cellulose. Protein bands were stained with Ponceau S and marked in Pencil. Primary antibody goat-anti-human CD5 (1:500) was added for 1 hour. Secondary antibody donkey anti-goat alkaline phosphatase conjugate (1:1000) was added for 1hour.

46.4.3 CD72

CD72 is the 45 kDa human homologue of murine Lyb-2 [306]. CD72 is a ligand for CD5 expressed upon both normal B-cells and leukaemia B-cells regardless of CD5 co-expression [307]. The CD5-CD72 ligand pair may be involved in an autostimulatory loop that may play a role in replenishment of all types of B-cell[308]. CD72 was highlighted as a possible target antigen for cytotoxicity against B-CLL B-cells [309]. Interestingly the rabbit anti- human, rat, mouse, CD72 antibody only detected a protein band at approximately 60kDa in T-cell lysates from normal healthy volunteers (Figure 52). This result was disappointing. Even when 100µg of lysate was, added to another gel and, blotted with the same combination of antibodies, CD72 was still not detected in B-cells. Primary and secondary antibody concentrations were increased and CD72 was still not detected in B cells from either normal healthy volunteers or B-CLL patients.

Figure 52: Western blot of various cell lysate using affinity-purified polyclonal rabbit anti-human, rat, mouse CD72



1- Broad Spectrum Molecular Weight markers

2- 50µg B-cell lysate from B-CLL Patient 26

3- 50µg B-cell lysate from B-CLL Patient 8

4- 50µg B-cell lysate from B-CLL Patient 10

5- 50 µg B-cell lysate from healthy volunteer 3

6- 50 µg B-cell lysate from healthy volunteer 4

7- 50µg T-cell lysate from B-CLL Patient 26

8- 50 µg T-cell lysate from healthy volunteer 7

9- lysis buffer

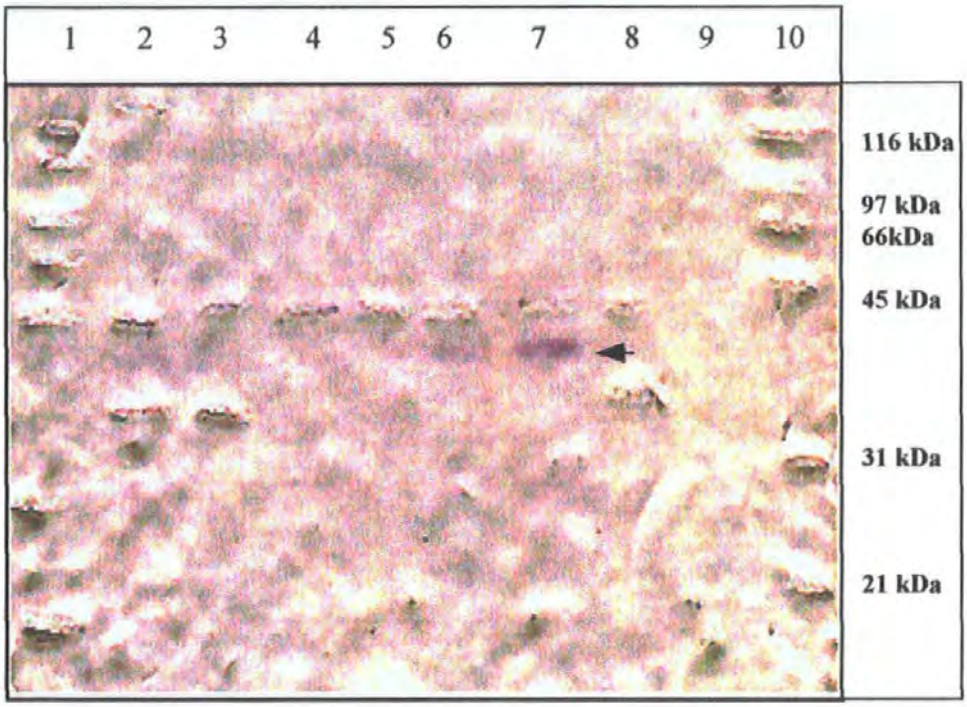
10- Broad Spectrum Molecular Weight markers

Figure 52: Lysates were run on a 4-15% Tris-HCL polyacrylamide gel under reducing conditions. Gel was left unstained and electro-blotted onto nitro-cellulose. Protein bands were stained with Ponceau S and marked in Pencil. Primary antibody rabbit-anti-human, rat, mouse CD72 (1:500) was added for 1 hour. Secondary antibody mouse anti-rabbit alkaline phosphatase conjugate (1:1000) was added for 1 hour.

6.4.4 CD23

CD23 is a 45 kDa type II integral membrane glycoprotein expressed on mature B-cells, monocytes, eosinophils, platelets and DCs [310]. Soluble forms of CD23 are increased in serum of B-CLL patients [311] and soluble CD23 may be an even stronger clinical marker than clinical stage [312]. However, CD23 expression on freshly isolated B-CLL cells was transient but was restored by cytokines secreted from activated T-cells such as IL-2, IFN-γ, TNF-α and IL-4 [313]. CD23 was therefore an ideal candidate for our protein of interest seen at approximately 42kDa. However, CD23 was only detected in T-cell lysates from a B-CLL patient (Figure 53). This result was disappointing. Even when 100μg of lysate, was added to another gel and, blotted with the same combination of antibodies, CD23 was still only detected in T-cell lysates from a B-CLL patient. Primary and secondary antibody concentrations were increased and CD23 was still only detected in T-cell lysates from normal healthy volunteers.

Figure 53: Western blot of various cell lysate using affinity-purified polyclonal goat anti-human CD23



LANES

1- Broad Spectrum Molecular Weight markers

2- 50μg B-cell lysate from B-CLL Patient 26

3- 50μg B-cell lysate from B-CLL Patient 8

4- 50μg B-cell lysate from B-CLL Patient 10

5- 50 μg B-cell lysate from healthy volunteer 1

6- 50 μg B-cell lysate from healthy volunteer 2

7- 50μg T-cell lysate from B-CLL Patient 26

8- 50 μg T-cell lysate from healthy volunteer 7

9- lysis buffer

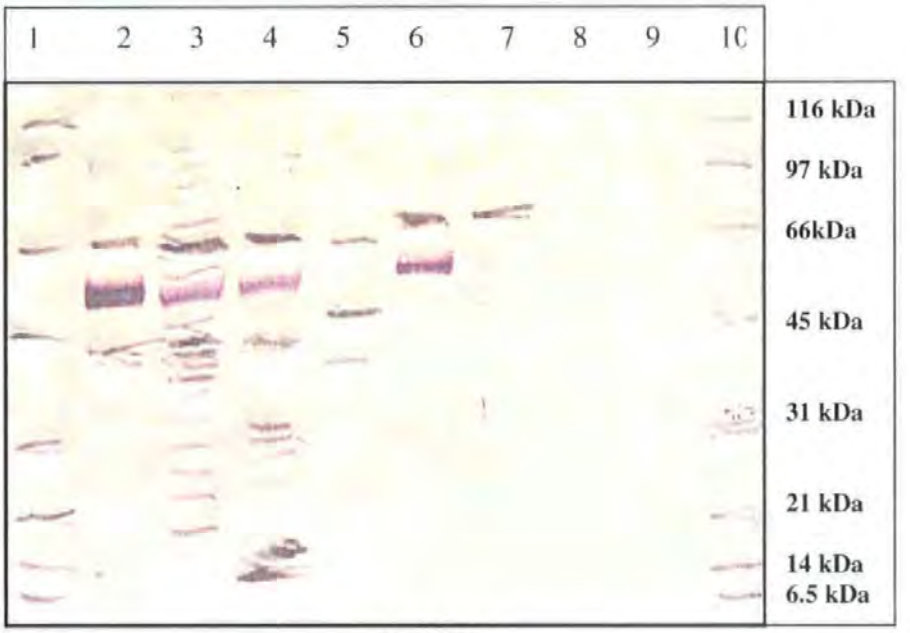
10- Broad Spectrum Molecular Weight markers

Figure 45: Lysates were run on a 4-15% Tris-HCL polyacrylamide gel under reducing conditions. Gel was left unstained and electro-blotted onto nitro-cellulose. Protein bands were stained with Ponceau S and marked in Pencil. Primary antibody goat-anti-human CD23 (1:500) was added for 1 hour. Secondary antibody donkey anti-goat alkaline phosphatase conjugate (1:1000) was added for 1 hour.

6.4.5 CD38

CD38 is a 45kDa type II integral membrane glycoprotein expressed on early and activated T and B cells [314]. CD38 is also found on monocytes and thymocytes [315]. Expression of CD38 by B-cells from B-CLL patients is correlated to poor survival and poor prognosis [316,317]. A strong protein band was detected at approximately 60 kDa by the mouse monoclonal anti-human CD38 antibody in the lanes loaded with 100µg of lysate from B and T-cells from B-CLL patient 26 and normal B-cells (Figure 54, Lanes 3,4 and 6). Interestingly, the detection of CD38 was strongest in the lane containing 50µg of B-cell lysate from B-CLL patient 10. There was no band detected by the anti-human CD38 antibody in the lane containing 50 µg B-cell lysate from B-CLL patient. This reflects the heterogeneity observed with CD38 expression in B-CLL patients.

Figure 54: Western blot of various cell lysate using mouse monoclonal anti-human CD38



1- Molecular Weight markers	6-100 µg B-cells healthy volunteer 1
2- 50µg B-cell B-CLL patient 10	7-50 µg B-cells healthy volunteer 2
3-100µg B-cell B-CLL patient 26	8-50 µg T-cells healthy volunteer 3
4- 100µg T-cell B-CLL patient 26	9- Lysis buffer
5- 50µg B-cell B-CLL patient 8	10-Molecular weight marker

Figure 54: Lysates were run on a 4-15% Tris-HCL polyacrylamide gel under reducing conditions. Gel was left unstained and electro-blotted onto nitro-cellulose. Protein bands were stained with Ponceau S and marked in Pencil. Primary antibody mouse monoclonal anti-human CD38 (1:500) was added for 1 hour. Secondary antibody rabbit anti-mouse alkaline phosphatase conjugate (1:1000) was added for 1 hour.

6.5 Protein sequencing

The 65kDa band and the 42 kDa band from the B-cell lysate of B-CLL patient and 65 kDa band from a normal healthy volunteer were separated by reducing SDS-PAGE and electro-blotted onto PVDF membrane. Proteins immobilised on the membrane were sent to Protein and Nucleic Acid Chemistry, University of Leicester, UK, and Proseq, Boxford, Massachusettes, USA, for sequencing. Results were generated from the 42kDa band from B-CLL patient and 65 kDa from normal healthy volunteers. However, the 65kDa from B-CLL patients did not yield any sequence data. The 65kDa band from normal healthy individuals yielded a sequence 35 amino acids long as shown in Figure 55 by N-terminal sequencing using an ABI 476 protein sequencer. A BLAST protein database search was carried out using the NCBI website [318]. The 35 amino acid peptide showed a strong homology to Human Serum Albumin as shown in Figure 55. The 42kDa band from B-CLL patients was an N-blocked protein. An *in situ* CNBr digestion revealed 2 strong signals and 4 weaker signals. O-phthalaldehyde (OPA) was added to the digest. This chemically selected the proline residue from the peptide mixture [319]. Peptides were then sequenced. Two strong peptide signals of 20 amino acids and 19 and one weaker peptide signal of 19 amino acids showed homology with Human actin (Figure 56). However, the remaining weak signal revealed a peptide of 13 amino acids to which no match could be found in the databases. Even when protein amino acid data was translated to nucleic acid sequence a corresponding match could not be found.

Figure 55: Sequence data from 65kDa band of B-cell lysate from normal healthy volunteer

Normal healthy volunteer 65kDa band.- 14 matches

1) Human Serum albumin precursor	97%
2) Bovine Serum albumin precursor	82%
3) Sheep Serum albumin precursor	80%
4) macmu Serum albumin precursor	82%
5) Rat Serum albumin precursor	77%
6) Horse Serum albumin precursor	74%
7) Felca Serum albumin precursor	71%
8) Pig Serum albumin precursor	77%
9) Canfa Serum albumin precursor	68%
10) Mouse Serum albumin precursor	70%
11) Rabbit Serum albumin precursor	68%
12) Merun Serum albumin precursor	69%
13) Chick Serum albumin precursor	65%
14) TRASC 67 KD Serum albumin (alb-1)	60%

1) Aligned with HUMAN Serum albumin precursor

DAHKSEVAHRFKDLGEENFKALVLIAFAQWLQQCP
25 DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCP 59

4) *Aligned with MACMU Serum albumin precursor*

DAHKSEVAHRFKDLGEENFKALVLIAFAQWLQQCP
17 DTHKSEVAHRFKDLGEEHFKGLVLVAFSQYLQQCP 51

8) Aligned with *PIG Serum albumin precursor*

DAHKSEVAHRFKDLGEENFKALVLIAFAQWLQQCP
23 DTYKSEIAHRFKDLGEQYFKGLVLIAFSQHLQQCP 57

15) Aligned with TRASC 67 kDa SERUM ALBUMIN (ALB-1)

DAHKSEVAHRFKDLGEENFKALVLIAFAQWLQQCP
4 HKSEIVHRFNDLKEEKFKGAALITFAQYLHKKP 36

Figure 55: Listed above are sequences showing homology from blast search of non-redundant Swissprot sequences. Percentages represent identical amino acids to test sequence. Below are shown examples of alignments. Sequence generated from blot is shown in blue. Red numbers give residue number from amino terminus. Green highlights amino acids not matching sequence data.

