UNIVERSITY OF SOUTHAMPTON

Murine Modelling of Adoptive Therapy For B Cell Lymphoma

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

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Doctor of Philosophy

MURINE MODELLING OF ADOPTIVE THERAPY FOR B CELL LYMPHOMA

By Claire Nicole Patrick

The interaction between CD40 and its ligand (CD154) plays a critical role in the activation of the immune system. Ligation of CD40 on antigen presenting cells (APC) enables them to process and present antigen efficiently and facilitates the generation of cytotoxic CD8+ T lymphocytes (CTL). Such a response is observed using anti-CD40 mAb to generate CTL in the treatment of syngeneic murine lymphoma. Adoptive transfer of CTL has proven effective in the clinic. However, a major barrier for the use of adoptive transfer is in obtaining the large numbers of CTL needed for effective treatment.

Our aim was to study means of expanding lymphoma specific CTL *ex vivo* and to determine the optimal cell surface signalling and cytokine environment required. In addition, we wished to investigate ways of prolonging the survival of these cells in the recipient mouse.

Here we show that $CD62L^{hi}$, $CD44^{low}$, $CD25^{low}$, $CD49d^{low}$, $41BB^{low}$ CD8+ T cells taken from the spleen of mice cured of the murine B cell lymphoma (BCL_1) via anti-CD40 treatment and not effector cells harvested from the peak of the CTL response nor naïve CD8+ T cells, can be used to generate CTL lines in vitro . We also demonstrate that the activation, proliferation and cytotoxic activity of in vitro generated CTL is dependent on the presence of antigen. We have shown that these CTL are specific for the tumour and are capable of long term expansion. Furthermore, specific polyclonal lines of CTL have shown to be therapeutic in an adoptive transfer model.

Furthermore we show that, like CD8+ T cells, CD4+ T cells taken from the spleen mice cured of BCL_1 can be cultured in vitro to generate tumour-specific lines which augment the efficacy of CD8+ CTL lines in adoptive therapy.

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This is dedicated to Alan Paul Monet.

Abbreviations

2-ME	2-mercapto-ethanol	**mAb**	Monoclonal antibody
AICD	Activation-induced cell death	**MACS**	Magnetically Assisted Cell Separation
APC	Antigen presenting cell or Allophycocyanin	**MHC**	Major Histocompatibility complex
		NP40	Nonidet P40
aAPC	Artificial antigen presenting cell	**OVA**	Ovalbumin
BrdU	5-Bromo-2-deoxyuridine	**PAMP**	Pathogen-derived molecular pattern
BSA	Bovine Serum Albumin	**PBMC**	Peripheral blood mononuclear cell
CDRs	Complementarity determining region	**PBS**	Phosphate Buffered Saline
CFSE	Carboxy Fluorescein Succinimidyl Ester	**PE**	Phycoerthyrin
		PMA	Phorbol 12-myristidate 13-acetate
CLIP	Class-II-associated Ii peptide	**PRR**	Pattern-recognition receptor
CTL	Cytotoxic lymphocyte	**PS**	Phosphotidylserine
CTLp	CTL precursors	**PTK**	Protein tyrosine kinase
DC	Dendritic cell	**RAG**	Recombination activating gene
ELISA	Enzyme-Linked Immunsorbant Assay	**RT-PCR**	Reverse transcription polymerase chain reaction
ER	Endoplasmic reticulum		
FCS	Foetal Calf Serum	**TAAs**	Tumour-associated antigens
FITC	Fluorescein isothiocyanate	**TCR**	T-cell receptor
FSC/SSC	Forward scatter/Side scatter	**T_H**	T-helper
HSP	Heat-shock protein	**TIL**	Tumour infiltrating lymphocyte
Ig	Immunoglobulin	**TLR**	Toll-like receptor/ Toll/ILR homologous region
Ii	Invariant chains		
IMDM	Iscove's modified Dulbecco's media	**TNF(R)**	Tumour necrosis factor (receptor)
ITAM	Immunoreceptor tyrosine-based activation motif	**Tr**	T-regulatory
		X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
LPS	Lipopolysaccaride		

1 INTRODUCTION

1.1 Cancer

Cancer is the spontaneous and uncontrolled new growth of abnormal cells. It is a major cause of mortality in the UK with more than 200,000 new cases (excluding non-melanoma skin cancer), registered in both 1998 and 1999 (Figure 1.1). In recent years whilst numbers of deaths from heart disease have fallen, deaths from cancer have remained stable. The result is that 1 in 3 people will be diagnosed with cancer during their lifetime and 1 in 4 people will die from cancer.

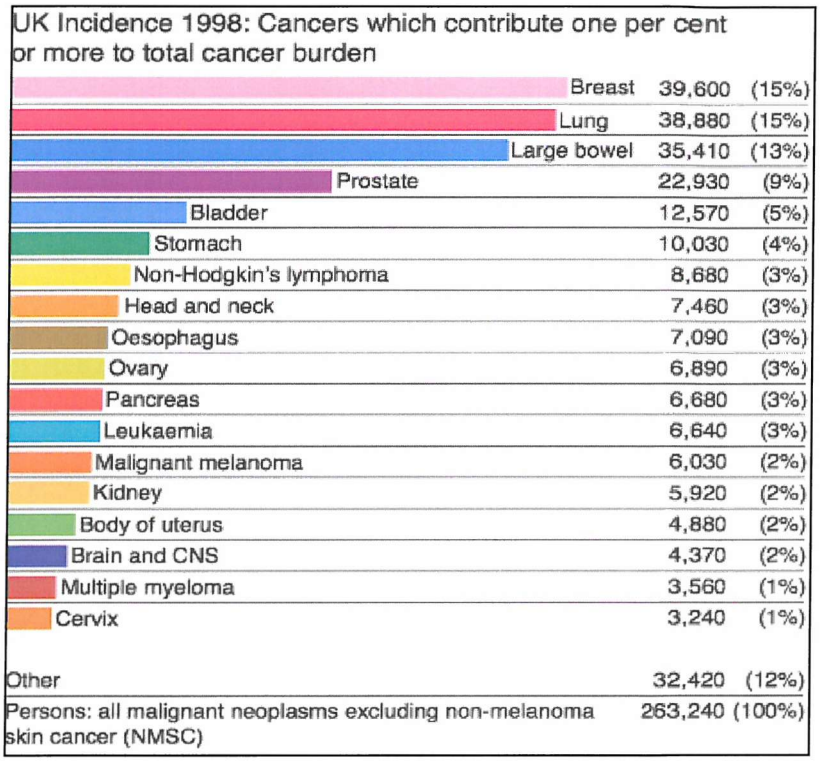
There are over 200 types of cancer, broadly characterised by the tissue from which they originate. Four major types, lung, breast, prostate and colorectal, account for over half of all new cases diagnosed.

Strategies presently used for eradicating cancerous tumours include surgery, chemotherapy, and radiotherapy. Although some cancers can be cured successfully by these regimes, in a significant proportion cure is not achieved (1). In addition, therapies such as chemotherapy, indiscriminately kill healthy cells causing unwanted side effects, therefore, it is evident that novel strategies should be explored. Immunotherapy is one such alternative.

This introduction aims to discuss how cellular responses are generated, why the immune system does not fully respond to and eradicate cancers and present strategies, with particular emphasis on adoptive cellular therapies, which aim to evoke a full and effective immune response against tumour.

1.2 The immune system - an overview

The vertebrate immune system protects individuals from invading pathogenic microorganisms and cancer. This host defence relies on both the innate immune system and the adaptive immune system.



UK Incidence 1998: Cancers which contribute one per cent or more to total cancer burden		
Breast	39,600	(15%)
Lung	38,880	(15%)
Large bowel	35,410	(13%)
Prostate	22,930	(9%)
Bladder	12,570	(5%)
Stomach	10,030	(4%)
Non-Hodgkin's lymphoma	8,680	(3%)
Head and neck	7,460	(3%)
Oesophagus	7,090	(3%)
Ovary	6,890	(3%)
Pancreas	6,680	(3%)
Leukaemia	6,640	(3%)
Malignant melanoma	6,030	(2%)
Kidney	5,920	(2%)
Body of uterus	4,880	(2%)
Brain and CNS	4,370	(2%)
Multiple myeloma	3,560	(1%)
Cervix	3,240	(1%)
Other	32,420	(12%)
Persons: all malignant neoplasms excluding non-melanoma skin cancer (NMSC)	263,240	(100%)

Figure 1.1 UK incidence 1998: Cancers which contribute one per cent or more to total cancer burden. Table taken from http:/www.cancerresearchuk.org. Sources : Office for National Statistics, Welsh Cancer Intelligence and Surveillance Unit, Information Statistics Division Scotland and Northern Ireland Cancer Registry

1.2.1 The innate immune system

The innate immune system is primarily involved in rapidly recognising foreign pathogens and then helping to activate the adaptive immune system. The innate immune system includes phagocytic cells, natural killer (NK) cells, complement and interferons (IFNs). The cells of the innate system use a number of pattern recognition receptors to recognise patterns shared between pathogens, known as pathogen-derived molecular patterns (PAMPs). These include lipopolysaccharide (LPS) and carbohydrates. If specific patterns on invading pathogens are recognised by the innate immune system this can lead to direct deletion of the invader, complement lysis, or tagging of the foreign pathogen (C3c coating) for a more robust response than of the adaptive system. However, the innate immune system cannot cope with molecular evolution we therefore developed adaptive immunity.

1.2.2 The Adaptive immune system

Unlike the innate immune response, adaptive immune responses are antigen-specific, show diversity and possess immunological memory, and are thus able to mount a more effective response to antigen on the second encounter. The adaptive immune system is potentially able to recognise billions of different antigens which may be subtly different from one another and can distinguish foreign antigens (non-self) from those expressed on normal cells.

1.2.2.1 An overview of the T cell response

T and B lymphocytes are central to the adaptive immune response. This is because each B or T cell bears a unique receptor on its surface capable of recognising a different antigenic surface. Therefore, when an antigen is recognised by an antigen receptor of a T cell, clonal expansion occurs, increasing the number of T cells with the ability to recognise the given antigen. Subsequent steps in the immune response cause T cells to mature and develop into effector cells capable of eradicating the pathogen. An important component of the adaptive response is that some of these expanded T cells subsequently differentiate into so-called memory cells, capable of mounting a more rapid response to the antigen upon a second encounter. Another important facet of the T cell response is that T cells cannot recognise antigens directly but instead recognise antigen in association with another molecule known as the major histocompatability complex (MHC). Prior to its association with MHC, antigen

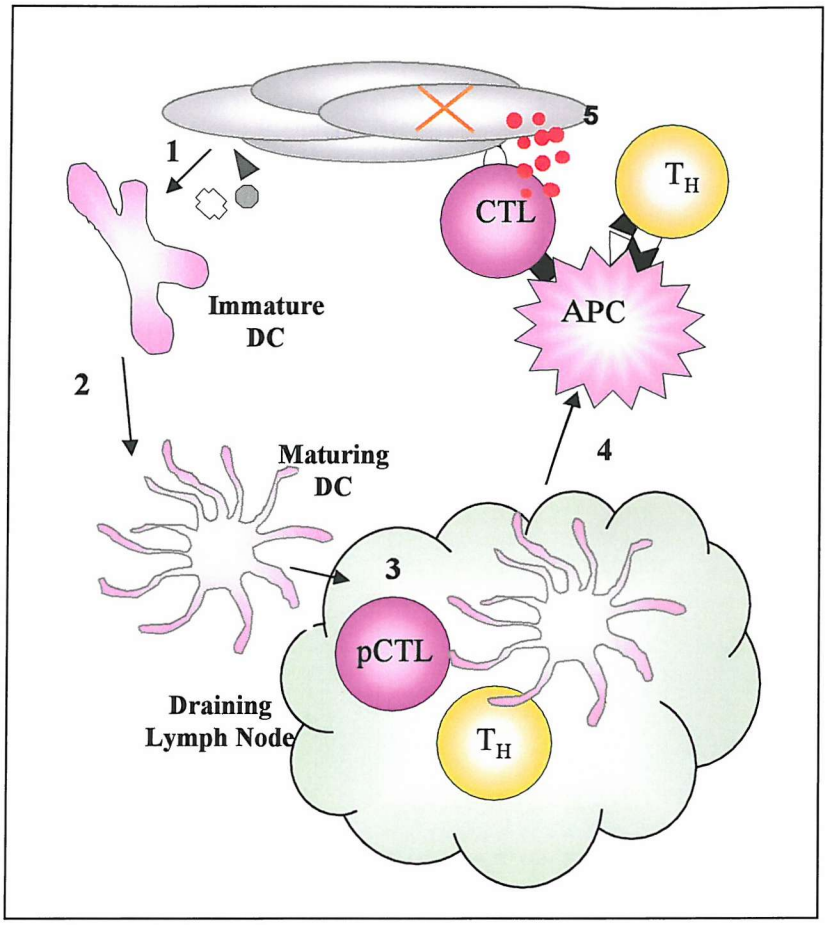


Figure 1.2 Overview of the T-cell Response
Immature tissue-resident or re-cycling DCs capture antigen from tissues and maturation is initiated by local pathogen-induced 'danger' signals, 1. Subsequently, maturing DCs migrate to and enter the local draining lymph node, 2. Here, DCs present class-I/II-restricted peptides derived from antigens captured in the tissues thereby initiating priming of CD4+ T_H cells and, subsequently, CD8+ CTL, 3. Signals derived from T_H cells upon antigen recognition (*e.g.*, CD40L) amplify DC maturation and promote T-cell priming. B-cells are also likely to be involved. Once primed, both T_H cells and CTL differentiate into effector cells and migrate to the inflammatory site (4) where local APCs are able to sustain the effector response, *e.g.*, CTL-mediated target cell lysis, 5.

Adapted from Smyth MJ, Godfrey DI, and Trapani JA, (2001) *Nature Immunology* **2** no 4 293-299

is first processed to a form recognisable by T cells in cells known as antigen presenting cells (APC). Although both B cells and macrophages are classed as APC, perhaps the most important APC is the dendritic cell (DC) (reviewed in (2)).DC progenitors in the bone marrow give rise to circulating DC precursors which track to tissues where they reside as immature cells with a high phagocytic capacity. If tissues are damaged these DCs capture antigens and migrate to the lymphoid organs maturing along the way. At this time DCs process the antigens and subsequently, present peptides derived from these antigens on MHC class I and II molecules (discussed in section 1.4.1). At the lymphoid organs DCs select CD4+ T cells which can recognise the processed antigen within the binding groove of the MHC class II molecule on the surface of the DC. This can lead to the initiation of an immune response by the CD4+ T_H cell specific for the antigen The CD4+ T_H response may be categorised as either T_H1 or T_H2, with the subsets being distinguishable by the different cytokines they produce; T_H1 development protects against intracellular pathogens and is characterised by the activation of macrophages and B-cells that produce complement-fixing or viral-neutralising immunoglobulin (Ig)G2a or of cytotoxic T-lymphocytes (CTL) of particular interest in the eradication of tumours and thus to this thesis; T_H2 responses, on the other hand, provide help for IgG1, IgE and IgA production and thereby aid protection against extracellular pathogens.

A central component of T cell immunity is the distinction between self and non-self, normal cells and cancer cells.

1.3 Thymic selection of T cells and T cell Tolerance

On antigen encounter, the adaptive immune system can either generate an immune response or become tolerant (enter a state of unresponsiveness). Both T cells and the humoral branch of the immune system (B cells) can exhibit tolerance. When tumour cells originate they must differ from autologous tissue, yet they are not detected by the immune system In some way then, the immune system is tolerant to the tumour cells in a way that it is tolerant to autologous tissue. The increased understanding of both central and peripheral tolerance by T cells may enable tolerance to be broken in therapeutic settings. In the next section how central tolerance is achieved in a normal setting will be considered.

Thymic development (an overview of which is shown in Figure 1.3) results in selection of T cells expressing a single TCR that interacts weakly with self-MHC complexes. Thus TCRs expressed by mature T cells are autoreactive but are of low enough affinity such that autoimmunity, that is the attack of self tissues, does not occur.

The first phase of development occurs before the expression of the CD4 and CD8 co-receptors during which T-lineage-restricted precursors give rise to αβ or γδ T-lineage cells. During this phase thymocytes display a double negative phenotype (DN; $CD4^-$ 8^-) which can be further subdivided based on their surface expression of CD25 and CD44 (DN1, CD44+CD25-; DN2, CD44+CD25+; DN3, CD44-CD25+; and DN4, CD44-CD25-) (3)

For those cells which eventually express an αβ TCR, at the DN3 stage a pre α TCR which has not been re-arranged is expressed (4). The invariant pre α TCR chain pairs with the TCR β chain which is the product of successful VDJ somatic re-arrangements of DNA at the β locus which is dependent of the expression of recombination activating gene-1 (RAG-1) and RAG-2. The pre-TCR αβ pair then becomes associated with the CD3/ζ complex and thus signalling can take place. Active signalling is required for further T cell maturation in the thymus. Successful assembly of the CD3-pre-Tα-TCRβ complex is accompanied by several rounds (6-8) of cell division and is sufficient to induce thymocyte maturation to the DN4 stage (5).

The second component chain (the variable α chain) of the αβ TCR is generated in the next phase of thymic development. DN4 thymocytes, having produced a functional β-chain, re-initiate RAG transcription and, thus produce a variable α chain able to pair with the existing β chain. This paring results in the formation of a diverse antigen-binding site. At this stage the expression of the co-receptors CD8 and CD4 begins to occur, forming a large population of double-positive (DP; CD4+CD8+) αβ-TCR-expressing immature cells.

The final stage involves TCR selection and concomitant entry to either the $CD4^+$ or $CD8^+$ T cell lineage(6).

The continuing development and maturation of the DP thymocytes and subsequent release into the periphery is dependent upon their selection. In fact, only 5% of this thymocyte population will be allowed to migrate. Four distinguishable processes characterise this selection, these are death by neglect, negative selection, positive selection and lineage-specific development (7).

Selection ensures that only those T cells expressing a productive TCR rearrangement, *i.e.* self-MHC restricted, are allowed to mature. Thus those cells which lack TCR ligation by self-MHC molecules during their brief life span of up to 4 days die via a death by neglect mechanism. Those, which signal via their TCR due to MHC ligation, are either negatively selected (induced to die via apoptosis) or positively selected (induced to proliferate) (8, 9). Which of these ensues, is discussed below.

1.3.1 Negative and positive selection

Engagement of MHC via the TCR leads to the cessation of both RAG transcription and α chain rearrangement and results in either positive or negative selection of the thymocyte. Negative selection, resulting in thymocyte deletion, occurs when the TCR engages MHC with high affinity. The most efficient mediators of negative thymic selection are known to be dendritic cells and macrophages. Negative selection eliminates those thymocytes which could cause autoimmune pathology if allowed to enter the periphery.

Conversely, positive selection occurs in response to low affinity binding by the thymocyte TCR leading to thymocyte survival and, subsequently, CD4/8 lineage commitment.

1.3.2 TCR co-receptor commitment

The commitment of the selected DP thymocytes to either the CD4 of CD8 lineage represents the last stage in thymic selection. TCR signalling (detailed in section 1.4.2) occurs when the TCR recognises its ligand leading to the activation of the Src family kinase lck which is both associated with the TCR CD3ζ complex and with the cytoplasmic tails of CD4 and CD8. This leads to downstream activation events, Ca++ influx into the cell and subsequent activation of transcription factors which direct the gene expression necessary for the function of mature cells. The lineage decision is controlled by the co-receptor-influenced duration of TCR-dependent signalling that is

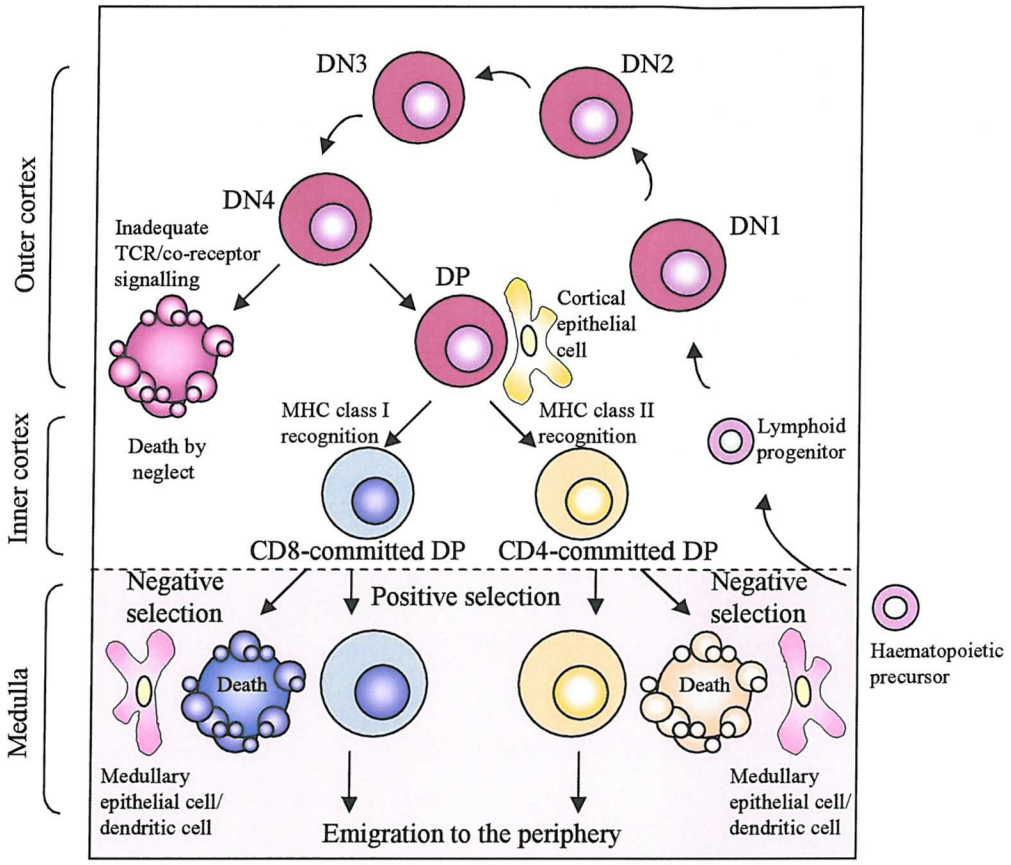


Figure 1.3 Thymic selection, an overview

Lymphoid progenitors arise in the bone marrow and migrate to the thymus where T cell development and tolerance to self MHC occurs. Early T cells lack expression of the TCR and either CD8 or CD4 and are called double negative (DN) thymocytes. As cells progress through the DN2 to DN4 stages, the pre-TCR is expressed which is comprised of the non-rearranging pre-Tα and a rearranged TCRβ chain. Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) stage and replacement of the pre-TCRα-chain with a newly rearranged TCRα-chain which yields a complete TCR. The αβ-TCR CD4+CD8+(DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self peptides. The fate of the DP thymocytes depends on signalling that is mediated by the interaction of the TCR with these self-peptide –MHC ligands. Too little signalling results in delayed apoptosis (death by neglect). Too much signalling can lead acute apoptosis (negative selection) ; this is most common in the medulla on encounter with strongly activating self-ligands on hematopoietic cells particularly dendritic cells (DCs). The appropriate intermediate level of TCR signalling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide-MHC-class-I complexes become CD8+ T cells whereas those which express TCRs that bind self-peptide-MHC-class-II ligands become CD4+ T cells. These single positive (SP) T cells are ready for export from the medulla to peripheral lymphoid sites.