Figure 56: Sequence data from 42kDa band of B-cell lysate B-CLL patient

42 kDa from B-CLL patient aligned with Human Actin

1) Strong signal (52%)

```
          E I L I I APPERKTYSVVPEIVR
320       K I K I I APPERRKYSVWIGGSI  342
```

2) Weak signal (30%, 79%)

```
          GTKDSYVDDEPXXXRGMLT
11        CKAGFAGDDAPRAVFPSIVG  33
42        GQKDSYVGDEAQSKRGILT   61
```

3) Strong signal (53%)

```
          KQATERGYSFYQNAFRSGS
185       KILTERGYSFTTTAEREIV  205
```

4)Weak signal

```
          ARGGQVTXQLTXXXEIXXH
```

Figure 56: Above are shown examples of alignments for 4 peptides generated by digestion. Alignment of sequence data and Genbank search (NCBI). Sequence generated from blot is shown in blue. Red numbers give residue number from amino terminus. Green highlights amino acids not matching sequence data. Percentages represent identical residues. Unidentified residues are symbolised by (X).

7. Discussion

7.1 Anti-Idiotype responses

Chronic B-cell malignancies represent a potential target for immunotherapy by virtue of the fact that they are derived from immune cells and thus have the capacity for interaction with T lymphocytes. In addition, each tumour is derived from a clonal expansion of one malignant B-cell, and thus each cell in the clone will share specific determinants, for example those encoded by variable region sequences of rearranged immunoglobulin genes. Clinical studies in which patients with lymphoma and myeloma were vaccinated with idiotype protein have demonstrated the generation of anti-idiotypic T-cell responses and a degree of clinical response [320,321, 239, 322,323]. An alternative approach has been to load DCs with idiotype protein and subsequently use the pulsed DCs as a form of immunotherapy. Such an approach has also been shown to induce anti-idiotypic immune responses in lymphoma, [324, 325, 326] and myeloma, [241,327] with some encouraging clinical results. Over-expression of surface IgM has been shown to possess possible prognostic significance in B-CLL patients[328]. A similar rationale can therefore be proposed for immunotherapy in B-CLL, and this study was designed to determine whether DCs loaded with B-CLL tumour protein could stimulate anti-B-CLL cell responses.

7.2. Phenotype of DCs

Initial problems of generation of adequate numbers of DCs from patients with B-CLL were overcome once the malignant B-cells were removed. These DCs were morphologically and numerically similar to those from healthy individuals. Immunophenotyping DCs generated from healthy individuals and patients with B-CLL demonstrated similar levels of expression of cell surface molecules, with the notable exception of CD40, which was increased, and CD86, which was decreased. A previous

study has demonstrated that dendritic cell generated from healthy individuals and patients with myeloma are phenotypically and functionally similar, however this did not include CD40 [329]. Although the significance of this finding is unclear at present, $CD40^+$ DCs have been found to be vital for the generation of anti-tumour responses in mice[330]. Establishment of T cell help via the CD40-CD40L interaction is essential for generation of antigen specific $CD8^+$ T-cells [156]. Ligation with anti-CD40 antibodies can replace the need for T cell help [331]. Stimulation of CD40 by increased expression of CD40-ligand has been shown to stimulate immune recognition of B-CLL B-cells *in vivo* [332] and *in vitro* [333]. The decreased levels of cell surface CD86 in B-CLL patients differ significantly from the findings in myeloma patients and may be linked to the disease. Soluble CD86 expression has been shown to be increased in serum from patients with AML and B-CLL. Both normal DCs and B-CLL B-cells express an alternatively spliced form of CD86 that encoded for the soluble form [334]. Since CD86 has been shown to be a key co-stimulatory molecule [335] its decreased expression upon the DCs may well be related to the increase in soluble CD86 levels seen in B-CLL patients.

7.3 Defect in B-CLL is T-cell dysfunction

The functional capabilities of DCs from B-CLL patients' were compared with those of normal healthy volunteers. Firstly, Tuberculin PPD and Tetanus Toxin were pulsed onto DCs and the number of activated T-cells and IFN-γ secretion was assessed. Numbers of activated T-cells were similar in both B-CLL patients' and normal healthy volunteers (Figure 16a and 16b). However, secretion of IFN-γ in response to antigen pulsed DCs was decreased in the B-CLL patient compared with the normal healthy volunteer (Figure 17a and 17b). The intracellular expression of IFN-γ by antigen activated T-cells in B-CLL patients has been shown to be significantly reduced and correlated to disease stage [336].

However, IFN-γ secretion from un-stimulated T-cells from B-CLL patients has been shown to be significantly higher (S.Scrivener unpublished observation) This may have a complex role, as changes in the proteasome are triggered by IFN-γ [123]. It is therefore possible that the presentation of tumour related MHC class I antigens has been altered in B-CLL patients. However, untreated T lymphocytes from B-CLL patients still release IFN-γ in response to exogenous IL-2 [337]. When the DCs ability to stimulate normal T-cells in the allogeneic culture system (Figure 18) was investigated, it was seen that the lower T-cell cytotoxicity levels were due to T-cell dysfunction in B-CLL patients. B-CLL patients have long been known to exhibit reduced T-cell helper function [338] and increased suppressor function [339]. The use of adoptive transfer has demonstrated that T-cells from early stage B-CLL patients inhibit the accumulation of malignant cells whereas in patients with late stage disease the T-cells could enhance the effects of the malignant B-cell [340].

7.4 Activation of T-cells by lysate pulsed DCs

There is a sub-population of peripheral human $CD4^+$, $CD25^+$ known to have regulatory properties [341]. However, these cells do not produce IL-2, IL-4 or IFN-γ. It is possible that the appearance of $CD25^+$ $CD3^+$ cells in our cultures in response to lysate pulsed DCs represent the stimulation of this regulatory subset. It seems unlikely as the same cultures secrete IFN-γ and the appearance of $CD3^+$, $CD25^+$ cells was validated by recall responses to Tetanus Toxin and Tuberculin PPD in both healthy volunteers and B-CLL patients. This regulatory T-cell sub-population also express CD45RO, HLA-DR and CTLA-4 (CD152). T-cells isolated from the blood of B-CLL patients and stimulated with OKT3 showed reduced expression of CD25 and CTLA-4 (CD152) [255]. It seems unlikely therefore that the regulatory sub-population of $CD4^+$ cells is the same population of dysfunctional T-cells observed in B-CLL patients.

7.5 T helper 1 response

Results from Chapter 3 demonstrate that patient derived DCs, when pulsed with soluble B-CLL lysate, stimulate autologous T-cells to secrete IFN-γ but not IL-4. This suggests that our culture system has primed a T helper 1. Antigen presenting cells, such as DCs, have the capacity to generate either a T helper 1 or a T helper 2 immune response [342]. T helper 1 responses are associated with the secretion of particular cytokines such as IFN-γ and IL-12 [343], and induce cell-mediated immunity, all of which are particularly important in the response against tumours. The levels of IFN-γ observed in this study are similar to those generated by superantigen-pulsed DCs cultured with T-cells [344] but are higher than those generated by CD40L-stimulated B-CLL B-cells cultured with autologous T-cells [345]. However, we have no evidence to suggest which cell type within the pulsed dendritic cell-T-cell co-culture is responsible for the production of IFN-γ. Immature resting DCs express relatively few CD80 and CD86 molecules. These molecules are up-regulated after contact with T-cells [346]. Contact with T-cells allows the establishment of CD40-CD40L interaction [111, 347]. CD40 up-regulates CD80 and CD86 and induces the p75 component of IL-12 [1, 348]. IL-12 has been shown to be a stimulator of IFN-γ production in T-cells [349]. However, IL-12 can also stimulate macrophages and DCs to produce IFN-γ [350]. T-cells of $CD57^+/CD28^-$ phenotype have been observed to be significantly increased in B-CLL patients and responsible for the secretion of IL-2, IFN-γ and TNF-α in response to anti-CD3 stimulation [351]. It is equally possible therefore that either $CD57^+/CD28^-$ T-cells or DCs or both are responsible for the increase in IFN-γ secretion in response to stimulation by lysate pulsed DCs.

7.6 Role of IL-12 in preparation of DCs

Studies have shown that IL-12 polarises DCs towards the induction of T helper 1

responses [352, 353, 354]. Pre-treatment of murine DCs with IL-12 was shown to elicit responses to relatively un-reactive tumour/self peptides, [355]. In view of this, we pre-incubated the DC with IL-12 just prior to the addition of antigen. Recently IL-12 responsiveness has been demonstrated in human monocyte derived DCs [356]. One preliminary experiment showed that without pre-treatment with IL-12, monocyte derived DCs pulsed with B-CLL lysate could not stimulate significant T cell responses. Therefore, all the experiments were carried out with an IL-12 pre-treatment before lysate was pulsed onto DCs. IL-12 could possibly reverse the effects of cytokines such as IL-10 and TGF-β which may have been encountered by the DCs from B-CLL patients. Dendritic cells do respond differently to the same stimuli because of previous exposure to cytokines and maturation agents [352].

7.7 Immunosuppression in leukaemia

T helper type 2 responses are associated with the secretion of cytokines such as IL-4 and IL-10 [357]. Tumours have been shown to secrete factors such as IL-10 [358,359, 360, 361, 362, 363], transforming growth factor-β (TGF-β) [364] and vascular endothelial growth factor (VEGF) [365], which suppress dendritic cell and/or T-cell function. Immature DCs treated with IL-10 have been shown to induce anergy in melanoma antigen specific $CD8^+$ cytotoxic T-cells *in vitro* [358]. IL-10 may modulate immune escape by two different mechanisms; anti-tumour antigens presented by IL-10 modulated immature DCs, with low co-stimulation markers, induce anergic anti-tumour specific T-cells and immature IL-10 modulated DCs induce regulatory T-cells which maintain the anergic nature of the anti-tumour specific T-cells [366]. However, the effect of IL-10 may be more complex as *in vitro* IL-10 can inhibit the proliferation of B-cells from B-CLL patients [367]. Clinical responses to dendritic cell vaccines have been affected by the cytokine profile of the individual

patient [368]. TGF-β is perhaps the most potent immunosuppressive factor [369]. TGF-β can inhibit the production of IL-12 by monocytes [370, 371]. TGF-β may also inhibit important cell surface receptors important for cell activation and growth [372]. TGF-β is a potent inhibitor of cytotoxic T-cell differentiation [370,373]. Administration of TGF-β *in vivo* can inhibit T-cell responses to viruses and allogeneic antigens [373]. However, TGF-β is also a potent inhibitor of proliferation of neoplastic cells *in vitro*, its effects *in vivo* may depend upon microenvironment [374]. In the majority of B-CLL patients TGF-β inhibits *in vitro* B-cell proliferation but loss of responsiveness is demonstrated in a subset of patients [375]. VEGF is mainly recognised by its angiogenic properties. VEGF inhibits the differentiation of $CD34^+$ cells into DCs [365]. B-cells from B-CLL patients have shown to secrete both TGF-β [376] and VEGF [377]. The T-cells used in our experiments were derived from patients with B-CLL and thus were exposed *in vivo* to immunosuppressive factors secreted by the B-CLL cells.

7.8 Role of IL-15 and reversal of T cell anergy

TGF- β secreted from tumour cells has been shown to suppress T-cells response to IL-2 [378] by blocking Signal Transducers and Activators of Transcription (STAT3) and STAT5 phosphorylation [379,380]. IL-15 has been shown to maintain STAT3 and STAT5 phosphorylation despite TGF-β secreted by multiple myeloma cells [286]. IL-15 may restore signalling of T cells, affected by *in vivo* exposure to inhibitory factors such as TGF-β, through STAT3 and STAT5 phosphorylation. T-cells isolated from B-CLL patients may be exposed to B-CLL specific antigens without co-stimulatory signals and induced into an anergic phenotype *in vivo*. Analysis of T-cell receptor B variable (TCRBV) genes, in patients' with B-CLL, has showed skewing of the T-cell population [255,381]. IL-15 may revert T-cells from an anergic phenotype and result in an increased response to tumour

revert T-cells from an anergic phenotype and result in an increased response to tumour antigens presented by autologous DCs. IL-15 is capable of selectively activating primed T-cells and naive $CD8^+$ cells but not naive $CD4^+$ cells [382]. IL-15 has also been shown to enhance responses of γδ T-cells to non-peptide antigens [383]. Therefore, the administration of IL-15, to T-cells from B-CLL patients, may enhance the survival of an additional population of T-cells responsive to the same or other antigens presented by the autologous tumour cell lysate-pulsed DCs. Further characterisation of the responding T-cell phenotype in IL-15 treated and untreated T-cells from B-CLL patients is required.

As well as its effects upon T-cells, IL-15 promotes differentiation and growth of B cells [384]. IL-15 has been shown to promote growth of B-CLL cells via the IL-2 receptor β and γ chain [385]. However, it is not likely that activated B-CLL cells are responsible for the increased T-cell responses observed, as separation methods sought to remove all $CD19^+$ cells from the dendritic cell-T-cell co-culture. IL-15 can also induce monocyte derived DCs to become mature cells resembling those generated with TNF-α [386]. The pre-treatment of T cells with IL-15 and subsequent culture with autologous immature DCs may increase measurable T-cell responses by an indirect effect upon dendritic cell maturation. Treatment of T-cells with IL-15 can stimulate IL-5 [387], IFN-γ and TNF-α [388] secretion that induces dendritic cell maturation. An increase in the stimulatory capacity of the DCs would then increase the magnitude of T-cell responses. However, in this study induction of CD83 expression within the dendritic cell population did not increase total specific cytotoxicity levels to the same extent as that seen with T-cell pre-treatment with IL-15.