Adapted from Ronald N Germain, T-cell development and the CD4-CD8 lineage decision (2002) *Nature Reviews* **2**:309-322.

postulated to lead to quantitative differences in p56lck activity. One possibility is that strong p56lck activation results in a CD4 lineage commitment, whilst weak activation leads to CD8 lineage commitment (10, 11).

Although intra-thymic selection is crucial, in order for the adaptive immune system to avoid all possible autoimmune reactions it is also necessary that T cells are tolerant of self proteins which are expressed outside the thymus after the initial T cell repertoire is formed. Thus there is also a role for peripheral tolerance (detailed in section 1.4.6). To understand the mechanisms of peripheral tolerance the generation of a T cell response must first be discussed.

1.4 The T cell response

Recognition of a foreign antigen by a T cell requires peptides derived from the antigen to be displayed within the cleft of an MHC molecule on the outer plasma membrane of a cell. This requires the protein antigen to be degraded within the cell into peptides in a sequence of events, known as antigen processing. The degraded peptides then associate with MHC molecules within the cell and complexes are transported to the membrane where they are displayed, a process known as antigen presentation.

MHC class I and II molecules are associated with peptides which have been processed in different intracellular compartments. MHC class-I molecules bind peptides processed largely from endogenous antigens for example normal cellular proteins, tumour proteins or bacterial or viral proteins produced within infected cells. MHC class II molecules in contrast, bind peptides resulting from the processing of exogenous antigens which have been internalised.

CD4+ T cells recognise peptide in the context of MHC class II and function as helper (T_H) cells, CD8+ T cells recognise peptide in the context of MHC class I and function as cytotoxic T lymphocytes (CTL).

Since all cells expressing class I or class II MHC molecules can present peptides to T cells they could all be designated as APC. However convention has it that cells which display peptide in the context of class-I molecules are target cell and cells which display peptide in the context of MHC class II are called APC and these cells include B cells, macrophages and DC. Importantly, DC and macrophages are also able to cross-present antigen derived from exogenous sources onto MHC class-I molecules

(12-14) This allows MHC class-I restricted responses to antigens derived from exogenous sources such as apoptotic cell corpses.

1.4.1 Antigen capture, processing and presentation by APC

Since, as previously outlined, DCs can induce a primary T cell response, antigen capture, processing and presentation by DC will be summarised below.

DCs can acquire antigen by phagocytosis, macropinocytosis and receptor-mediated endocytosis. In the immature state DCs express a wide variety of endocytic receptors (15-17) these include the FcγR (I-III), CD36 and the integrin αvβ5 . DCs can also capture antigen by carbohydrate-binding C-type lectins.

1.4.1.1 Presentation on MHC class II

Usually, as is the case in B cells and macrophages, newly synthesised class II molecules associate with an invariant chain (Ii) in the endoplasmic reticulum (ER). Once translocation has occurred via the golgi apparatus class-II/Ii are located in endocytic compartments where, in the acidic environment, Ii can be degraded by proteases leaving only the class-II-associated Ii peptide (CLIP) within the peptide binding groove of the MHC molecule. Subsequently the CLIP is exchanged for peptides derived from exogenous proteins and the class-II-peptide complex is translocated to the cell surface (18) Alternatively, peptides can be exchanged between internalised class-II-peptide complexes in this compartment.

The presentation of exogenous antigen by DCs differs from the presentation by other APC; it is regulated such that surface peptide MHC class II complexes are only efficiently presented after DC maturation (19).

1.4.1.2 Presentation on MHC class I

Presentation of endogenous peptides occurs when proteins are degraded and transported into the ER of the cell in a TAP dependent manner. Within the ER is a loading complex containing tapasin, calnexin and calreticulin which allows the binding of processed peptides onto newly synthesised MHC class I molecules (20). From there the peptide-class I complex is transported to the cell surface via the golgi apparatus.

In contrast to the class-I-restricted presentation of peptides derived from endogenous proteins, a CTL response can be primed after cross-presentation of exogenous-derived peptides. Cross presentation is predominantly and efficiently carried out by DC but evidence of cross-presentation by other APC such as macrophages has been demonstrated (21,22).

1.4.2 Antigen recognition by TCR and TCR signalling

As previously outlined the TCR recognises antigen which has been processed and placed in the context of MHC class I or class II molecules on APC. The pairing of the immunoglobulin-like folds within the TCRα and β chains during thymic processing results in the formation of an antigen-binding site such that three hypervariable loops, known as complementarity determining regions (CDRs), from each chain are bought into close proximity. CDR3 regions from both chains provide the greatest diversity within the TCR antigen-binding site.

The TCR initiates signal transduction by activating many cytoplasmic protein tyrosine kinases (PTKs) and these transduction events are critical for the activation of T cell functions during an immune response (23). The TCR can be divided into two distinct functional parts, the ligand binding subunit and the signal transducing subunit, which consists of the CD3 complex (δ, γ, ζ, and two ε chains) The signal transducing unit contains conserved immunoreceptor tyrosine-based activation sequence motifs (ITAMs) within its cytoplasmic domain and following TCR engagement these ITAMs become phosphorylated by Lck (a Src family PTK), creating binding sites for SH2 domain containing proteins such as ZAP70, which is a critical step for further downstream events (reviewed in (24)). The activation of Lck itself is via the CD45 mediated de-phosphorylation of a C-terminal tyrosine residue and is initiated upon the redistribution of the receptor complex within the lipid bilayer which is thought to be achieved by the compartmentalisation of the fluid membrane into distinct domains known as lipid rafts. A number of the proteins intrinsic to the TCR signalling pathway including the key signallers Lck and LAT are found in lipid rafts in unstimulated cells (25,26) Indeed, recent work has shown that targetting of Lck to lipid raft domains seems crucial for its function (27,28).

The stability of rafts may explain how TCR engagement can be sustained for long periods of time and may also act to amplify the signal.

1.4.3 DC maturation

For effective priming of a T cell the APC must be mature. As previously outline in section 1.2.2.1 immature DCs reside in peripheral tissue until there is tissue damage or cell death at which point they mature and increase their rate of migration to lymphoid organs. Upon tissue damage, DCs respond to both pathogen–derived molecular patterns (PAMPs) and indirect indicators of infection such as heat shock proteins (HSPs) and type I interferons, resulting in DC maturation (these signals are known as "danger" signals - discussed in section 1.4.3.1). Maturing DCs increase the expression of MHC class II and co-stimulatory molecules (detailed in section 1.4.4.2), cytokines such as IL-10/12 and 18, stimulatory molecules such as CD40 (detailed in section 1.4.4.1) and chemokine receptors, whilst decreasing antigen uptake. Maturing DCs migrate to secondary lymphoid organs where they encounter T cells. Their morphology also changes, with the elongation of so-called "dendritic processes" which increase to allow the efficient interaction with T cells.

1.4.3.1 Danger

The idea that thymic selection is the sole method of defining self from non-self, is now thought of as rather simplistic. The more contemporary "danger model" suggests that it is not the "foreignness" of an antigen, which triggers an immune response, but whether the said antigen is "dangerous" and associated with a damaging response (29, 30). Thus "danger" is a mechanism by which immunogenicity can be put in to context; *i.e.* it addresses whether a T cell response really needed. The previous section outlined that "danger" signals are required to mature DC and that danger can be detected in a number of ways. DC maturation can occur due to ligation of pattern-recognition receptor (PRRs). To date these include Toll-like receptors (TLRs), cytokine receptors and Fc receptors and sensors of cell death or stress. TLRs are highly conserved receptors able to recognise and mediate immunological responses

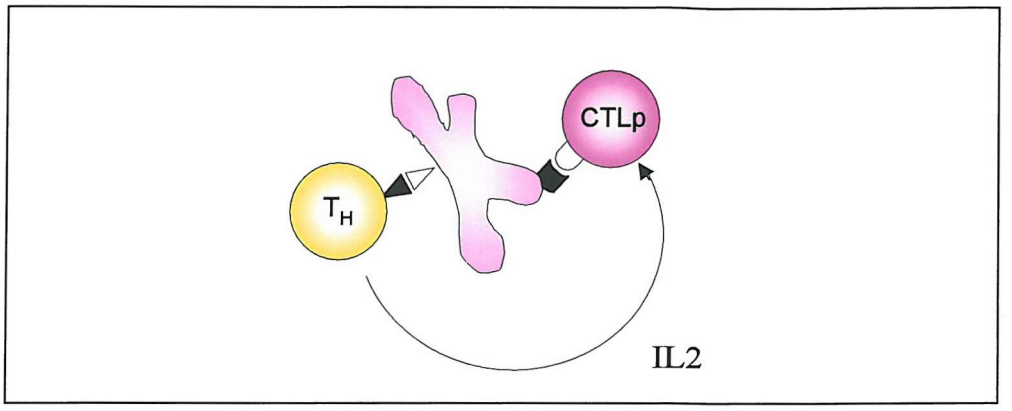


Figure 1.4a T_H mediated induction of primary CTL responses was initially proposed to occur via the delivery of cytokines to CTLp following MHC class II epitope binding.

Adapted from Polly Matzinger (1994) Tolerance Danger and the Extended Family. *Annu. Rev. Immunol.* **12**:991-1045

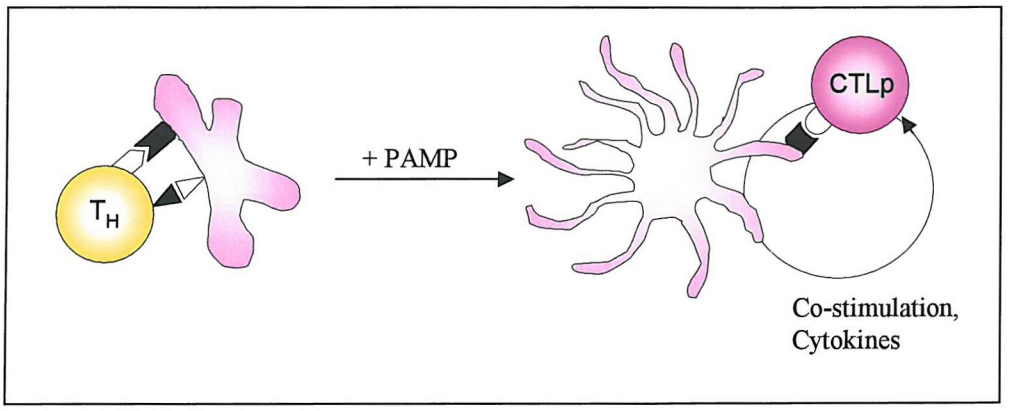
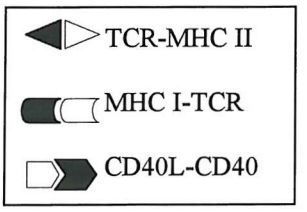


Figure 1.4b The sequential model, it is now appreciated that help from TH cells is affected by the licensing of DCs via T_H derived molecules such as CD40L. T_H derived signals amplify the PAMP initiated DC maturation

Adapted from Ridge, J.P., Di Rosa, F. & Matzinger, P. (1998) A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature*, **393**: 474-477



against distinct pathogen-derived products. It is known that TLR2 mediates immunological response against yeast and gram-positive bacteria, TLR4 against LPS and gram-negative bacteria. TLR9 recognises CpG motifs contained within viral oligonucleotides (31-36). Additional complexities exist in this system which reflect the requirement for additional checkpoints to prevent autoimmune pathology within the host. These include the requirement for co-receptors involved in TLR-ligand interactions, and redundancy between TLR family members (37).

HSPs and other soluble factors such as , IFNα, TNFα, IL-1, and mature IL-18 are also able to activate DCs (38,39).

1.4.4 T cell priming

The DC having received a "danger signal" and having matured to efficiently present antigen migrates to T cell areas of the lymphatics where contact with T cells occurs.

The generation of a T cell immune response involves the coming together of an antigen specific CD4+ T helper cell (T_H) with a cytotoxic T lymphocyte precursor (CTLp) and the APC (shown simplistically in Figure 1.2). Importantly, the CTLp and T helper cell must recognise antigen on the same APC and it is through these interactions that a naïve CD8 T cell can mature into an effector CTL (40-42).

The immune response was originally thought to occur due to a simultaneous three-cell interaction (shown in Figure 1.4a). However, this model posed a problem due to the improbability of the APC, T_H and CTLp being in the same place at the same time. More recently a sequential two-cell interaction model (40) (Figure 1.4b) has been proposed by Matzinger *et al*, whereby the APC is first activated by the T_H cell and then at a latter time is able to activate the CTLp after the T_H cell has left. As such, the DC serves a temporal and spatial bridge in T cell priming.

The experiments reported by Ridge *et al* and others also strongly suggests a role for CD40-CD40ligand (CD40L) interactions in licensing CTL, discussed in detail in section 1.4.4.1.

In simple terms, T cell activation by the APC occurs through a two-step process. Activation is initiated by the interaction of the TCR-CD3 complex with the processed peptide bound to MHC molecules resulting in so called signal 1. The recognition of

the MHC bound peptide causes the upregulation of CD40L (CD154) on the surface of the CD4+ T_H cell. This crosslinks the CD40 found on the surface of the APC and the resulting signal transduction leads to the transcription of genes for co-stimulatory molecules such as B7.1 and B7.2 (detailed in section 1.4.4.2) as well as adhesion molecules such as ICAM-1. These co-stimulatory molecules then present signal 2 to the T cell through interaction between B7 and CD28 on the T cell. The up-regulation of co-stimulatory molecules which provide signal 2, therefore can be said to prime or license the APC, thus the APC can present to the CTLp. Additionally, a signal induced by the action of IL-2 on its high affinity receptor (CD25) on the CTLp, is thought to lead to proliferation and differentiation into an effector CTL (43). The emerging role of soluble factors as a postulated signal 3 for CTL activation is discussed in section 1.4.4.3.

1.4.4.1 CD40-CD40L interactions

CD40 is expressed constitutively on APC such as DC and is a type I transmembrane glycoprotein which is a member of the TNF- receptor (TNF-R) superfamily The TNF-R superfamily also includes molecules such as Fas, CD27, CD30, OX-40 and 41BB (44, 45). Its ligand, CD40L (CD154), is a type II glycoprotein which forms a homotrimer. CD40L is expressed on CD4+ T_H cells upon activation. It was at first thought that CD40L expression was restricted to activated CD4+ T cells however it is now known to be expressed on many other cell types and its expression has many functional consequences (46-48). An important role of the interaction between CD40 and CD40L is to promote the proliferation, differentiation and immunoglobulin class switching of B cells (49) Importantly the interaction can be mimicked by anti-CD40 mAb. In addition, CD40 ligation increases the cytokine production e.g. IL-6, IL-10 and TNFα and expression of co-stimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) and of ICAM-1 on APC as previously mentioned. Indeed CD40L-/- CD4+ T_H cells are unable to up-regulate B7-1 on B cells (50). The expression profile of both CD40L and CD40 is also wider than first thought and therefore CD40/CD40L interactions are thought to play a central role in the immune system (45). The importance of CD40L is also supported by observations in several murine experimental models including the LCMV model where, without a functional CD40L, mice are unable to mount a normal CTL response to some LCMV epitopes and where

the secondary response to LCMV is greatly reduced (51) Indeed, exposure of CTLp to antigen without CD40 stimualtion via CD4+ T_H cells or, importantly, anti-CD40 mAb in some cases does not lead to activation (52, 53). Furthermore, many studies have demonstrated that the licensing effect of activated T_H cells may be attributed to the up-regulation of CD40L on these cells following epitope recognition, followed by the subsequent binding of CD40 on professional APCs and the licensing of these cells to prime CTLp. Thus in the afore mentioned sequential, two-cell interaction model (Figure 1.4) it is now appreciated that T-help is provided by the licensing of DCs via T_H-derived molecules, such as CD40L and the ligation of CD40 acts to amplify the the PAMP-initiated DC maturation.

All of these data together reveals the importance of CD40-CD40L interactions at the pivotal point of DC activation leading to an effective T cell response to antigen. However, it is important to note that the CD40-CD40L interaction is not the only interaction which can lead to the licensing of DCs. It seems likely, therefore, that members of the TNF receptor superfamily other than CD40 may account for at least some of the CTL responses that occur independently of CD40 and, furthermore, that TNF/TNFR member pairs act at multiple levels during the priming and effector phases of a T-cell response, for example 41BB/41BBL (54, 55).

Other members of the TNF family, such as LIGHT and TRANCE, are expressed on activated T_H cells and may contribute to DC-mediated CTLp priming by either increasing DC survival (56), secretion of pro-inflammatory cytokines or up-regulating MHC, adhesion and co-stimulatory molecules(57-59).

1.4.4.2 Signal 2

As previously mentioned, a T cell can only react to the presence of pathogen when two molecular events occur simultaneously (signal 3 will be discussed in section 1.4.4.3). The molecular events are termed signal 1 and signal 2. Signal 1 is the detection of the processed pathogen in the context of MHC by the TCR, leading to a signalling cascade (section 1.4.2). Signal 2 occurs when a co-stimulatory molecule on the T cell is ligated. Maturation of DCs as previously discussed leads to the expression of co-stimulatory molecules on the DC. The two signal system is therefore important, acting as a check point for danger signals. That is to say the co-ordinated

triggering of the two separate receptors is to ensure that the ligation of the TCR is due to a real immunological threat to the host. Co-stimulation leads to the production of IL-2, an autocrine growth factor able to expand the population of effector T cells. The effect of TCR triggering without co-stimulation usually leads to clonal anergy (detailed in section 1.4.6).

The best studied co-stimulatory molecule isolated to date is CD28, a member of the Ig superfamily, expressed as a disulphide-linked homodimer present on CD4+ T cells and many CD8+ T cells. CD28 is ligated by both B7-1 and B7-2 which are also Ig family members expressed as monomers on APC upon maturation and up-regulated by the ligation of CD40 on these cells. CD28 ligation by either B7-1 or B7-2 is required for and is sufficient for successful T cell priming and avoidance of T cell anergy (60) In addition B7-1 and B7-2 can bind a CD28 homologue called cytotoxic T lymphocyte antigen 4 (CTLA-4) with an avidity more than 40-50 times that of CD28. Unlike CD28, CTLA-4 triggering inhibits T cell activation and therefore dampens the T cell response. Importantly, it is only expressed on T cells after a period of activation, whilst CD28 is constituitively expressed (61). The function of B7 is therefore more than one of co-stimulation, since B7-CTLA-4 interactions provide a critical down-reguatory signal for T cells (62, 63). Many other members of the CD28 family have recently been identified including the inducible co-stimulator (ICOS). Triggering through ICOS and TCR leads to the proliferation of T cells, and the production of IL-4, IL-5, IFNγ and TNFα at comparable levels to co-stimulation via CD28. However the levels of IL-2 produced via ligation of ICOS are lower. Signalling via ICOS also enhances the expression of CD40L on T cells. The B7 family of co-stimulatory ligands, their receptors on T cells and functions are shown in Table 1.1.

As well as the molecular interactions shown in Table 1.1, T-cell priming and differentiation is reliant on other co-stimulatory pathways. Indeed, even though a number of T cell responses are compromised in CD28-deficient mice including IL-2 production, many other T cell responses are un affected such as those mediated via 4-1BB a member of the TNF-R superfamily expressed on activated T cells (64)(reviewed in (65) and (66)) Seminal investigations into the 4-1BB /4-1BBL interaction focused on their role in co-stimulation using either transfected ligand or the soluble form to block receptor/ligand interactions. These studies have

demonstrated an important role for 41BB/4-1BBL in T cell activation (67) including the production of IL-2 and IL-4 aswell as T cell proliferation and survival (68) both for CD8+T cells and more recently for CD4+ T cells (69). Importantly, 4-1BBL can co-stimulate resting T cells in both CD28 positive and CD28 negative mice operating in a CD28 independent manner at optimal Ag densities (70-72).

In addition to its co-receptor functions, 4-1BB has also been shown to be able to signal in both directions back into the cell which expresses the ligand, a function which has also been described for other members of the TNF family members (73) Other TNF-R family members have also been shown to have a role in co-stimulation. For example, signals via CD30 have been shown to enhance sub-optimal proliferative T cell responses to immobilised anti-CD3 (reviewed in (74)) signalling through human CD27 was shown to enhance suboptimal proliferation of both CD4+ and CD8+ T cells induced by PHA and other TNF-R family members upregulate the pro-survival protein Bcl-xL in murine T cells (personal communication T.F.Rowley) and several groups have shown that OX40L expressed on APCs can provide co-stimulation to CD4+ T cells (75) and break peripheral T cell tolerance (76).