7.9 Class II restricted killing of B-CLL cells

An abnormally high number of CD8 positive T cells with a cytotoxic cell surface

demonstrated [390]. Triggering B-CLL B-cells with a CD40L-transfected 3T6 cell line stimulated allogeneic T-cells from B-CLL patients to make proliferative and cytotoxic responses [333]. However, untreated B-CLL cells are not capable of stimulating similar results [391]. B-CLL B cells stimulated by CD40L when cultured with allogeneic T cells from B-CLL patients demonstrated $CD8^+$ cytolytic T cell responses. Whereas, when B-CLL B cells stimulated by CD40L were cultured with autologous T cells from B-CLL patients $CD4^+$ mediated release of IFN-γ was observed [345]. The capacity for B-CLL B cells to appear more antigenic to B-CLL patient T-cells was linked to an upregulation of CD80 and CD86 on the B-CLL B cell surface. T-cell abnormalities such as the oligoclonal / monoclonal expansions of $CD4^+$ TCRBV subsets that have been observed [392,256] may reflect previous attempts *in vivo* of T cells to recognise tumour cells [393]. Antigen-non-specific activated $CD4^+$ T cells have the potential to inhibit the proliferative response of B-CLL B-cells [394]. Spontaneously occurring T lymphocytes in B-CLL patients have been isolated that were capable of responding to CD40L activated B-CLL B cells [429]. However, in all of these experiments increased T cell signalling via CD80 and CD86 is required to generate an adequate measurable T cell response. Therefore, presentation of B-CLL antigens in the correct context of co-stimulatory molecules is required by T-cells from B-CLL patients in order to make an anti-tumour response.

Although the levels of specific cytotoxicity (24%), generated in our cultures at an effector: target ratio of 40:1, seem low when compared to those of other *in vitro* studies [232, 254, 235, 236, 395], they are comparable to levels shown by others [231, 396, 237, 238, 397, 398, 399]. These differences could be due in part to the fact that different diseases were studied. However, in a study of B-CLL, similar levels of specific cytotoxicity were generated when CD40L stimulated B-CLL cells were cultured with allogeneic T-cells but not autologous T-

cells [345]. Of particular interest was the nature of the responding cell population. The responding cells in two patients were predominantly $CD4^+$. Antibody blocking experiments in the three patients tested demonstrated significant inhibition of cytotoxicity with anti-class II, anti-pan TCR αβ and anti-CD4 but not with anti-class I or anti-CD8 monoclonal antibodies. Overall this suggests that $CD4^+$ HLA class II restricted cytotoxic T-cells are responsible for the majority of lysis of B-CLL cells in our culture system. MHC Class II-restricted cytotoxicity results from exogenous antigen processed and presented by DCs via the classical MHC class II pathway (Figure 7). HLA class II molecules are only expressed on antigen presenting cells such as B cells. [400]. Some tumour cells such as metastatic melanoma express MHC class II. Hyper-expression of MHC class II molecules can be a triggering factor for autoimmune diseases [401]. Class II restricted cytotoxic T cells form a major part of anti-viral responses to herpes simplex virus and measles [402, 403]. As B-CLL is a malignancy of B cells it may be that the MHC class II molecules are highly expressed on the B-CLL B cell surface and this is why a MHC class II-restricted response is generated. However, if this were the sole reason, other B cell malignancies would show similar MHC class II-restriction.

In general, it is thought that $CD8^+$; classes I restricted T-cells are responsible for anti-tumour immunity [236, 238, 398, 404]. However, $CD4^+$ cytotoxic T-cells have been reported in hepatocellular carcinoma [405] and p21-ras (12Val) mutated cells [406]. $CD4^+$ T cells which secreted IFN-γ were shown to eliminate UV light-induced tumour 6132A-PRO by indirect cell killing mechanisms in a mouse model. In animal tumour systems, transfection of MHC class II molecules into the tumour cell resulted in rejection whereas transfection with MHC class I had no effect [407]. MHC class II-restricted $CD4^+$ T cells have been isolated against tumour antigens such as tyrosinase, MART-1/Melan-A, gp100, MAGE-3,

NYESO-1/CAG3 which have all previously been presented by MHC class I molecules [408]. Normally cytotoxic $CD4^+$ T-cells represent 1-6% of the $CD4^+$ T-cell population [409]. $CD4^-$ T-cells with an anergic cytotoxic phenotype have been expanded from PBMC of B-CLL patients [410, 411]. Therefore, the stimulation of cytotoxicity against autologous B-CLL B-cells by lysate pulsed DCs may be a result of the activation of this anergic population of T-cells. However, B-CLL patients with decreased the numbers of $CD4^+$ T-cells due to recent therapy with fludarabine [412], may not exhibit a specific cytotoxic response in our culture system.

7.10 Maturity of DCs

However, the levels of cytotoxicity (Figure 23a) may have been because of the relatively immature state of the monocyte derived DC (CD83 negative) used to present lysate antigens to the T-cells. CD83 shares its homology with the members of the IgG superfamily[413]. $CD83^+$ cells possess a distinct cellular phenotype, cytokine gene expression profile [414] and greater stimulatory capacity [415] compared with $CD83^-$ cells. After loading of antigen onto the dendritic cell surface a further 'danger signal' is required to achieve maximal presentation of that antigen to the T-cell [273]. Maturation agents therefore have been cytokines or bacterial products associated with infection such as LPS [109,276, 277], IFN-α [274], TNF-a [110], Poly(I:C) [275], CpG DNA [416].

Results in Chapter 5 illustrate that the addition of TNF-α, Poly(I:C) and LPS to autologous DCs after pulsing with B-CLL lysate does result in the emergence of $CD83^+$ DCs. However, the highest percentage of $CD83^+$ DCs after addition of any maturation agent was merely 12%. Higher $CD83^+$ expression levels have been observed where the cells were cultured with 10% Fetal calf serum [110] or under serum free conditions [415] but

not when cells were cultured in medium containing 5% human AB serum [417]. Hence our levels of CD83 expression were probably limited by the presence of human AB serum in the culture. It could be argued that LPS and TNF-α are required for longer incubation periods in order to induce CD83. This is unlikely, however, as the effects of TNF-α (10ng/ml), such as increased MHC class I and II, ICAM-1, B7 and CD40 expression and down regulated Ii and FcγRII expression, have been observed after 24 hours [417]. In contrast to LPS, TNF-α and Poly (I:C), IFN-α did not stimulate CD83 expression in our system. This is probably because maturation effects with IFN α and β require the presence of TNF-α [274].

The addition of TNF-α (10ng/ml) or Poly(I:C) to monocyte derived DCs after pulsing with B-CLL lysate did not increase the level of specific cytotoxicity at the highest effector: target ratio. However, at lower effector: target ratios the addition of maturation agents to DCs stimulated effectors to exhibit maximal specific cytotoxicity levels. This indicated that although the absolute number of targets killed by specific cytotoxicity did not increase with maturation agents, the frequency of specific cytotoxic cells within the effector cell population was increased. The absolute levels of specific cytotoxicity may be limited by the expression of antigen/s upon the targets in the context of MHC Class-II molecules. *In vivo* TNF-α matured lysate-pulse-DCs were able to generate clinical responses in patients' with medullary thyroid carcinoma whereas immature DCs did not[418].

LPS maturation of monocyte derived DCs in serum free conditions acts through inducing secretion of high levels of TNF-α [413]. Whether LPS induced maturation of monocyte derived DCs is entirely due to TNF-α is unclear. Although Chapter 5 showed a difference in the degree of specificity of T-cell responses generated using LPS or TNF-α,

this may be due to differences between individual B-CLL patients. LPS, TNF-α and Poly (I:C) activate NF-κB [413,419,420]. Expression of many genes which are important in antigen presentation by DCs involves NF-κB, such as CD86[421] and CD83 [413]. Other genes contain a NF-κB binding site in their regulatory regions such as CD80, MHC class I (H-2Kb), MHC class II (HLA-B7), CCR5, IL-12, macrophage inflammatory protein (MIP)-1α, MIP-1β, IL-1α, IL-1β, IL-6, IL-8, CD54, Fas, Fas Ligand, RANTES and TNF-α [422, 423]. Dendritic cells, induced to maturation by LPS or TNF-α, exhibit increased stimulatory capacity because of the accumulation and persistence of antigen MHC class-II complexes upon the cell surface[424]. Matured DCs also exhibit MHC class-I up-regulation that stimulates additional subsets of T-cells[425], hence increasing the magnitude of the overall T-cell response.

7.11 Natural Killer cells

Although there is evidence that DCs can directly trigger anti-tumour responses by Natural Killer (NK) cells [426], the effector cells in our assay did not express the NK markers CD16 and CD56. Although our work demonstrated that NK cells were not present within the responding T cell population; a better way to demonstrate that NK cells were not responsible for the killing of B-CLL targets was to use a cell line K562. K562 cells are lysed by NK cell activity [289]. No specific killing of K562 cell line was demonstrated by T-cell effectors generated by co-culture with B-CLL lysate-pulsed autologous DCs (Figure 40). In addition, B-CLL B-cell targets have been shown to be resistant to anti-FAS mediated cytotoxicity [427] and to lysis by normal or autologous LAK cells [428]. IL-15 can also induce NK cell development [287] and increase cytotoxic activity of NK cells [288]. K562 cells are sensitive to NK cell mediated cytotoxic activity and in this study IL-15 did not increase the amount of NK cell activity.

K562 cells are sensitive to NK cell mediated cytotoxic activity and in this study IL-15 did not increase the amount of NK cell activity.

7.11 *In vivo* priming of T-cells and deletion

Perhaps the modest level of tumour specific cytotoxicity observed is due to the relatively low frequency of B-CLL-reactive cells within the patients' T-cell repertoire. The lack of a specific response by T-cells from healthy volunteers presented B-CLL lysate antigens by their own DCs suggests the presence of a pre-existing pool of reactive T-cells in B-CLL patients not found in healthy volunteers. Recently the existence of autologous T lymphocytes capable of spontaneous specific recognition of B cells from B-CLL patients has been demonstrated [429]. However, there is also evidence that there are considerable decreases in the ratio of TH1 to TH2 helper T cell phenotypes [336]. FasL expression has been found on melanoma [430], lymphoma [431], glioma [432], lung tumour cells, colon cancer [433] and breast adenocarcinoma. The expression of FasL by some tumours may confer immune-privileged status by inducing deletion by apoptosis of autologous tumour-specific effector lymphocytes [434].

7.12 Antigen processing and cell fusion

One of the main aims of the optimisation experiments was to generate MHC class I restricted responses by the stimulation of CD8 positive T cells. Most of the studies that have previously been able to generate CD8 responses have used peptides to pulse onto DCs. Many of the properties of intrinsic to peptides (the size and method of production) may result in the DCs processing them via the MHC class I pathway. In general it is thought that DCs process and present exogenous proteins by the HLA class II pathway and that endogenously synthesised protein generate MHC class I restricted cytotoxic T lymphocytes [435]. However, this is not to say that DCs cannot present exogenous protein

I molecules [438]. However, the method of entry into the dendritic cell has been shown to influence the most common pathway by which the proteins are processed through [439]. Fusion of B-cells and monocyte derived DCs from a B-CLL patient was still attempted to try and overcome any problems related to 'cross-priming' such as; lack of appropriate bone-marrow derived APC, limiting nature of the antigen, inadequate $CD4^+$ help and inadequate levels of transportor associated with antigen processing (TAP).

Dendritic cells transfected with full-length cDNA have resulted in MHC class I restricted T cell responses being generated [440]. However, the fusion of DCs and carcinoma cells has been shown to generate both MHC class I and class II responses [441, 442]. Although initial attempts with *in vivo* vaccination did not result in clinically significant outcomes [443]. Use of human allogeneic DCs fused with tumour cells in metastatic renal cell carcinoma has shown more promising results *in vivo* [444]. When compared *in vitro*, either allogeneic or autologous DCs fused to ovarian carcinoma cells could generate cytotoxic T cell activity but allogeneic DCs fused to tumour cells produced higher proliferative responses [445].

Membrane fragmentation and amalgamation are common effects of applying electric pulses to cells. Membrane resealing occurs rapidly and spontaneously after cessation of the electric pulse[446]. There is a significant difference between 'membrane mixing' and the establishment of a stable growing hybrid cell [447]. True fusion between human tumour cells and $CD34^+$ cells have resulted in hybrid cells capable of efficient growth [262]. However, attempts to produce hybrids with monocyte derived DCs have been unsuccessful in producing stable hybrid populations because of their low replicative potential [229, 448]. Analysis of fusion rates, by flow cytometric dyes and antibody staining, overestimates hybrid efficiencies [449] but, for the purposes of this study it provided relevant information

Analysis of fusion rates, by flow cytometric dyes and antibody staining, overestimates hybrid efficiencies [449] but, for the purposes of this study it provided relevant information about 'membrane mixing' between two specific cell types in a heterogeneous mix of cells. Fusion rates for B-CLL cells were low but this was probably due to the well-known spontaneous apoptosis of B-CLL cells *in vitro*. Dendritic cells mixed with B-CLL B-cells without an electric current showed a 50% decrease in cell viability. Flow cytometric analysis showed this cell death to be located in the $CD20^+$ cell population. Perhaps the addition of cytokines such as IL-2, IL-4 and IL-15, known to increase *in vitro* viability [385] of B-CLL B-cells, may increase fusion yields. It was not possible to bring the cells into preliminary alignment before applying the membrane destabilising voltage, due to the limitation of the gene pulser apparatus. This has been shown to increase fusion yields by the formation of 'pearl-chained' cells [446]. By increasing the voltage and pulse duration higher fusion rates and higher purity were achieved in separated, fused dendritic-B-CLL B-cells. However, voltages and current duration were not increased greatly as irreversible membrane breakdown results in cell death due to electric pulses of excessive strength and duration [446]. Further optimisation of the voltage and current duration could possibly increase yields of fused dendritic-B-CLL B-cells. For these experimental purposes, introduction of antigens into the cytosol of the DCs by 'membrane mixing' was the main objective rather than the establishment of a proliferating hybrid cell. It has been demonstrated that short-term fusion or co-culture can result in the generation of anti-tumour T-cell responses [450].