Table 1.1 The B7 Family of Co-stimulatory Ligands (adapted from (77))

B7 Family Member	Expression	Receptor(s)	Function
B7-1 and B7-2	Predominantly lymphoid expression; induced on monocytes, DCs and B cells	CD28 CTLA-4	Priming of naive T-cells, IL-2 production Inhibition of T-cell responses
B7-RP1 (B7h, B7-H2)	Lymphoid and non-lymphoid expression (heart, kidney, testis, lung); LPS-inducible in non-lymphoid tissue; induced on monocytes and DCs	ICOS	Enhancement of cytokine production in peripheral tissue without biasing
PD-L1 (B7-H1)	Lymphoid and non-lymphoid expression (heart, lung, kidney); induced on monocytes by IFNγ	PD-1	Inhibits T-cell proliferation and cytokine production
PD-L2 (B7-DC)	Lymphoid and non-lymphoid expression (heart, lung, kidney, pancreas, liver); predominantly DC expression	PD-1	Inhibits T-cell proliferation and cytokine production and/or enhances IFNγ and IL-2 production
B7-H3	Lymphoid and non-lymphoid expression (heart, kidney, testis, lung, liver); induced on monocytes and DCs	?	IFNγ, TNFα and IL-8 production

Thus co-stimulation acts to provide a checkpoint in T cell priming in response to antigen and causes the T cell response to occur in situations which may be dangerous to the host. However, the requirement for co-stimulation will be different depending on whether the cell has encountered an antigen previously. Co-stimulation can also modulate the T cell differentiation into T_H1 vs T_H2 cells (78).

1.4.4.3 Signal 3

The recent identification of the requirement of the so called "signal 3" has major implications for tumour immunology. Recently, Curtsinger *et al* have reported that the activation of naïve CD8+ T cells to undergo clonal expansion and to develop effector function depends on not two, but three signals, (1) Ag, (2) co-stimulation and (3) IL-12 or adjuvant. In their murine model system, when physiological Ag levels and costimulation was used to stimulate naïve CD8+ T cells, IL-12 was required in addition to IL-2 for proliferation and cytotoxic function to occur. At high Ag density IL-2 alone could induce a proliferative response but IL-12 was still required for development of cytotoxicity in these cells. Furthermore, naïve CD8+ T cells expanded without IL-12 were functionally tolerant to re-challenge with Ag. The further investigation in to the requirement for a third signal hopes to fully elucidate the mechanisms by which tolerance to Ag occurs even when B7 expression levels on mature DC are increased (79, 80).

1.4.5 CTL Effector functions

Once priming and differentiation has occurred mature CTL can migrate to inflammatory sites. Here they are able to bind target cell MHC and cause target cell apoptosis by either of two methods both of which require direct contact between the effector and target cell (reviewed in (81). The methods are via a cytotoxic granule-exocytosis pathway involving perforin and granzyme proteases or via the Fas:FasL system (82, 83). The granule-exocytosis pathway involves the release of cytoplasmic granule toxins, perforin, a membrane-disrupting protein and granzymes, which are a family of structural related serine proteases. Together, these induce apoptosis of the target cell by activation of apoptotic cysteine proteases called caspases. (84, 85).

The second method of target cell killing is via the engagement and aggregation of target-cell death receptors such as Fas by their cognate ligands such as FasL. Following TCR engagement FasL is synthesised by CTL and is subsequently able to ligate Fas on the target cells thereby resulting in classical caspase-dependent target cell apoptosis (86). The main role of the Fas-FasL pathway is to control the expansion of lymphoid cells and eliminate any cells which are self-reactive. The Fas-FasL interaction is also used particularly by $CD4^+$ CTL (detailed in Chapter 5), to cause target cell lysis.

CTL may also secrete IFNγ and TNFα in the vicinity of target cells which are able to induce apoptosis directly in some cell types, and which activate and recruit phagocytes.

1.4.6 Peripheral T cell Tolerance.

Peripheral tolerance needs to be constantly maintained to avoid immune responses directed against "self" antigens, which are only encountered in the periphery. Although intrathymic selection is crucial it is incomplete and thus all self-proteins, which are expressed after the T cell repertoire is formed, must be tolerated in the periphery. Numeruous studies using many models have shown that peripheral tolerance can occur in more than one way. These include, clonal ignorance clonal deletion and T cell non-responsiveness (87, 88). Regulatory lymphocytes may also stop the activation of T cells in response to Ag. As discussed in section 1.4.4.3 it has been recently proposed that IL-12 may be needed, in combination with signal 1 and signal 2 to fully activate naïve CD8+ T cells and thus where IL-12 is lacking upon recognition of Ag even with co-stimulation, tolerance can ensue.

The term anergy has been used to define: a state of long-lasting, partial or total unresponsiveness induced by partial activation. In the absence of IL-12 in the system described above T cells may be therefore described as anergic.

Figure 1.5 shows four alternative and established ways in which induction of T cell anergy has been observed *in vitro*, these are 1) The recognition of peptide in the context of MHC but in the absence of co-stimulation thus signal 1 is provided without signal 2. 2) Full signaling through MHC-TCR interactions and signal 2 provided via

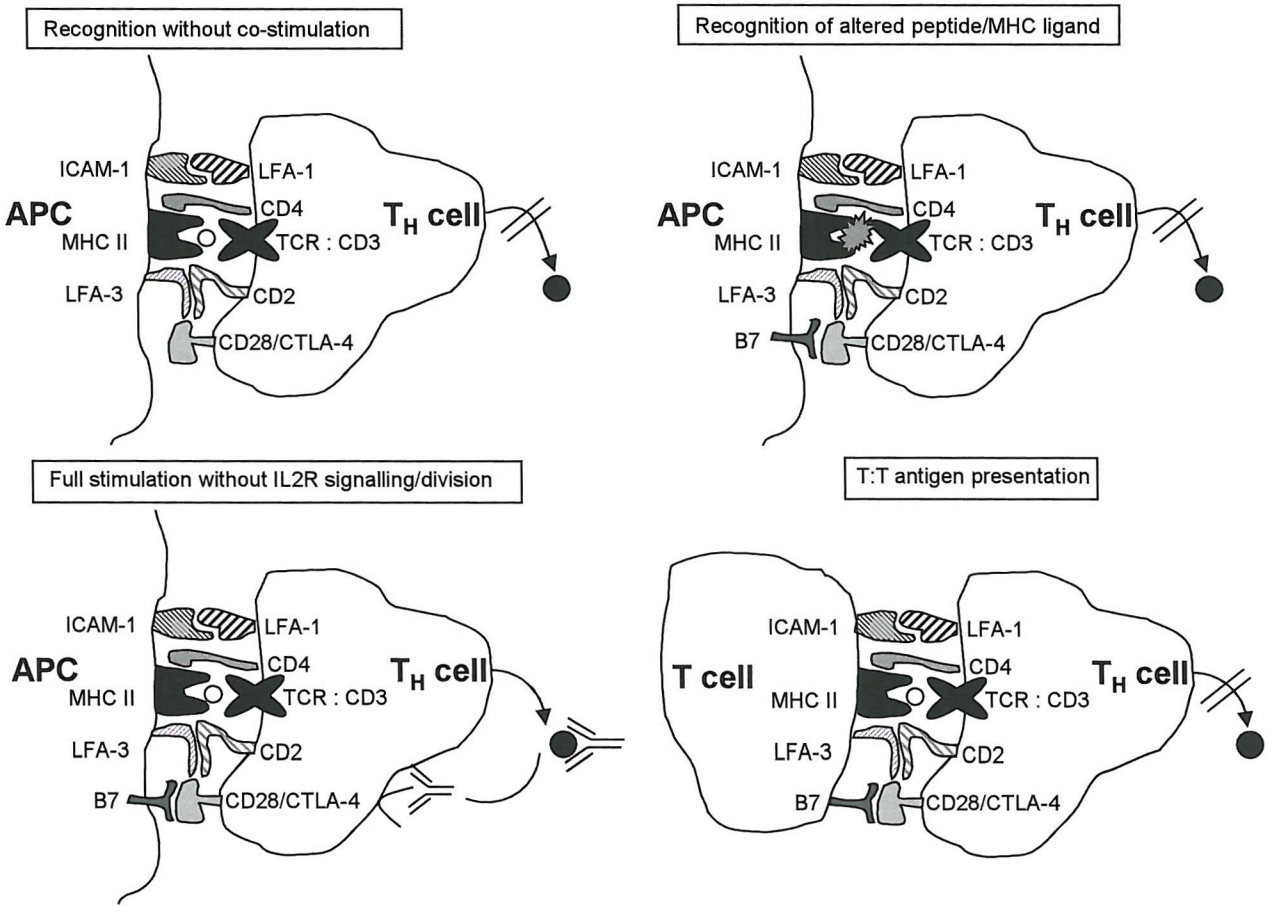


Figure 1.5 Mechanisms of T cell anergy, maintenance of peripheral tolerance.

Adapted from The contributions of T cell anergy to peripheral tolerance, Lechler R, Chai J-G, Merelli-Berg F,Lombardi G, *Immunology*,(2001) **103**: 262-269

B7-CD28 interactions but in the absence of IL-2R signaling 3) The recognition of altered peptide/MHC ligand or 4) T cell to T cell presentation. (89)

1.5 Immune Response to Tumour.

We have previously discussed a normal immune response, here we discuss evidence for an immune response to cancer. For a long time the role of the ability of the immune system to detect and destroy cancer cells was disputed,, as tumours derived from the host's own cells were originally thought to avoid immune recognition altogether. This theory was supported by the observation that many immunocompromised mouse strains failed to develop spontaneous tumours (90). However, spontaneous responses to human tumours such as melanoma and renal cell carcinoma were observed nearly three decades ago (91) and coupled with the recent increased understanding of the molecules which regulate the immune system, the identification of tumour associated antigens (TAAs) (detailed in section 1.5.3) and tumour escape mechanisms, there is new hope that not only does immunsurveillance exist but that the immune response can be augmented to create effective immunotherapies (92).

1.5.1 Immunosurveillance of Tumours

Cells are continually exposed to environmental stresses and neoplastic transformations can occur, thus cells must either repair themselves or recognise that repair has failed and enter into apoptosis. If cells are not repaired and do not undergo apoptosis they have the potential for malignant transformation. In our life-time many cells should undergo neoplastic transformation via this mechanism yet it is relatively rare that tumours develop. Evidence to support immunosurveillance of tumours includes the fact that perforin$^{-/-}$ and IFN$\gamma^{-/-}$ mice (mice with deficient IFNγ signalling or T cells which are unable to kill target tumour) (93) show an increased incidence of both spontaneous and carcinogen induced tumours (94,95) and Rag2-/- mice (deficient of T and B cells) get spontaneous tumours by 18 months of age (96). Evidence points to immunosurveillance both by the innate immune system involving NK and NKT cells (97,98) and the adaptive immune system, with much focus on DCs initiating adaptive immunity to tumours (92).