Despite sub-optimal electrofusion conditions, it was possible to generate hybrid monocyte derived DCs that could stimulate both MHC class I and II-restricted effector T-cells. This is in contrast to previous findings where only HLA class-II restricted T-cell

and ineffective stimulation of anergic dysfunctional T-cells, were overcome by electrofusion of monocyte derived DCs with B-CLL B-cells.

7.13 Autoimmunity versus anti-leukaemia effect

One concern that has been raised with regard to dendritic cell vaccination is the possibility of inducing autoreactivity. In our culture system, reactivity was not demonstrated against autologous B-CLL T-cells and granulocytes or HLA matched and allogeneic B-cells from healthy individuals. Ideally we would wish to test for cytotoxicity against autologous non-malignant B-cells from B-CLL patients but due to the overabundance of the malignant B-cells in these patients we found this impossible. Although T-cells make poor targets we did not wish to introduce anti-viral epitopes into our system in the form of EBV-transfected targets. Recently, T cells generated by stimulation with CML pulsed DCs showed proliferation against autologous CML cells but not an HLA-identical sibling [451]. Clinical studies with dendritic cell vaccination have so far not reported evidence of autoimmune disease, [324, 240, 241, 243,246]. Autoimmunity has been induced by DCs pulsed with peptides eluted from tumour cells in mice [452, 453]. One report states that blast cell lysates pulsed onto human DCs mediate inhibitory effects [454]. However, it seems more likely that immature DCs were generated and that these were able to induce tolerance as this has been demonstrated [105,455, 456].

7.14 Possible B-CLL antigen(s)

Our experiments have demonstrated specific T-cell responses to B-CLL. Although cytotoxicity was induced with some allogeneic B-CLL lysates this was not the case with others. This suggests that, although a common antigen may be present in a proportion of B-CLL patients, other B-CLL patients may have unique antigens. This should not be surprising, as B-CLL is not a homogenous disease. 2-D analysis of proteins from B-cells of

CLL patients, other B-CLL patients may have unique antigens. This should not be surprising, as B-CLL is not a homogenous disease. 2-D analysis of proteins from B-cells of B-CLL patients revealed a group of proteins between 50-60kD that were not found in B cells from normal individuals [295]. Analysis of the B-cell lysates from B-CLL patients confirmed the presence of a B-CLL common antigen and patient specific antigens (Figure 45a).

Due to the use of CD19 Dynabeads during the production phase of the lysate it would also be useful to show that similar levels of CD19 are present in the lysates from B-CLL patients and B cells from healthy volunteers. CD19 was detected at 64kDa and 31kDa to varying degrees in B-cell lysates from both B-CLL patients and normal healthy volunteers (Figure 50). CD5 has been demonstrated on the cell surface of B cells from B-CLL patients along with the expression of CD72 (a CD5L) [457]. CD5 and CD6 have been linked to Bcl-2/ Bax ratios in a role of apoptosis protective mechanisms [458]. CD5 was detected at in B-cell lysates from B-CLL and normal healthy volunteers (Figure 51). However, CD72 was only detected upon T-cell lysates from normal healthy volunteers (Figure 52). This was in disagreement with studies analysing cell surface expression of these molecules. CD5 expression in T-cell lysates from B-CLL patients correlated with the reduced cell surface expression previously demonstrated [255]. Molecules such as CD23 [459] and CD38 [300] are increased upon the B-CLL B cell surface. CD23 was not detected in B-cell lysates from B-CLL patients but was detected in T-cell lysates from 1 B-CLL patient. CD38 was detected in some but not all B-cell lysates from B-CLL patients and in 1 normal healthy volunteer. However, the normal healthy volunteer also showed detection of CD5. This may be for two possible reasons; a lack of symptoms resulting in a no diagnosis or detection of protein by western blot cannot differentiate between constitutive expression

capacity. Bcl-2 is an intracellular signalling molecule which has been reported to be greatly up-regulated in B cells from B-CLL patients [459]. Another inhibitor of apoptosis protein, Survivin has recently been shown to be inducible in B-CLL cells [460]. High CD20 expression has been related low life expectancy [461]. However, none of these proteins are of a molecular weight corresponding to the 41kDa or 65 kDa bands of interest. There is a relatively uncharacterised B lymphocyte surface molecule (p42) with a molecular weight of 42kDa that shows no reactivity to anti-CD23, CD40 or CD72 antibodies [462].

Sequencing revealed the 65kDa band from normal healthy volunteers to have a close homology with human serum albumin. The 65kDa band from B-cell lysates of B-CLL patients could not be sequenced probably because it was naturally N-blocked. The 42kDa band from B-cell lysates from B-CLL patients showed a homology with human actin. However, this was not a close homology and so may be a protein similar in structure to actin. The production of actin-containing and vimentin–containing intermediate filaments in response to 12-o-tetradecanoyl-phorbol-13-acetate (TPA) have been observed in B-cells from B-CLL and hairy cell leukaemia (HCL) and not normal B cells [463]. There is also evidence of an atypical relationship between CD5-CD21 and actin in B-CLL B-cells [464]. A subset of genes have been isolated from B-CLL patients using DNA chip microarrays which are specifically expressed [465]. Analysis of the B-cell lysates using DNA chip microarrays would prove very interesting.

7.15 Implications for the clinic

There are three possible sources of DCs for use in immunotherapy. Firstly, DCs can be directly purified from the peripheral blood. Their low density allows physical separation [466, 467]. Purity can easily be increased by depletion of T-cells, B-cells, Natural Killer cells, and monocytes from the separated fractions of blood or the positive selection of HLA-DR^{++} [468], CD83^{+}, or CMRF-44^{+} [469] cells using magnetic antibody coated beads or a cell sorter.

However, DCs from the peripheral blood are still at a relatively low density. Secondly, $CD34^+$ purified cells from peripheral blood [470] or bone marrow [471, 472] can be cultured with GM-CSF and TNF-α. Larger numbers can be generated using this method although the DCs are often only 50% pure. It is difficult to separate the antigen capture process from antigen presentation. However, DCs grown from $CD34^+$ precursors do have high cell rates which make them ideal candidates for gene transfers with retroviruses and cell fusions. Apheresis can be used to increase the number of $CD34^+$ progenitor cells[473] or $CD14^+$ precursors [474] by *in vivo* administration of G-CSF or GM-CSF followed by large scale isolation of DCs by immunomagnetic bead separation. Thirdly, monocyte derived DCs can be generated from $CD14^+$ cells cultured with GM-CSF and IL-4. 90% of these DCs are immature and so at the optimal stage for antigen uptake. Maturation can be initiated by culture with TNF-α [110](in a serum free environment).

Culture conditions are important for a product that will be returned to the patient's body. The use of fetal calf serum would involve the risk of uptake and presentation of xenogeneic antigens by DCs. However, the use of xenogeneic antigens has shown that slight side-effects associated with cross-species interactions may be beneficial in breaking tolerance to self antigens [475]. Serum contains proteases that would alter the structure and therefore antigenicity of proteins and peptides added *in vitro* to DCs [476]. Autologous serum from patients with a malignancy could contain antibodies which could interfere with loading of the tumour antigen onto the DCs. This has been demonstrated with a anti-Gala(1,3)Gal antibodies that cross-react with MUC-1 [477]. Serum from B-CLL patients has been shown to contain an undefined serum factor that may play a role in the pathogenesis of the disease [478]. Therefore, the use of serum free mediums such as AIM-V [229, 479, 480], X-VIVO 15 [481] are favoured for the production of DCs of a clinical grade.

has been shown to contain an undefined serum factor that may play a role in the pathogenesis of the disease [478]. Therefore, the use of serum free mediums such as AIM-V [229, 479, 480], X-VIVO 15 [481] are favoured for the production of DCs of a clinical grade.

The nature of the antigen used to prime DCs has considerable implications for therapeutic studies. Many *in vivo* studies have used well-defined synthetic peptide sequences with some success [240,243,244,406,480,482, 483, 484]. A previous study in melanoma had demonstrated the generation of equivalent anti-melanoma responses after vaccination with either melanoma peptide- or tumour lysate-pulsed DCs [240]. Longer peptides or complete proteins in cell lysates may prove more effective as the DCs endocytose and process the antigen so that the optimal 9-mer peptide is presented [236]. A mix of peptides also guards against loss of 1 dominant tumour antigen and tumour escape. Where self-proteins are also tumour-associated proteins they can be modulated to become more antigenic with some success [485]. In many malignancies there is limited amount of autologous antigen. This is not the case in B-CLL as the malignant B-cells are commonly found in the periphery. Total RNA from tumour cells has been shown to be effective at generating anti-tumour responses [486]. Where antigen is limited, vaccination with RNA after amplification of RNA by PCR may provide an alternative [487]. DNA transduction of DCs as a strategy possesses several advantages; responses are not restricted by MHC haplotypes, unknown peptides can still be presented and presentation of antigens is more prolonged [488].

The site of injection for DCs may prove vital. Sub-cutaneous injection of dendritic cell vaccines was more effective than intra-venous injections in murine experiments [489]. Intralymphatic injection of DCs ensures contact between DCs and T-cells at the lymph node [490] and has resulted in more effective responses [240].

In vitro generation of tumour-specific T-cells by DCs and injection of T-cell clones

illustrated in a study of 1 melanoma patient [491]. T-cell clones and DCs have been combined with other therapies such as cytokines [492] and bone marrow transplantation [493].

The data presented here would favour a manipulation of the T-cells of B-CLL patients by cytokine modification to overcome the anergic nature of the T-cells from B-CLL patients. Fusion hybrids of autologous DCs and B-CLL B-cells were the most effective stimulus for both MHC class I and II restricted responses. Generation of autologous reactive T-cell clones within the B-CLL patient would be subject to immunosuppression. De-bulking of the tumour prior to immunotherapy would be vital in order to prevent the large number of peripheral B cells interfering with dendritic cell homing and subsequent T-cell interaction. However, the choice of drug therapy or radiotherapy would have to avoid immunosuppression particularly of the $CD4^+$ T-cells. The potential of the peptide sequences already isolated could be explored so that a defined protein (gene) could be targeted. Eventually this could lead to generation of new drug therapies. The reverse immunology approach has been successful in other malignancies at identifying optimal antigens. Testing of tumour associated proteins using the DC system could lead to more effective therapies for B-CLL patients particularly those with drug resistant disease.

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cancer [21]. *In vivo* studies using DC loaded with tumour antigens have demonstrated encouraging clinical anti-tumour responses against B-cell lymphoma [22], melanoma [23], myeloma [24], parathyroid carcinoma [25], prostate cancer [26–28] and renal carcinoma [29]. In this study, we evaluated whether *in vitro* B-CLL-specific T-cell responses could be generated using autologous tumour cell lysate-pulsed DC.

MATERIALS AND METHODS

Volunteer selection

Local research ethics committee permission and individual informed consent were obtained for these studies. A group of 16 patients who were either untreated or who had not received treatment in the last 6 months were selected for the study. Patient details are given in Table 1. Another group of five healthy volunteers was used as a control. Protocols for isolation of cells from the blood from patients and healthy volunteers were identical. Selection of patients for antibody blocking and mixed lysate experiments was random.

Dendritic cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from peripheral blood. PBMC from patients with B-CLL and healthy volunteers were depleted of $CD19^+$ cells using Pan B Dynabeads (Dynal, Merseyside, UK). The CD19-depleted PBMC were cultured in a 24-well tissue culture plate (GIBCO, Life Technologies, Paisley, UK) at 37°C in 5% CO_2 for 2 h. Culture medium consisted of RPMI 1640 (GIBCO), 10% human AB serum, 2 mM glutamine (Sigma, Dorset, UK), 500 U/ml penicillin (Sigma) and 500 μg/ml streptomycin (Sigma). Non-adherent cells were removed by vigorous washing in culture medium. Adherent cells were then cultured in culture medium with 800 U/ml granulocyte macrophage colony stimulating factor (GM-CSF) (Cambridge Bioscience, Cambridge, UK) and 1000 U/ml interleukin-4 (IL-4) (Cambridge Bioscience) at 37°C in 5% CO_2 for 6 days. The cultures were fed every 2 days with fresh culture medium containing IL-4 and GM-CSF. On day 6, the culture medium was removed and the cells cultured in fresh culture medium with 800 U/ml GM-CSF and 100 μg/ml interleukin-12 (IL-12) (Cambridge Bioscience) for 16 h, washed, and resuspended in culture medium.

Table 1. Patient profile

Patient	IWCLL stage	WBC count $\times 10^9$/l	Previous treatment
	A/0	25	None
	A/0	61·7	None
	A/0	18	None
	A/0	14·9	None
	A/0	23·2	None
	A/0 previously A/I	9·8	Chlorambucil
	A/0 previously CIII	13·5	Chlorambucil
	A/0 previously CIII	15	Chlorambucil
	A/I	57	None
	B/II	119·2	None
	C/IV	118	None
	C/IV	122	Chlorambucil and Fludarabine
	C/IV	167	Chlorambucil and splenic radiotherapy
	A/0	20	None
	A/I	26·4	None
	B/I	41·2	Chlorambucil and Cyclophosphamide

Preparation of soluble cell lysate

The $CD19^+$ B-cells from the PBMC fraction were removed from Dynabeads using Pan B Detachabeads (Dynal). In the B-CLL patient, these cells were 97% $CD5^+$ and 92% $CD20^+$. B-cells were resuspended in 2 ml lysis buffer (10 mM bicarbonate buffer pH 7·1 and 0·5 mM phenyl methyl sulphonyl fluoride) (Sigma) on ice. The cells were homogenized on ice using a Dounce Homogeniser (Jencons, Leighton Buzzard, UK) and then ultrasonicated using two 10 s bursts with a 15 s rest from a 50 W Vibracell (Sonics and Materials Inc, Jencons). Soluble protein was collected after ultracentrifugation at 55 000 rev/min (100 000 *g*) for 1 h at 4°C. The protein concentration was quantified by the Bradford protein assay method using a protein determination kit (Biorad, Hemel Hempstead, UK). Soluble protein lysates were sterile-filtered using 0·4 μm filters (Nalgene, Marathon Laboratory Supplies, London, UK) and stored at − 70°C. All cell lysates were exposed to $CD19^+$ Dynabeads. Lysates were defined as allogeneic if they originated from a different individual to the effector T cells. Lysates were defined as non-B-CLL if they were made from B-cells or T-cells from healthy volunteers, or cell types unaffected by B-CLL such as granulocytes.