1.5.2 Evidence for a T cell response to tumour.

Perhaps the most compelling evidence that there is an immune response, in particular an adaptive T cell response, to tumours is the fact that many tumours employ mechanisms in order to escape immune recognition by these cells. Tumour cells which are not recognised as dangerous by the immune system (Figure 1.6), or which can induce tolerance, survive; tumour cells which are recognised by the immune system will be killed. If a cell transforms and is not detected by the innate immune response and has established a tolerant environment in which to grow, the maintenance of evasion of the immune system can be achieved by the tumour cells' genetic instability. These mechanisms may include down-regulation of MHC Class I molecules (definitive indication of the importance of CD8+ T cells in the immune response to tumour) (99) or the tumour antigens themselves (reviewed in (100)), a decrease in the expression of co-stimulatory molecules, secretion of immunosuppressive TGF-β1 which can inhibit DC migration and reduce the ability of the DC to mature into a potent APC, or secretion of the inhibitory cytokine IL10 (101). It is also thought that tumour cells may also kill those T cells attacking it, by expression of FasL on their surface (102). In addition to tumour evasion there may be other ways in which tumours manage to avoid an immune response. These may include safety mechanisms employed by the immune system itself to avoid auto-immunity. Recently, regulatory or suppressor T cells have been described. These include CD4+CD25+ T cells (reviewed in (103)) and NKT cells. CD25+CD4+ T cells are implicated in the suppression of the activation and proliferation of other CD4+ and CD8+ T cells through either direct contact with APCs (Figure 1.6c) or via soluble factors. Depletion of CD25+CD4+ T cells lead to the rejection of syngeneic tumours *in vivo* in mouse studies (104). Similarly NKT cells were shown to prevent tumour regression in a mouse model by inhibiting CTL-mediated anti-tumour immunity in an IL-13-dependent manner (Figure 1.6) (105).

1.5.2.1 Evidence of the CD8+ T cell response to tumour.

Tumour immunology has focused on CD8+ CTL responses for two reasons 1) the majority of tumours are MHC class I positive but MHC class II negative and 2) CTL are able to directly kill tumour cells which express relevant peptide antigens in the

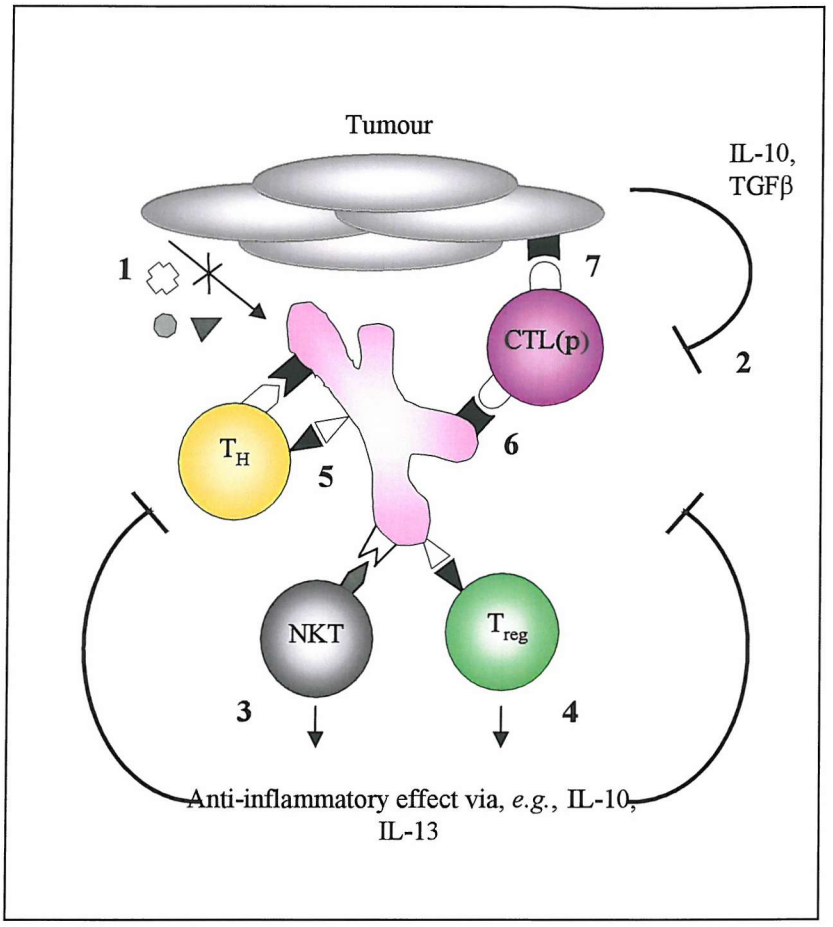


Figure 1.6 Active and Passive Mechanisms that Prevent Spontaneous Tumour Rejection.
Several barriers exist that may hinder the elicitation of an effective anti-tumour T-cell response. Insufficient 'danger' signals derived from or induced by the tumour may result in ineffectual DC maturation, 1. This effect may be supplemented by active suppression of an appropriate T-cell response by the production of, for example, IL-10 and TGFβ, 2. Immunoregulatory cells such as CD1-binding NKT cells, γδ T-cells (not shown) or Treg cells may exert anti-inflammatory effects via cytokine production or cell-cell contact (not shown), 3 and 4. Absence of TAEs, weak MHC binding by TAEs or low avidity of cognate T-cells may result in blunted T-cell priming or differentiation, 5 and 6. Finally, mechanisms that directly prevent granule-mediated or FasL-mediated apoptosis protect tumour cells from CTL-mediated lysis, 7.

Adapted from Smyth MJ, Godfrey DI, and Trapani JA, (2001) *Nature Immunology* **2** no 4 293-299

context of MHC class I. In a number of studies both CD8+T cell lines and CD8+ T cell clones specific for tumour associated antigens TAAs (1.5.3) have been stimulated *in vitro* and have been shown to mediate anti-tumour immunity when transferred back in to tumour-bearing hosts (106). Also in support of CD8+ mediated anti-tumour responses, immunisation using dendritic cells in conjunction with tumour peptides can result in MHC I restricted anti-tumour responses (107,108). Furthermore, the loss of CD8+ T cells either by using depleting antibodies or using genetic knock out, abrogates the anti-tumour immunity induced by many cancer vaccines (109,110). This evidence in support of CD8+ T cells in anti-tumour immunity has lead to the definition of many MHC class I- restricted TAAs using tumour-reactive CD8+ T cells which exhibit anti-tumour activity *in vivo* (111,112). The targeting of these TAAs has lead to some success in the clinic in terms of tumour regression and has facilitated the development of cancer vaccines (113). Known TAAs and their impact on immunotherapy are discussed in section 1.5.3.

1.5.2.2 Evidence of the CD4+ T cell response to tumour

Despite the reasons, detailed above, for preferential investigation of the role of CD8+ T cells in anti-tumour immunity, the role of CD4+ T helper cells must not be ignored. CD4+ helper T cells are known to play a central role in the regulation of most antigen-specific responses and optimal vaccination or adoptive therapy of cancer may indeed require the participation of both CD8+ and CD4+ T cells (discussed in section 1.6.2.2.4). As with CD8+ T cells, experiments using antibodies to deplete CD4+ T cells or the use of CD4- knockout mice have shown a role for CD4+ T cells in various vaccine/challenge experiments including recombinant bacterial vaccines (114), cell-based vaccines (115), recombinant viral vaccines (110), and in experiments where interleukins were transfected into tumour cells (116,117). Furthermore, early adoptive transfer experiments using specific CD4+ T cell clones demonstrated anti-tumour immunity in tumour bearing hosts (detailed in section 1.7.2) (118,119).

1.5.3 Tumour Associated Antigens (TAAs)

Tumour associated antigens (TAAa) are recognised a small tumour-associated peptides in the context of MHC class I or class II molecules by CD8+ or CD4+ cells respectively on the tumour cell surface (120). Since the identification by Boon *et. al.* of such antigens there has been a shift in tumour immunology towards the belief that immunotherapy against cancer can become an established treatment (121). Different categories of MHC I restricted tumour antigens which can induce CTL responses have been identified and isolated (122) (reviewed in (112) and (123)) TAA which can induce CTL responses *in vivo* and *in vitro* can be classified as:- (1)"Cancer testis" (CT) antigens which are expressed in melanomas and several other tumour but in no normal tissue except testis, (2) Differentiation antigens which are expressed by cells at a particular point in their differentiation but could be a potential target for an immune response on clonally expanded tumour cells, (3) Tumour specific antigens which are unique to the tumour and could be normal proteins which are point mutated or products of gene fusions, (4) Self antigens which are over expressed in tumour , (5) Viral antigens, some human tumour are associated with virus and the viral proteins expressed on tumour cells can provide specific targets for immunotherapy (124). Examples of defined TAA recognised by CD8+ T cells are shown in Table 1.2.

Reactive CD4 + T cells restricted by MHC class II are also well documented against tumours including melanomas, leukemias, colon cancers and breast cancers. (125)From this preliminary work it is evident that there is a role for CD4+ T cells in cancer vaccines and that the further identification of MHC class II-restricted tumour antigens would facilitate these vaccine approaches. Thus, in addition to MHC class I restricted TAA MHC class II restricted peptides recognised by CD4+ T cells have also been isolated (126-130). Examples of TAA which are recognised in the contest of both MHC class I and MHC class II are shown in Table 1.3.

1.5.3.1 Tumour rejection antigens

Despite the fact that there may be a specific immune response elicited by both CD8+ and CD4+ T cells to a particular TAA, this does not mean that the response will reject tumour *in vivo*. Thus, from a vaccination standpoint it is of importance to choose those antigens which generate an effective immune response which will eradicate

tumour these TAA have been named "tumour rejection antigens"(reviewed in (131), (123) and (132)).

Table 1.2 TAAs recognised by human CD8+ T cells

Category	Tumour Antigen	Cancer Expressing the Antigen
Cancer Testis Antigen	MAGE-1	Melanoma, Breast, Head and Neck,
	MAGE-2	Bladder, Gastric and Lung cancer
	MAGE-3	
	MAGE-12	
	BAGE	
	GAGE	
	NY-ESO-1	
Differentiation Antigens	Tyrosinase	Melanoma
	TRP-1	
	TRP-2	
	gp100	
	Mart-1	
	MC1R	
Tumour Specific Antigens	Immunoglobulin Idiotype (Id)	B-cell NHL, MM
	CDK4	Melanoma
	Caspase-8	Head and Neck cancer
	β-Catenin	Melanoma
	CIA 0205	Bladder cancer
	BCR/ABL	CML. (133)
	Mutated p21/ras	Pancreatic, Colon, Lung cancer
	Mutated p53	Colorectal, Lung, Bladder, Head and Neck cancer
Over-expressed self Antigens	Proteinase 3	CML
	WT 1	CML, ALL, AML
	MUC-1	Breast Adenocarcinoma
	CEA	Colon, Breast, and other cancer
	Normal p53	Breast, Colon and other cancer
	Her2/neu	Breast, Ovary and Lung
	PAP	Prostate cancer
	PSA	Prostate cancer
	PSMA	Prostate cancer
	α-Fetoprotein	Liver cancer
	G250	Renal cell carcinoma
Viral Antigens	HPV E6/E7	Cervical and Penile cancer
	EBV LMP2a	Hodgkins disease
	HCV	Liver cancer
	HHV-8	Kaposi sarcoma

NHL, non-Hodgkin's lymphoma; MM, multiple myeloma; CML, chronic myeloid leukaemia; ALL, acute myeloid leukaemia; CEA, carcinoembryonic antigen; HPV, human papilloma virus; EBV, Epstein-Barr virus; HCV, hepatitis C virus; HHV, human herpes virus

Taken from: Cancer vaccines and immunotherapy *British Medical Bulletin* **2002**;62 149-162(134)

Table 1.3 TAAs recognised by CD4+ and CD8+ T cells

Antigens	MHC Class I restrictions	MHC Class II restrictions
Differentiation antigens		
Tyrosinase	A1, A2, A24, B44	DR4
MART-1/Melan-A	HLA-A2, B45	DR4
gp100	A2, A3	DR4
Tumour-specific shared antigens		
MAGE-3	A1, A2, B44	DR11, DR13, DP4
NY-ESO-1/CAG3	A2, A31, Cw	DR4, DP4

Adapted from Wang R-F *et al* T cell-mediated immune responses in melanoma : implications for immunotherapy; Critical Reviews in Oncology/ Hematology 43 (2002) 1-11(135).

1.6 Cancer immunotherapy

Cancer immunotherapy can be defined as either active or passive. Active immunotherapies involve the generation or augmentation of the patient's own anti-tumour response, passive (or adoptive) immunotherapies involve the transfer of immune cells or antibodies with anti-tumour reactivity into patients. Furthermore immunotherapies can be sub-classified into specific and non-specific regimes (136). These general characterisations are illustrated in Table 1.4.

An obvious advantage of evoking or augmenting the bodies' own immune response to tumour is that, theoretically, the immune response would be targeted specifically to the cancerous cell. Therefore damage to normal cells would be reduced or eradicated, decreasing the long-term side effects of therapy.

Table 1.4) General types of cancer immunotherapy

	ACTIVE	PASSIVE
Specific	- Tumour vaccines (immunisation against known tumour antigens)	- Monoclonal antibodies ("naked", conjugated) - Radioimmunotherapy - Engineered antibody derivatives e.g. bispecific antibodies, single chain Fv fragments - "T bodies" - Antigen specific killer cells expanded *ex vivo* (adoptive transfer)
Non-specific	- Bacillus Calmette-Guérin (BCG) - Corynebacterium parvum - IFNγ - IL-2 - IL-12 - Other cytokines	- Lymphokine activated killer cells (LAK) - Tumour infiltrating lymphocytes (TIL)

Table 1.4 Taken from Davis, I D, *Immunology and Cell Biology* (2000) **78**, 179-195 (136)

1.6.1 Active Immunotherapy

Active immunotherapy includes the use of cytokines or vaccines to augment the host immune response in both specific and non-specific strategies. The first indications that active immunotherapy strategies could cause regression of established invasive human tumours came from studies in metastatic kidney cancer and melanoma and involved the administration of IL-2 (137) (reviewed in (138)). The IL-2 had no effect directly on tumour growth but anti-tumour responses were induced through the expansion of the host's immune cells following the activation by specific antigen (139,140).

Vaccinations strategies aim to combine selected tumour antigens and appropriate routes of administration into the host and the use of TAA is hoped to optimise vaccinations in humans. Vaccines (reviewed in (134) and (141)) can be tumour cell based (Figure 1.7a) where non viable whole tumour cells are injected in to the host

(142). The fact that the tumour itself is not able to induce an adequate immune response has lead to attempts to overcome this by introduction of cytokine genes or co-stimulatory molecules in to the tumour *e.g.* granulocyte macrophage colony-stimulating factor (GM-CSF) known to contribute to the maturation of DCs (143). Peptide based vaccines rely on the *in vivo* loading of empty MHC molecules on APC. Peptide vaccinations at present in clinical trials include the use of gp100 and MAGE-3 for the treatment of melanoma (144,145). TAA vaccines can be delivered by recombinant viral vectors. These have the advantage of delivering a signal for danger (perhaps from viral destruction of the host cell or CpG motifs) as well as a TAA. The same has been argued for DNA-based vaccines where DNA encoding for TAA is injected directly intramuscularly. Dendritic cell vaccines (Figure 1.7b) involve the loading of TAA on to DC and many strategies have been employed to accomplish this including loading with peptides (146), whole proteins (147), tumour cell lysates (148) and tumour-derived RNA (149). In addition, DCs have been transfected with viral vectors expressing tumour antigen or fused with tumour cells (150, 151). DC based vaccines have been employed in human trials against lymphoma, prostate cancer and melanoma.

1.6.2 Passive immunotherapy

Passive immunotherapy includes the use of antibodies (Ab) or Ab derivatives such as radiolabelled Ab or drug conjugated Ab, or effector cells.

1.6.2.1 Antibody Therapy

Monoclonal antibody (mAb) therapy involves targeting of malignant cells with specific mAb. The application of this therapy has increased in recent years due to the refinement of production techniques including engineering mouse-human chimerised antibodies with human Fc regions and production of fully humanised mAb (152). This is important as previously the failure of therapy by mAb treatment was due, at least in part, to the production by the host of human anti-mouse antibodies (HAMA) against the injected mAb (153). A selection of mAb currently in clinical development for cancer are shown in Table 1.5.

Monoclonal Ab work through a variety of mechanisms. It was thought that the major mechanisms operated through the recruitment of effectors, as mAb can bind and

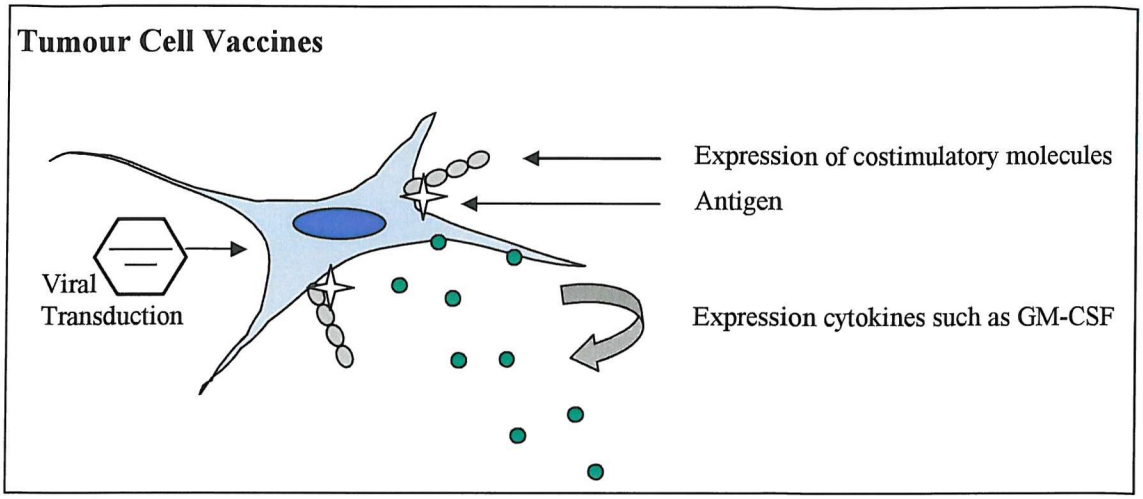


Figure 1.7a Tumour cell vaccines. Immunogenicity of tumour cell vaccines can be improved by transducing the tumour cell with genes that encode key components of the immune response for example cytokines such as GM-CSF and costimulatory molecules

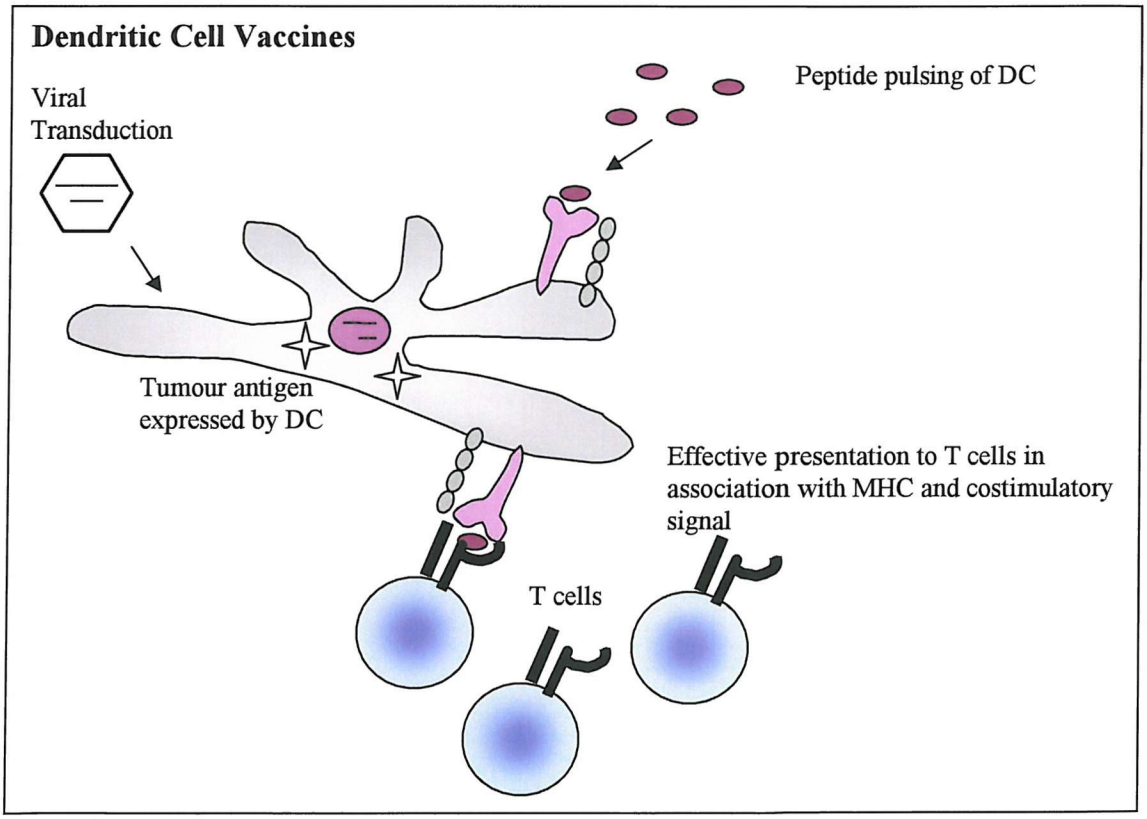


Figure 1.7b Dendritic cell vaccines. DCs generated *ex vivo* from patients peripheral blood monocytes or CD34 haemopoietic stem cells which can be loaded with tumour antigens in a number of ways such as pulsing DCs with antigenic peptide or infecting the cells with recombinant viral vectors.

Adapted from: Armstrong et al, Cellular immunotherapy for cancer *BMJ* 2001; **323**: 1289-1293

activate complement and cytotoxic cellular effectors via their Fc region. There is evidence that this the case from Fc blocking studies and in the treatment of the murine BCL_1 model of lymphoma with anti-CD40 mAb (154). Treatment of BCL_1 by anti-CD40 is only achieved when an intact IgG mAb is used and not when the Fc region is removed. In addition, the mAb Campath is more effective in therapy as an IgG2b molecule compared to IgG2a as each different isotype displays a different affinity for Fc receptors (155).