Pulsing dendritic cells with tetanus toxin and tuberculin PPD

The known antigens tetanus toxin and tuberculin purified protein derivative (PPD) were used as positive controls to validate the proliferation and ELISA assays. Tetanus toxin and tuberculin PPD were added to DC culture medium at 0·4 U/ml and 900 U/ml, respectively, and incubated for 4 h at room temperature.

Pulsing dendritic cells with soluble B-cell lysate

DC were pulsed by the addition of soluble lysate to the DC culture

Table 2. Dendritic cell surface markers. Analysis of markers for CD19 depleted PBMC cultured in RPMI 1640 + 10% AB serum + IL-4 (1000 U/ml) + GM-CSF (800 U/ml) for 6 days at 37°C, 5% CO_2 from two normal volunteers and four B-CLL patients. *P*-values were calculated using single-tailed unpaired Student's *t*-test

Cell surface marker	Normal dendritic cells (Mean percentage + s.d.)	BCLL patient dendritic cells (Mean percentage + s.d.)	
HLA-DR	56·8 + 17·1	54·0 + 8·4	
CD83	1·4 + 0·9	1·2 + 0·8	
CD40	12·9 + 5·9	26·7 + 8·4	$P = 0·02$
CD86	27·8 + 1·8	15·7 + 3·8	$P = 0·003$
CD16	2·2 + 1·2	12·4 + 16·0	
CD56	0·3 + 0·3	0·2 + 0·1	
CD3	10·7 + 5·2	9·2 + 5·6	
CD14	19·3 + 18·2	11·2 + 7·0	
CD11c	4·2 + 4·5	10·1 + 6·0	
CD20	4·1 + 2·7	4·7 + 3·0	
CD1a	9·6 + 7·3	9·6 + 8·3	
CD45	92·5 + 3·5	89·0 + 4·0	

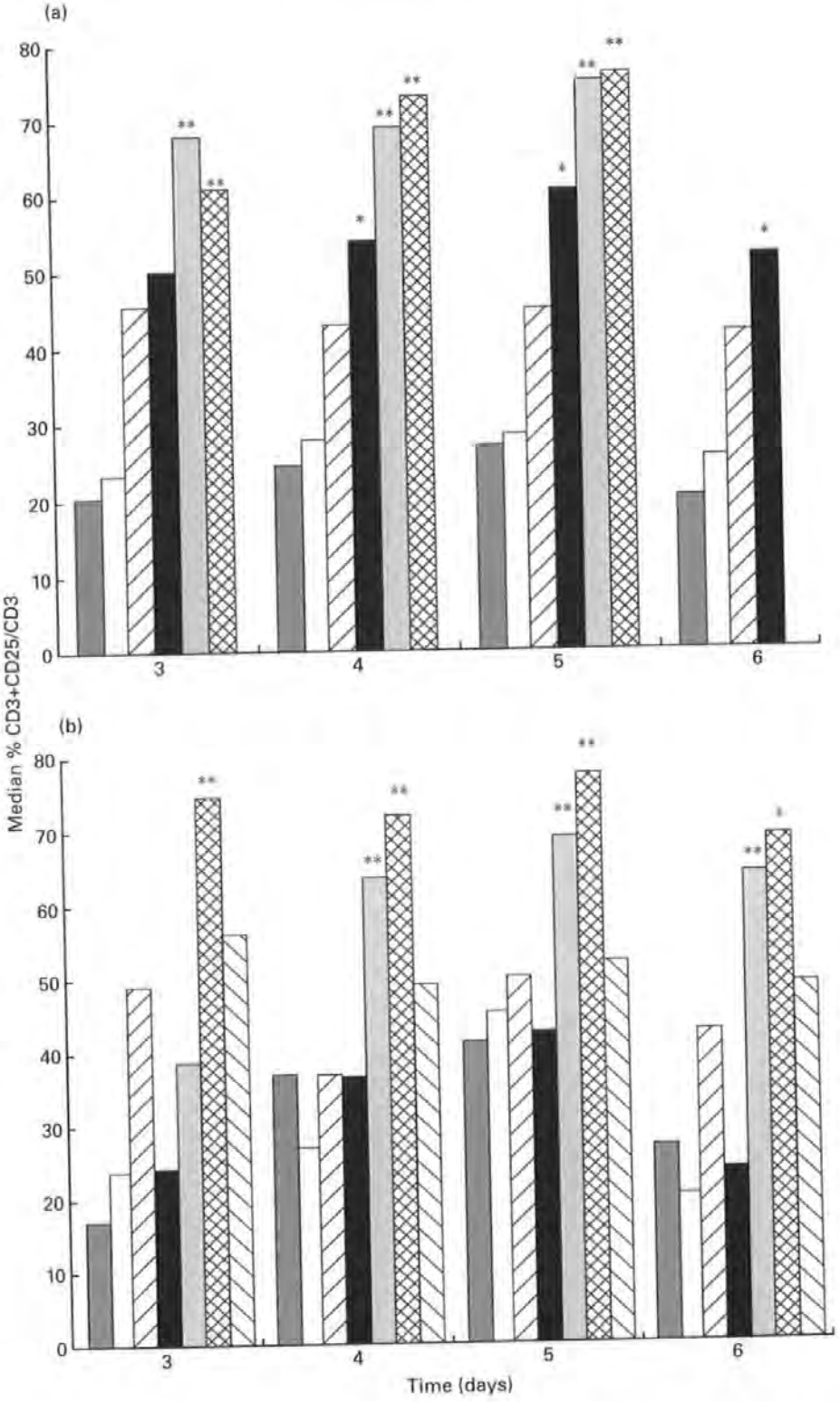

Fig. 1. Proliferation. Numbers of CD3/CD25-positive T-cells from (a) five B-CLL patients and (b) five healthy volunteers were measured. T-cells were cultured alone (■), with autologous dendritic cells pulsed with B-CLL lysate (■), allogeneic lysate from healthy volunteers (non-B-CLL) (▨), autologous lysate from healthy volunteers (non-B-CLL) (▨), or no lysate (□) were measured. One measurement per patient was made. Positive controls of autologous dendritic cells pulsed with tetanus toxin (□) or tuberculin PPD (▩) and cultured with autologous T-cells were measured in duplicate in one patient and one healthy volunteer. Median ranked values, calculated from duplicates, were tested using the Kruskal–Wallis test. (*) Indicates a significant increase in double positive cells, with $P = 0{\cdot}03$ (**) with $P = 0{\cdot}01$ (a) and $P = 0{\cdot}007$ (b), when co-cultures of pulsed dendritic cells were compared with unpulsed dendritic cells.

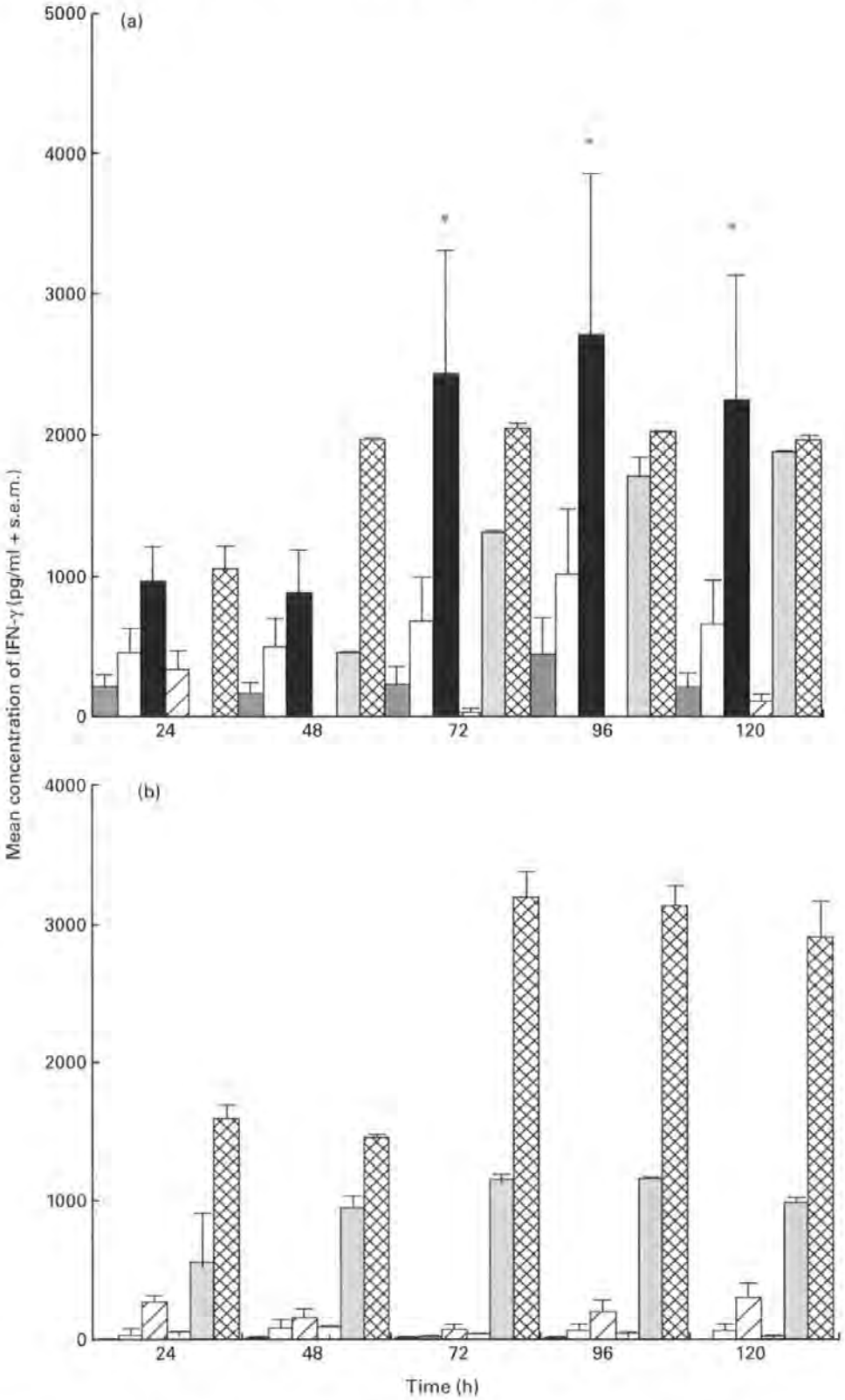

Fig. 2. Cytokine production. Tissue culture supernatant fluids from cultures of (a) 10 B-CLL patients and (b) five healthy volunteers were measured for IFN-γ by ELISA in duplicate. T-cells were cultured alone (▩), with autologous dendritic cells pulsed with B-CLL lysate (■), allogeneic lysate from healthy volunteers (non-B-CLL) (▨), autologous lysate from healthy volunteers (non-B-CLL) (▨), or no lysate (five patients with lysis buffer) (□). Positive controls of autologous dendritic cells pulsed with tetanus toxin (□) or tuberculin PPD (▨) and cultured with autologous T-cells were measured in duplicate in one patient and one healthy volunteer. Mean values were expressed and data analysed by one-way ANOVA. (*) Indicates an overall significant effect of treatment with $P = 0{\cdot}0004$.

medium at 100 ng/ml per 10^6 cells, and incubated for 4 h at room temperature. Control, unpulsed dendritic cells were incubated at this time with lysis buffer, except in Patient's 5, 7, 9, 10 and 13.

T-cell isolation and T-cell cultures

T-cells were isolated indirectly from the PBMC fraction by depleting adherent and $CD19^+$ cells. The purity of the T-cells was, on average, 60% when assessed by flow cytometry using anti-CD3-FITC-conjugated antibodies. Lysate-pulsed or unpulsed dendritic cells were aliquoted at a concentration of 10^3 cells per well to 96-well round-bottom tissue culture plates. T-cells were then added to give a T-cell:DC ratio of at least 20:1. Cultures destined for cytotoxicity assays were fed with 5 U/ml of interleukin-2 (IL-2) (Cambridge Bioscience) on days 3, 7, 10, 14 and 17. DC in cultures destined for cytotoxicity assays were re-stimulated by the addition of 100 ng/ml soluble B-cell lysate or lysis buffer on days 7 and 14. Cultures were continued for a total of 21 days at 37°C in 5% CO_2 in culture medium + 5% AB serum. Cultures used to assess cytokine secretion were not fed IL-2 or re-stimulated with soluble lysate.

Immunophenotyping

The following monoclonal antibodies were used for immunophenotyping studies of DC and effector cells in the cytotoxic assays: CD4-FITC (Serotec, Oxford, UK), CD8-PE (Serotec), CD3-FITC (Serotec), CD16-FITC (Serotec), CD56-FITC (Serotec), HLA-DR-PE (Serotec), CD83-FITC (Immunotech, Coulter, Luton, UK), CD40-PE (Serotec), CD86-FITC (Serotec), CD14-PE/CD45-FITC (Becton Dickinson, Oxford, UK), CD11c-PE (Serotec), CD20-PE (Serotec), CD5-PE (Serotec), CD19-FITC (Serotec), CD1a-FITC (Serotec), anti-IgG1-PE and anti-IgG1-FITC (Serotec). Cells were washed twice in PBS and then twice in PBS + 0·05% BSA (Sigma). Directly-conjugated antibodies were added at 10 μl per 10^6 cells and incubated for 15 min at room temperature. Cells were washed twice in PBS and twice in PBS + 0·05% BSA. Positive antibody binding was assessed in terms of gates set at 2% of relative isotype controls using an Epics Elite flow cytometer (Coulter, Luton, UK).

Proliferation assay

T-cell activation was measured, quantifying cells co-expressing CD3 and CD25 (IL-2 receptor) by double-labelled flow cytometry. The methodology outlined by Loken and Wells [30] was employed, using anti-CD3-FITC-/anti-CD25-PE-conjugated monoclonal antibody (Immuno Quality Products, Mast Diagnostics, Merseyside, UK). Positive antibody binding was assessed in terms of gates set at 2% of anti-IgG1-PE- and anti-IgG1-FITC-labelled cells. Anti-CD3-FITC- (Serotec) and anti-CD25-PE (Serotec)-conjugated monoclonal antibodies were added individually to allow compensation.

Quantification of cytokine secretion

Cell-free tissue culture supernatant fluids were harvested on days 1–5 and stored at −70°C until required. When convenient, the supernatant fluids were thawed and the concentrations of IFN-γ and IL-4 measured in duplicate by ELISA (Pelkline, Eurogenetics, Hampton, UK). Sensitivity limits for the assays were 2–6 pg/ml for IFN-γ and 0·2–0·4 pg/ml for IL-4.

Cytotoxicity assay

Cytotoxicity was measured by a flow cytometric method, LIVE/DEAD cell mediated cytotoxicity (Molecular Probes, Cambridge Bioscience) [31]. Target cells were labelled with 4 μl per 5×10^5 cells of $diOC_{18}$, for 2 h at 37°C in 5% CO_2, and then washed twice in culture medium. Effector cells were harvested from the tissue culture and placed in flow cytometry tubes (Falcon, Marathon Laboratory Supplies, London, UK) at the appropriate effector:-target ratios. A minimum of 1×10^4 labelled targets was added. Propidium iodide was added to each tube. Targets and effectors were gently mixed and centrifuged at 1000 rev/min for 30 s. Targets and effectors were incubated together for 4 h at 37°C in 5% CO_2. Flow cytometry standard gates were set on unlabelled targets stained with propidium iodide, and $diOC_{18}$-labelled targets without propidium iodide. Non-specific cell death (spontaneous apoptosis) was measured by the cytotoxicity of $diOC_{18}$-labelled targets stained with propidium iodide without effectors. Cytotoxicity was expressed as the number of dead targets (cells staining positive for propidium iodide and $diOC_{18}$) divided by the total number of targets (cells staining positive for $diOC_{18}$). Percentage specific cytotoxicity was measured as follows:

$$\% \text{ specific cytotoxicity} = (\text{total cytotoxicity} - \text{spontaneous cytotoxicity}) \times 100$$

As a control, effectors consisting of unpulsed dendritic cells were used to detect any non-specific uptake of the $diOC_{18}$ dye from target cells by dendritic cells.

Target cells in cytotoxicity

The B-CLL B cell targets were 97% $CD5^+$ and 92% $CD20^+$. The B-CLL T cell targets showed binding for CD20-PE < 2%. Allogeneic targets were defined as those originating from another individual to the effector T cells. Targets were defined as non-B-CLL if they were made from B cells and T cells from a healthy volunteer, or granulocytes and T cells from B-CLL patients.

Antibody blocking studies

Antibody blocking experiments involved the addition of anti-HLA class I (Serotec), anti-HLA class II (DR, DP, DQ) (Serotec), anti-Pan TCR αβ (Serotec), anti-CD4 (Serotec) and anti-CD8 (Serotec) monoclonal antibodies at 100 μg/ml at the commencement of the 4 h incubation of effectors and targets.

Statistics

Effects of treatment upon cytotoxicity and cytokine secretion were analysed by one-way analysis of variance (ANOVA). Direct comparisons between treatment groups were analysed using the Student's *t*-test. Differences between median values were compared using the Kruskal–Wallis test. Statistics were generated using Statsgraphics Plus software (Manugistics, Maryland, USA).

RESULTS

Characterization of dendritic cell immunophenotypes

The results of immunophenotyping studies of DC derived from normal volunteers and patients with B-CLL are shown in Table 2. There were no significant differences in mean cell-surface marker expression of HLA-DR, CD1a, CD3, CD4, CD11c, CD14, CD16, CD20, CD45, CD56 and CD83, between patients and healthy volunteers. However, CD40 was found to be significantly increased in patients compared with healthy volunteers

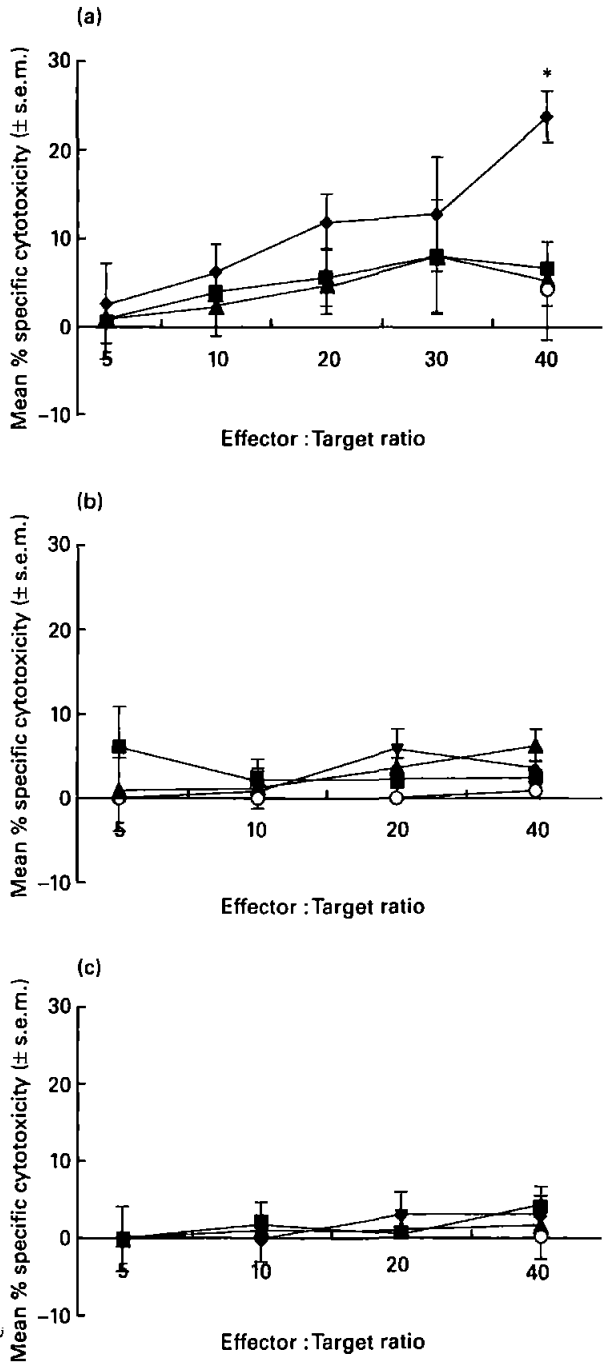

Fig. 3. Cytotoxicity of B-CLL T-cells. Effectors from 10 B-CLL patients were cultured for 21 days (see Materials and Methods for conditions) and cytotoxicity against autologous B-CLL B-cell targets (a), allogeneic B-cell targets from healthy volunteers (non-B-CLL) (b) and autologous B-CLL T-cell targets (c) was measured. T cells had been cultured alone (▲) or with autologous dendritic cells pulsed with autologous B-CLL lysate (◆), allogeneic lysate from healthy volunteers (non B-CLL) (O) or lysis buffer (except in patients 7 and 13 no lysate) (■). Data were analysed by one-way ANOVA. (*) Indicates an overall significant effect of treatment with $P = 0.0008$. Individual results can be seen in Table 3.

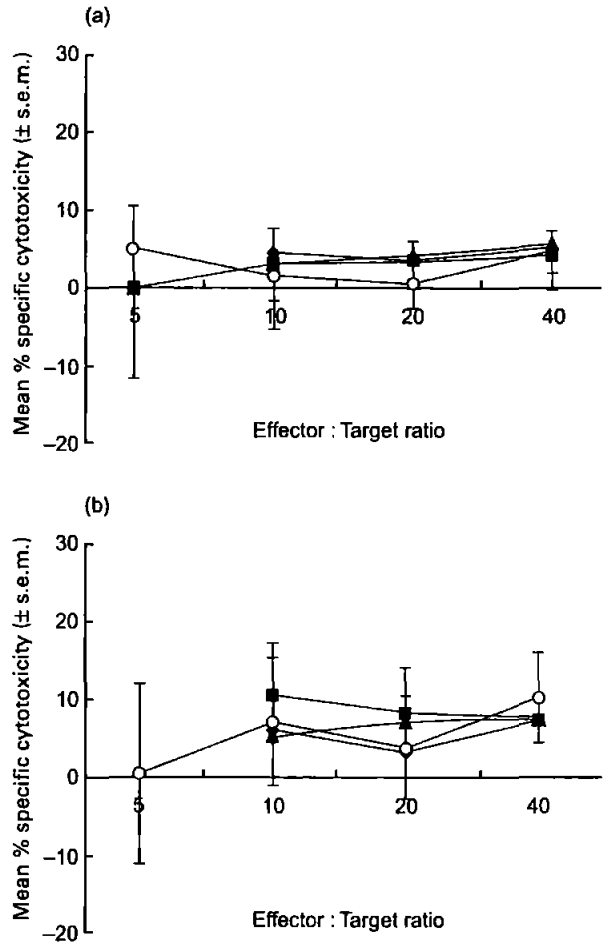

Fig. 4. Cytotoxicity of normal T cells. Effectors from five healthy volunteers were cultured for 21 days (see Materials and Methods for conditions) and cytotoxicity against autologous B-cell targets from healthy volunteers (non-B-CLL) (a) and allogeneic B-CLL B-cell targets (b) was measured. T-cells were cultured alone (▲) or with autologous dendritic cells pulsed with allogeneic B-CLL lysate (▨), autologous lysate from healthy volunteers (non-B-CLL) (O) or lysis buffer (■). One measurement was made per patient and treatment group.

($P = 0.02$), and CD86 was found to be significantly decreased in B-CLL patients compared with healthy volunteers ($P = 0.003$).

Proliferation assays

T-cell activation was observed when autologous dendritic cells, pulsed with either tetanus toxin or tuberculin PPD, were cultured with autologous T cells in a B-CLL patient ($P = 0.01$) (Fig. 1a) and a healthy volunteer ($P = 0.007$) (Fig. 1b). T-cells derived from patients with B-CLL were cultured alone or with autologous B-CLL lysate-pulsed or unpulsed dendritic cells. A significant increase in T-cell activation was found after 4 days of culture by T cells cultured with lysate-pulsed dendritic cells, compared with T cells cultured with unpulsed dendritic cells ($P = 0.03$) (Fig. 1a). Although there was an increase in the percentage of activated B-CLL T-cells after co-culture with autologous B-CLL dendritic cells pulsed with an allogeneic non-B-CLL lysate from a healthy volunteer, this was not significant (Fig. 1a). Similarly, when an allogeneic lysate was pulsed onto dendritic cells from a healthy volunteer, the percentage of activated autologous T-cells was

Table 3. Individual specific cytotoxicty to B-CLL targets for 10 patients in Fig. 3(a) at 40:1 effector:target ratio. Individual cytotoxicity results after effectors have been incubated for 4 h with B-CLL B-cell targets at 37°C,5% CO_2 at a ratio of 40:1. Effectors had been cultured for 3 weeks with two rounds of restimulation

Patient number	T-cells (% + s.d.)	Dendritic cells + lysis buffer +T-cells (% + s.d.)	Dendritic cells + B-CLL lysate + T-cells (% + s.d.)	Dendritic cells + non-B-CLL lysate + T-cells (% + s.d.)
1 *	4·2	7·7	21·7	6·9
2	3·2 + 4·5	1·34 + 4·9	20·6 + 3·7	5·05 + 4·3
3	6·3	6·6	22·30	n.d.
4	0	11·09 + 0·13	30·76 + 1	n.d.
5	4·40	2·90	21·52	n.d.
7 †	9·70	9·70	28·60	n.d.
8	0·00	2·50	18	0·17
11	6·60	5·00	14·10	n.d.
13 †	3·40	2·13	17·70	n.d.
16	3·15 + 2·3	4·45 + 3	13·1 + 4·7	n.d.

*Indicates non-B-CLL lysate is lysate from granulocytes. †Indicates patients' who did not receive lysis buffer as a control. Results showing no standard deviation were not performed in duplicate due to limited numbers of effectors.

increased but not significantly (Fig. 1b). There was no increase in activated T-cells cultured with autologous dendritic cells pulsed with an autologous non-B-CLL B cell lysate from healthy volunteers (Fig. 1b).

Quantification of cytokine secretion

T cells from both B-CLL patients and healthy volunteers secreted increased amounts of IFN-γ when cultured with autologous dendritic cells pulsed with either tetanus toxin or tuberculin PPD (Fig. 2a, b). T cells derived from patients with B-CLL were cultured alone or with lysate-pulsed or unpulsed (lysis buffer added in five patients) dendritic cells. A significant increase of IFN-γ secretion in culture supernatant fluid was found after 72 h by T cells cultured with B-CLL lysate-pulsed autologous dendritic cells compared with both T cells cultured with unpulsed dendritic cells and T cells cultured alone ($P = 0{\cdot}0004$) (Fig. 2a). In addition, there was no secretion of IFN-γ by T cells cultured with autologous dendritic cells pulsed with soluble allogeneic B-cell lysate derived from healthy volunteers (non-B-CLL) (Fig. 2a). T cells derived from healthy volunteers and cultured with autologous dendritic cells pulsed with autologous B cell lysate from healthy volunteers (non-B-CLL) or with allogeneic B-CLL lysate did not secrete significant amounts of IFN-γ (Fig. 2b). Concentrations of IL-4 in tissue culture supernatant fluids were measured in two patients with B-CLL and found to be less than 50 pg/ml.

Cytotoxicity assays

T cells derived from patients with B-CLL were cultured alone, or with lysate-pulsed or unpulsed (lysis buffer added in eight patients) dendritic cells, and then tested for cytotoxicity against healthy and B-CLL-derived B- and T-cell targets. A significant increase in cytotoxicity against B-CLL targets was generated by T cells cultured with B-CLL lysate-pulsed autologous dendritic cells, compared with both T cells cultured with unpulsed dendritic cells and T cells cultured alone ($P = 0{\cdot}0008$) (Fig. 3a). However, no significant cytotoxicity was demonstrated against allogeneic B-cell targets from healthy volunteers (non-B-CLL targets) (Fig. 3b), or against autologous T cells derived from B-CLL patients (non-B-cell targets) (Fig. 3c). In one patient, effectors were tested against B-cells, PBMCs and T-cells from two HLA class I- and II-matched healthy volunteers and one HLA class II-matched healthy volunteer. No cytotoxicity to these targets was demonstrated.

T-cells derived from healthy volunteers cultured with autologous dendritic cells pulsed with autologous B-cell lysate from healthy volunteers (non-B-CLL lysate), or with allogeneic B-CLL lysate, did not demonstrate significant cytotoxicity against B-cell targets from healthy volunteers (non-B-CLL targets) (Fig. 4a) or B-CLL targets (Fig. 4b).

T-cells derived from patients with B-CLL cultured with soluble B-CLL lysate in the absence of dendritic cells did not show significant cytotoxicity against B-CLL or B-cell targets from healthy volunteers. In addition, T cells cultured with autologous dendritic cells pulsed with an autologous granulocyte lysate from B-CLL Patient 1 showed no significant increase in cytotoxicity against autologous B-CLL cells (see Table 3) and autologous granulocytes, or allogeneic B-cell targets from a healthy volunteer (non-B-CLL).

T-cells derived from Patient 14 and cultured with autologous dendritic cells pulsed with an allogeneic B-CLL lysate from patient 15 did not demonstrate cytotoxicity to B-CLL targets from patient 14 (Fig. 5a). However, T cells derived from patient 6 and cultured with autologous dendritic cells pulsed with allogeneic B-CLL lysates from patients 2 and 13 demonstrated significant cytotoxicity to B-CLL targets from patient 6 ($P = 0{\cdot}003$ *and* $P = 0{\cdot}009$). Allogeneic B-CLL lysates from B-CLL Patient 7 did not stimulate cytotoxicity to B-CLL targets from patient 6 (Fig. 5b). The allogeneic lysates used above had been shown to generate specific cytotoxicity when used as autologous lysates with autologous effectors and targets. These results are summarized in Table 4.

Characterization of responder cell immunophenotypes

Effector cells from B-CLL patients 14 and 16 were immunophenotyped after 21 days in culture. The mean cell surface expressio

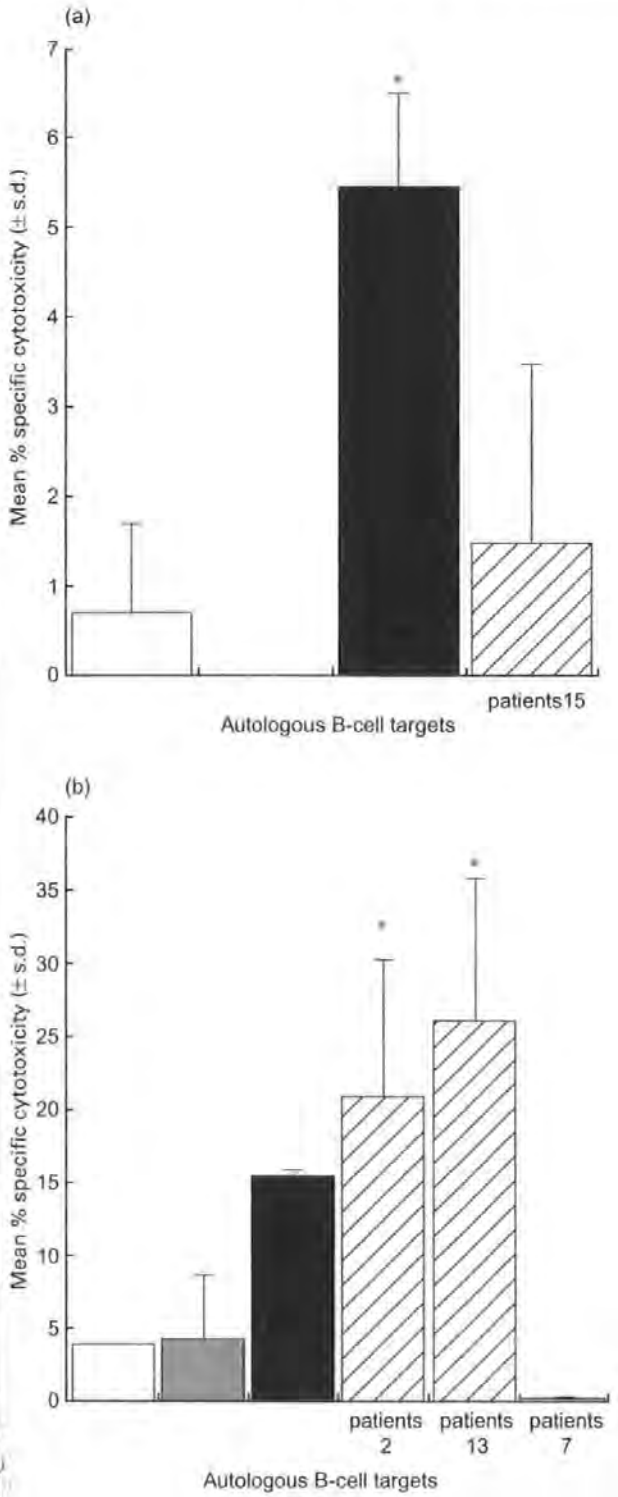

Fig. 5. Effect of allogeneic B-CLL lysate. Effectors from B-CLL Patients 4 (a) and 6 (b) were cultured alone (□), with autologous dendritic cells pulsed with autologous B-CLL lysate (■), B-CLL allogeneic lysates from Patients 15, 2, 13 and 7 (▨), and lysis buffer (□) for 21 days. Cytotoxicity shown was performed at a target:effector ratio of 40:1. (*) Indicated a significant increase in mean specific cytotoxicity compared with autologous dendritic cells with lysis buffer, with $P < 0.01$, when analysed by Students *t*-test. No significant specific cytotoxicity was measured against autologous T cells, allogeneic B cells or allogeneic T cells. Each treatment group was measured in duplicate per patient.

was found to be 47% $CD4^+$ cells and 13% $CD8^+$ cells, the remaining cells being CD3 negative. CD16 and CD56 expression was absent.

Antibody blocking studies

Antibody blocking experiments were performed at the effector stage of the cytotoxicity assay on three patients chosen at random. Significant inhibition of cytotoxicity was demonstrated with anti-class II but not with anti-class I monoclonal antibodies in patients 2 (Fig. 6a) ($P = 0.006$) and 12 ($P = 0.031$) (Fig. 6b). Significant inhibition of cytotoxicity was demonstrated in patient 4 with anti-pan TCR αβ and anti-CD4, but not with anti-CD8 monoclonal antibodies ($P = 0.03$ *and* $P = 0.046$) (Fig. 6c).

DISCUSSION

Chronic B cell malignancies represent a potential target for immunotherapy by virtue of the fact that they are derived from immune cells and thus have the capacity for interaction with T lymphocytes. In addition, each tumour is derived from a clonal expansion of one malignant B cell and thus, each cell in the clone will share specific determinants, for example, those encoded by variable region sequences of rearranged immunoglobulin genes. Clinical studies in which patients with lymphoma and myeloma were vaccinated with idiotype protein have demonstrated the generation of anti-idiotypic T-cell responses and a degree of clinical response [32–36]. An alternative approach has been to load DC with idiotype protein and subsequently use the pulsed DC as a form of immunotherapy. Such an approach has also been shown to induce anti-idiotypic immune responses in lymphoma [22,37] and myeloma [24,38], with some encouraging clinical results. A similar rationale can therefore be proposed for immunotherapy in B-CLL, and this study was designed to determine whether DC loaded with B-CLL tumour protein could stimulate anti-B-CLL cell responses. Since a previous study in melanoma had demonstrated the generation of equivalent anti-melanoma responses after vaccination with either melanoma peptide- or tumour lysate-pulsed DC [23], we decided to load DC with soluble B-CLL cell lysate.

Initial problems of generation of adequate numbers of DC from patients with B-CLL were overcome once the malignant B-cells were removed. These DC were morphologically and numerically similar to those from healthy individuals. Immunophenotyping DC generated from healthy individuals and patients with B-CLL demonstrated similar levels of expression of cell surface molecules, with the notable exception of CD40 and CD86. A previous study has demonstrated that DC generated from healthy individuals and patients with myeloma are phenotypically and functionally similar; however, this did not include CD40 [39]. Although the significance of this finding is unclear at present, $CD40^+$ DC have been found to be vital for the generation of anti-tumour responses in mice [40]. Stimulation of CD40 by increased expression of CD40-ligand has been shown to stimulate immune recognition of B-CLL cells *in vivo* [41]. The decreased levels of CD86 in B-CLL patients differ significantly from the findings in myeloma patients and may be linked to the disease. Since CD86 has been shown to be a key co-stimulatory molecule [42], its decreased expression upon the dendritic cells from B-CLL patients may affect the optimal presentation of antigens to T-cells.

Our results demonstrate that patient-derived DC, when pulsed with soluble B-CLL lysate, stimulate autologous T cells to

Table 4. Summary of cytotoxic T cell generation and responses.

T cells	Dendritic cells	Lysate	Targets	Response
B-CLL	Autologous B-CLL	Autologous B-CLL B-cells	Autologous B-CLL	Increase in IFN-γ. Increase in specific cytotoxicity. Blocked by anti-MHC-class II, anti-TCR, anti-CD4.
			Autologous B-CLL T-cells	No response
			Allogeneic healthy B-cells	No response
B-CLL	NONE	Autologous B-CLL B-cells	Autologous B-CLL B-cells	No response
Healthy volunteer	Autologous healthy volunteer	Autologous healthy B cells	Autologous healthy B cells	No response
			Allogeneic B-CLL B-cells	No response
		Allogeneic B-CLL B-cells	Autologous healthy B cells	No cytotoxic or cytokine response, non-significant increase in percentage activated T cells
			Allogeneic B-CLL B-cells	
B-CLL	Autologous B-CLL	Autologous B-CLL granulocytes	Autologous B-CLL B-cells	No response
			Allogeneic healthy B-cells	No response
			Autologous B-CLL granulocytes	No response
B-CLL	Autologous B-CLL	Allogeneic healthy B-cells	Allogeneic healthy B-cells	No cytotoxic or cytokine response, non-significant increase in percentage activated T cells
B-CLL	Autologous B-CLL	Allogeneic B-CLL B-cells	Autologous B-CLL B-cells	2/4 patients showed increased specific cytotoxicity

proliferate, secrete IFN-γ (but not IL-4) and lyse autologous B-CLL targets. This suggests that our culture system has primed both a T helper 1 and a cytotoxic T-cell immune response. Antigen-presenting cells, such as DC, have the capacity to generate either a T helper 1 or a T helper 2 immune response [43]. T helper 1 responses are associated with the secretion of particular cytokines, such as IFN-γ and IL-12, and induce cell-mediated immunity, which is particularly important in the response against tumours. T helper 2 responses are associated with the secretion of cytokines such as IL-4 and IL-10. Tumours have been shown to secrete factors, such as IL-10 [44], transforming growth factor-β (TGF-β) [45] and vascular endothelial growth factor (VEGF) [46], which suppress DC and/or T cell function. Immature DC treated with IL-10 have been shown to induce anergy in melanoma antigen-specific $CD8^+$ cytotoxic T-cells *in vitro* [47]. The T-cells used in our experiments were derived from patients with B-CLL and thus, were exposed *in vivo* to possible immunosuppressive factors secreted by the B-CLL cells. Recent data in our group suggests that the T cells of B-CLL patients show dysfunctional cell surface molecule expression [48]. Studies have shown that DC can be polarized towards stimulatory or inhibitory phenotype, IL-12 polarizing towards the induction of T helper 1 responses [49–51]. Pre-treatment of murine dendritic cells with IL-12 was shown to illicit responses to relatively unreactive tumour/self peptides [52]. In view of this, we pre-incubated the DC with IL-12 just prior to the addition of antigen. Recently, IL-12 responsiveness has been demonstrated in human monocyte-derived dendritic cells [53]. One preliminary experiment showed that without pre-treatment with IL-12, monocyte-derived dendritic cells pulsed with B-CLL lysate could not stimulate significant T-cell responses. The specific T-cell responses to B-CLL generated in our culture system suggest that, if a state of anergy to the tumour existed *in vivo*, the *in vitro* culture with DC and pre-treatment with IL-12 has reversed this state.

The levels of IFN-γ observed in our study are similar to those generated by superantigen-pulsed DC cultured with T cells [54], but are higher than those generated by CD40L-stimulated B-CLL B cells cultured with autologous T cells [55]. Although the levels of specific cytotoxicity (24%), generated in our cultures at an effector:target ratio of 40:1, seem low compared with those of other *in vitro* studies [15,17–19,56], they are comparable with levels shown by others [14,20,21,57–59]. These differences could be due, in part, to the fact that different diseases were studied. However, in a study of B-CLL, similar levels of specific cytotoxicity were generated when CD40L-stimulated B-CLL cells were cultured with allogeneic T cells, but not autologous T cells [55].

We attempted to increase the immunogenicity of our B-CLL lysate by heat-shock treatment of the B-CLL cells before preparation of the lysate, and by using combined fractions of soluble and membrane-bound lysate. Neither of these treatments resulted in increased percentages of specific cytotoxicity. So far

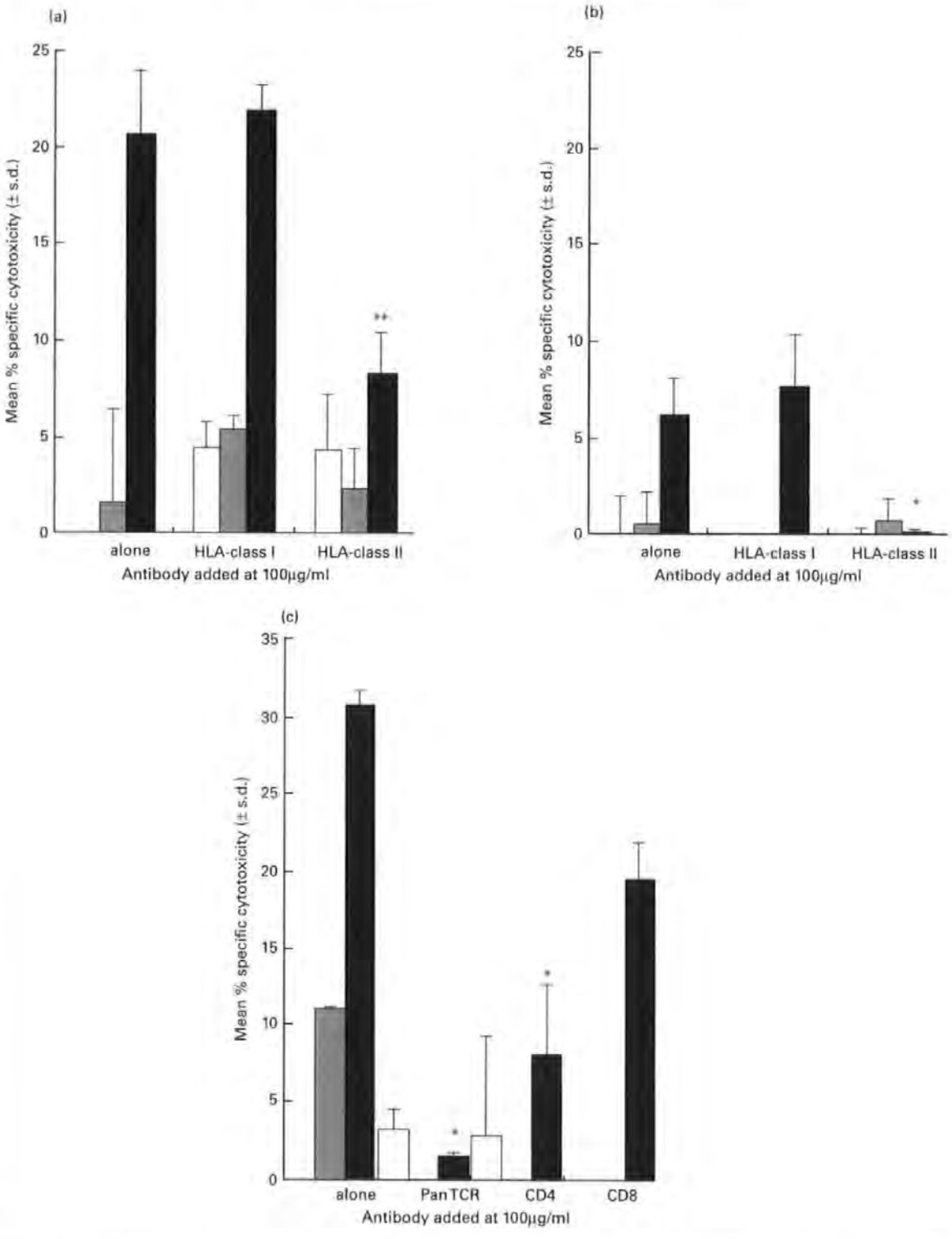

Fig. 6. Antibody blocking experiments. T-cell effectors from B-CLL patient 2 (a) and B-CLL patient 12 (b) were cultured alone (□), with autologous B-CLL dendritic cells pulsed with B-CLL lysate (■) or lysis buffer (▨) for 21 days (see Materials and Methods). Anti-human HLA-class I and anti-human HLA-class II antibodies were present whilst effectors were incubated at a 40:1 effector:target ratio with autologous B-CLL B-cell targets (a and b). T-cell effectors from B-CLL patient 4 (c) were cultured alone (□), with autologous B-CLL dendritic cells pulsed with B-CLL lysate (■) or lysis buffer (□) for 21 days (see Materials and Methods). Anti-Pan TCR $\alpha\beta$, Anti-CD8 and Anti-CD4 antibodies were incubated with autologous B-CLL B-cell targets at a target:effector ratio of 40:1 (c). The (*) and (**) indicate a significant inhibition of specific cytotoxicity in the presence of antibody compared with none, with $P < 0.05$ and $P < 0.01$ when analysed by Student's *t*-test. Each treatment group was measured in duplicate per patient.

we have been unable to generate stronger T-cell responses *in vitro* with additional immune manipulation during the cell culture period. Perhaps the modest level of tumour-specific cytotoxicity observed is due to the relatively low frequency of B-CLL-reactive cells within the patients' T-cell repertoire. However, the low levels of cytotoxicity may be due to the relatively immature state of the monocyte-derived DC ($CD83^-$) used to present lysate antigens to the T cells. After the loading of antigen onto the dendritic cell surface, a further 'danger signal' is required to achieve maximal presentation of that antigen to the T cell [9]. Current work is testing whether treatment with lipopolysaccharide and polyriboinosinic polyribocytidylic acid can enhance further DC maturation after tumour lysate loading, and therefore stimulate higher levels of specific cytotoxicity to B-CLL cells.

Of particular interest was the nature of the responding cell population. The responding cells in two patients were predominantly $CD4^+$. Although there is evidence that DC can directly trigger anti-tumour responses by Natural Killer (NK) cells [60], the effector cells in our assay did not express the NK markers CD16 and CD56. Antibody blocking experiments in the three patients tested demonstrated significant inhibition of cytotoxicity with anti-class II, anti-pan TCR $\alpha\beta$ and anti-CD4, but not with anti-class I or anti-CD8 monoclonal antibodies. Overall, this suggests that $CD4^+$ HLA class II-restricted cytotoxic T cells are responsible for the majority of lysis of B-CLL cells in our culture system. In general, it is thought that $CD8^+$ class I-restricted T cells are responsible for anti-tumour immunity [19,21,61]. However, $CD4^+$ cytotoxic T cells have been reported in hepatocellular carcinoma [62] and p21-ras (12Val) mutated cells [63]. In general, it is thought that DC process and present exogenous proteins by the HLA class II pathway, and that internalizing antigen from apoptotic cells generates MHC class I restricted cytotoxic T lymphocytes [9]. B-CLL patients with decreased the numbers of $CD4^+$ T cells due to recent therapy with fludarabine [64] may not exhibit a specific cytotoxic response in our culture system.

One concern that has been raised with regard to dendritic cell vaccination is the possibility of inducing autoreactivity. In our culture system, reactivity was not demonstrated against autologous B-CLL T-cells and granulocytes, or HLA matched and allogeneic B-cells from healthy individuals. Recently, T-cells generated by stimulation with CML-pulsed dendritic cells showed proliferation against autologous CML cells, but not an HLA-identical sibling [65]. However, our system was unable to demonstrate strong T-cell responses to allogeneic lysates. We can only surmise that our system is not optimized for the presentation of allogeneic antigens via the indirect pathway. However, the system was able to present well characterized antigens such as tetanus toxin and tuberculin PPD. The lack of a specific response by T-cells from healthy volunteers presented with B-CLL lysate antigens by their own dendritic cells suggests the presence of a pre-existing pool of reactive T-cells in B-CLL patients not found in healthy volunteers. Recently, the existence of autologous T-lymphocytes capable of spontaneous specific recognition of B-cells from B-CLL patients has been demonstrated [66]. Ideally, we would wish to test for cytotoxicity against autologous non-malignant B-cells from B-CLL patients, but due to the overabundance of the malignant B-cells in these patients, this was found to be impossible. Although T-cells make poor targets, we did not wish to introduce anti-viral epitopes into our system in the form of EBV-transfected targets. Clinical studies with DC vaccination have so far reported no evidence of autoimmune disease [22–24,26,29], although autoimmunity has been induced in mice in one study [67].

Our experiments have demonstrated specific T-cell responses to B-CLL. Although cytotoxicity was induced with some allogeneic B-CLL lysates, this was not the case with others. This suggests that, although a common antigen may be present in a proportion of B-CLL patients, other B-CLL patients may have unique antigens. This should not be surprising, as B-CLL is not a homogenous disease. However, despite the obstacles this heterogeneity may present, we believe that it is important to identify the antigen(s) within the B-CLL lysate responsible for the immune responses we have demonstrated, and this work is currently in progress. At the same time, this study provides evidence that a clinical trial of immunotherapy in B-CLL is feasible.

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Generation *in vitro* of B-cell chronic lymphocytic leukaemia-proliferative and specific HLA class-II-restricted cytotoxic T-cell responses using autologous dendritic cells pulsed with tumour cell lysate

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SUMMARY

Immunotherapy using dendritic cells has shown encouraging results in both haematological and non-haematological malignancies. In this study, monocyte-derived dendritic cells from patients with B-CLL were cultured for 6 days in the presence of IL-4 and GM-CSF. Autologous B-CLL T-cells were cultured alone or with B-CLL lysate-pulsed and unpulsed autologous dendritic cells. IFN-γ secretion was assessed using ELISA. Cytotoxicity was assessed, after 21 days in culture and re-stimulation, using flow cytometry with and without blockade by anti-HLA class I, anti-HLA class II, anti-CD4, anti-CD8 and anti-TCRαβ monoclonal antibodies. B-CLL T cells stimulated with B-CLL lysate-pulsed autologous dendritic cells showed a significant ($P = 0{\cdot}0004$) increase in IFN-γ secretion and a significant ($P = 0{\cdot}0008$) increase in specific cytotoxicity to autologous B-cell targets, but none to autologous T cell or B cell targets from healthy individuals. B-CLL T cells cultured with (non-B-CLL) B-cell lysate-pulsed B-CLL dendritic cells showed no significant response. Pulsing dendritic cells from healthy volunteers with an autologous (non-B-CLL) B-cell lysate did not stimulate proliferation, cytokine production or cytotoxicity by autologous T cells. Pulsing B-CLL dendritic cells with allogeneic B-CLL lysates and culturing with autologous T-cells elicited cytotoxicity against autologous B-CLL targets in some cases, but not in others. Cytotoxicity was significantly reduced by blocking with anti-HLA class II ($P = 0{\cdot}001$), anti-TCRαβ ($P = 0{\cdot}03$) and anti-CD4 ($P = 0{\cdot}046$) antibodies. Phenotyping of the responding T-cell population demonstrated the majority to be CD4 positive. Our data demonstrate that HLA class II-restricted proliferative and cytotoxic T-cell responses to B-CLL can be generated using autologous dendritic cells pulsed with tumour cell lysate.

Keywords dendritic cells tumour lysate B-cell chronic lymphocytic leukaemia HLA class II

INTRODUCTION

B cell chronic lymphocytic leukaemia (B-CLL) is characterized by the accumulation of a clone of malignant B cells in lymphoid tissue, the bone marrow and the peripheral blood. Although chemotherapy can induce partial or complete remission [1], long-term disease-free survival is unusual. Recent trials of bone marrow transplantation or monoclonal antibodies are promising [2]. However, new treatment modalities for this disease are required. In view of the fact that B-CLL is a tumour of the immune system, it seems challenging and logical to attempt to harness that system to treat this disease.

Early attempts at cancer immunotherapy were relatively crude, including injecting patients with killed tumour cells or adjuvants such as *Bacillus Calmette Guerin* and *Corynebacterium parvum*.

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The recent marked growth in our understanding of the immune system and its interaction with malignant disease includes the mechanism by which antigen is presented to T lymphocytes [3,4]. Dendritic cells (DC) are now known to be essential for the initiation of primary immune responses and are particularly efficient at capturing and presenting antigens to T cells [5–7]. By priming them *in vitro* with tumour antigens, these 'professional' antigen-presenting cells (APCs) may bypass the state of ignorance in which the immune system appears to co-exist with most tumours [8]. Studies of DC vaccines in both animal models and man have demonstrated the generation of anti-tumour immune responses [9–11].

Reliable methods for generating immature DC from peripheral blood mononuclear cells have facilitated their use in immunotherapy [12,13]. *In vitro* studies in man have demonstrated that DC loaded with tumour antigens can induce cytotoxic T-lymphocyte (CTL) responses against melanoma [14–16], chronic myeloid leukaemia [17–19], acute myeloid leukaemia [20] and pancreatic

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