Another mechanism by which mAb could effect cancer killing is by the induction of apoptosis in the tumour cell. This is the suggested mechanism of action of both anti-idiotype mAb and rituximab an anti-CD20 mAb (reviewed in (156)). Studies in syngeneic mouse lymphomas have shown that signalling anti-Id antibodies have greater efficacy than non- signalling ones.

Monoclonal Ab can also work by blocking growth factor signals for example trastuzumab which blocks human epidermal growth factor – 2 (HER-2) (157).

Importantly, mAb can also target the immune system rather than the tumours themselves. The aforementioned anti-CD40 mAb is the most extensively studied mAb of this type and is able to bypass T cell help in antigen presentation to CD8+ T cells. Thus anti-CD40 mAb mimics CD40L in this system, discussed further in the introduction to Chapter 3. Furthermore, anti-CD40 could target the tumour itself inducing direct apoptosis of CD40+ tumours, enhancement of presentation of TAA by malignant cells, as well as effector recruitment as previously discussed.

Table 1.5. Selection of mAb in clinical development for cancer

Indication	Target Ab	Antibody Name	Product Type
Ovarian	CA125	OvaRex	Murine
Breast	HER2/neu	Herceptin	Humanised (IgGl)
CLL	CD52	Campath lH/ (LDP-03)	Humanised (IgGl)
AML	CD33	SmartM195	Humanised (IgG)
NHL	CD20	Rituximab	Chimeric (IgGl)
	CD22	LymphoCide	Humanised (IgG)
	HLA	Smart ID10	Humanised
	HLA DR	Oncolym (Lym-1)	Radiolabelled murine

Table 1.5 Taken from MJ Glennie and PWM Johnson, *Immunology Today* (2000), **21**, no. 8, 403-410. (158)

1.6.2.2 Adoptive Immunotherapy of Cancer

Adoptive therapy is a passive cellular immunotherapy by which allogeneic donor T lymphocytes are removed, primed against tumour, expanded and injected back into the patient. It is a promising strategy for the treatment of a variety of malignancies such as leukaemia, Non-Hodgkin's lymphoma and melanoma. Adoptive transfer of tumour-specific immune cells provides the opportunity to overcome tolerance mechanisms in the host by the *ex vivo* priming step, achieved by manipulation of donor cells.

Different cells have been employed for adoptive therapy regimes including LAK (lymphokine activated killer cells) TIL (tumour infiltrating lymphocytes) and both CD8+ and CD4+ polyclonal lines and clones (reviewed in (140)).

Tumour infiltrating lymphocytes (TILs) were of particular interest in early studies by Rosenberg *et al.* Whilst investigating the mechanism of regression of cancer with the administration of IL-2 in mice, it was revealed that lymphocytes within the stroma of growing tumours could recognise tumour in *in vitro* assays(159-161). These lymphocytes were termed tumour infiltrating lymphocytes (TILs). However, trials in which patients with metastatic melanoma were treated with their own TILs which had

been grown *ex vivo* in high concentrations of IL-2 gave rise to only moderate responses with short duration. In addition to this, high doses of IL-2 were required for cell survival and the concurrent administrations of IL-2 produced toxic effects which showed that this was not a viable prospect for the clinic (162)(reviewed in (138)).

A basic lesson from the early studies using TIL was that not enough tumour-specific cells were killing tumour. This may have been due to poor definition of TAA, sub-optimal specificity or survival of TIL or poor localisation of TIL into tumour sites.

This has led to many groups trying to alter the balance of effectors (CTL) to targets (tumour) in the tumour sites of the host by either increasing the number or efficacy of the effectors, decreasing the tumour cell numbers or by improving tracking of effector cells to sites of tumour. Thus, studies aimed to test the efficacy of TIL in cases where tumour has been manipulated by medical or surgical procedures are ongoing (163, 164).

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In addition to TIL, both polyclonal T cells and cloned T cell subsets from blood, lymph nodes and spleen have been investigated in adoptive therapy regimes

The identification of the previously discussed tumour rejection antigens has allowed adoptive therapy to move forward, taking lessons from those therapies which have worked, such as adoptive transfer of viral antigen specific T cells in the treatment of virally induced tumours, as well as viral infections. Indeed, transfer of CD8+ CMV (cytomegalovirus)–specific T cell clones isolated from an immunocompetent bone marrow donor has been shown to restore protective immunity against CMV in the recipient. Likewise EBV (Epstein-Barr virus) reactivation occurs in some bone marrow transplant patients and this has been overcome in some cases by the transfer of EBV reactive T cells isolated from the donor and expanded *in vitro* (165) (reviewed in (166)).

1.6.2.2.1 *Ex vivo* manipulation of donor cells

Ex vivo manipulation of donor cells aims to generate effective tumour-specific CTL or increase their efficacy. A variety of strategies have been employed to try and achieve this. Dendritic cells have been used as an effective APC for *ex vivo* CD8+ T cell induction (167). In the cases where TAA are known DC s can be isolated from PBMC in humans (or bone marrow in mice in model systems), matured *in vitro* and pulsed with TAA for use in co-culture with T cells from donor blood (eg. (168)).

Since DCs are efficient at generating a primary response to antigen, donor T cells used for co-culture can be naïve. Unfortunately, this method has been seen to produce low avidity T cells *in vitro* (169). However, variation of peptide dose and cytokine mixtures has been used to try and overcome this problem and CTL recognising both dominant and sub-dominant epitopes have been generated using this approach, including CTL specific for MART-1/Melan A, MAGE-1, MAGE-3, tyrosinase and gp100(170-174).

Other strategies currently being assessed include methods where genes coding for tumour proteins are introduced into APC. This is being achieved by transduction with retroviral vectors, infection with recombinant viral vectors (175) or transfection with RNA(149) or DNA using lipid complexes (176). Again, in all of these cases the dominant TAA has been identified (Reviewed in (177)).

The observation that DCs can present antigens derived from apoptotic cells (12), (178) has allowed the generation of tumour-specific CTL using DC co-culture methods even in the absence of defined MHC class I restricted TAA, for example by using apoptotic leukaemic blasts as the source of TAA. In these studies the correct cytokine milieu and culture conditions have been investigated for efficient TAA presentation and DC maturation to occur, for example with the addition of CD4+ T cells in culture or more recently with ligation of CD40 (Reviewed in (179)).

CTL lines can be generated by this method, however many groups have created clonal CTL from a single precursor (180). Although CTL clones have advantages in the further identification of TAA of polyclonal CTL lines may be advantageous in adoptive therapy strategies. If tumour escape occurs such that one clone is no longer effective *in vivo*, others will still be present to eradicate tumour. Polyclonal T lymphocytes have been used, to treat patients with leukaemia after allgeneic bone marrow transplant. This technique, however, has had the complication of Graft vs Host disease (GVHD) (181).

In special cases where the tumour itself can also potentially function as an APC for example B cell lymphoma, co-culture of CD8+ T cells and irradiated tumour can generate tumour-specific CTL (detailed in section 1.6.2).

Attempts have been made to enrich host PBMC for CD8+ T cells specific for known TAA by the use of peptide-bound tetramers of MHC class I molecules (182,183). MHC tetramers can be used to sort specific CD8+ T cells but have limitations as they require knowledge of which TAA is involved and thus are not applicable to all tumours. In addition, individual tetramers select only single specificity CD8+ T cells and so if only one clone was expanded for use in adoptive therapy the probability of tumour escape would be high. Furthermore, tetramers select according to the affinity of the MHC Class I-peptide complex for TCR and not for function of the T cell and therefore T cell clones selected by this method may found to be anergic when isolated from cancer patients.

Another method for increasing the specificity of CTL to be adoptively transferred involves purifying CTL according to IFNγ secretion (this method could be combined with other methods, for example enriching TIL or CTL lines generated on pulsed DCs). The capture assay system means that cells responding to tumour as indicated by IFNγ production, can be isolated not by affinity but by response to tumour. Thus isolated CTL would not be anergic (184).
This idea can also be extended to the purification of CTL according to markers of activation such as CD62L (L-selectin). In mouse studies tumour induced $CD62L^{high}$ and $CD62L^{low}$ T cells from lymph nodes have been separated and effective therapies of murine tumours have been carried out using the $CD62^{high}$ depleted CD8+ T cells. Subsequent analysis revealed that the increased efficacy of the adoptive transfers was due either to the increase in the specific, reactive $CD62L^{low}$ T cells or decrease in the suppressive $CD62L^{high}$ portion and secondly, that $CD62L^{low}$ cells are postulated to track to the anatomical sites previously refractory to treatment (185).

The IL-2 gene has also been transfected into human melanoma-reactive cloned lymphocytes (186). The effect of this was that the lymphocytes remained viable even after IL-2 had been withdrawn from culture without losing specificity (illustrated in Figure 1.8(3)).
In addition TCR gene transfer (Figure 1.8(1)) has been carried out in mouse models with great success. A virus specific TCR was introduced into peripheral blood T cells

which when transferred back into recipient mice expanded upon viral infection and homed to effector sites (187). This technique is now being applied to human tumours

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The genetic transfer of specific TCR means that these transferred CD8+ T cells recognise TAA in the context of MHC class I. The introduction of a chimeric receptor for antigen (CAR) which could signal via the CD3 ζ chain into human T cells by Brentjens *et al* however, means that antigen can be seen as the whole peptide present on cells without the need for effective presentation on MHC class I. This could be advantageous in the cases where MHC class I is down regulated on tumour cells (Figure 1.8(2)). This recent study showed that anti-CD19 CAR transfected CD8+ T cells effectively lysed CD19 positive tumour cells and could be expanded (section 1.6.2.2.1)

Like T_H cells CD8+CTL can be sub-divided into two distinct populations based upon their cytokine profile and these are called T_C1 and T_C2 cells (188). T_C1 and T_C2 cells are important in the context of adoptive therapy since studies have shown that differing cytokines can affect the growth kinetics of different tumour cell populations as well as MHC class I, adhesion molecule and chemokine receptor expression on tumour cells. Thus it is likely that T_C1 and T_C2 cells differ in their modes of effecting tumour regression (189). In one study in transgenic mice, relative effects of adoptively transferred OVA specific T_C1 and T_C2 populations were assessed against an OVA-transfected B16 melanoma. Both T_C1 and T_C2 mediated a decrease in lung tumour growth with subsequent prolonged survival times, and both T_C1 and T_C2 cells accumulated in the site of tumour. However T_C1 cells persisted for up to three weeks at the tumour site after it had been cleared whereas T_C2 numbers rapidly diminished and protection was shown to be five times more effective with T_C1 cells (190). Interestingly the efficacy of T_C1 but not T_C2 cells was eradicated when CD8+ T cells from $IFN^{-/-}$ mice were used, suggesting differing mechanisms of action (191). Another study has revealed that efficacy of the CD8+ T cells is limited by migration away from the site of tumour and by down-regulation of certain adhesion molecules which make entry back to the site of tumour difficult (192). This was shown to be true of T_C2 populations in the EG7 thymoma model (193). Thus T_C1 CD8+ cells may be the optimal CTL to use in adoptive therapy.

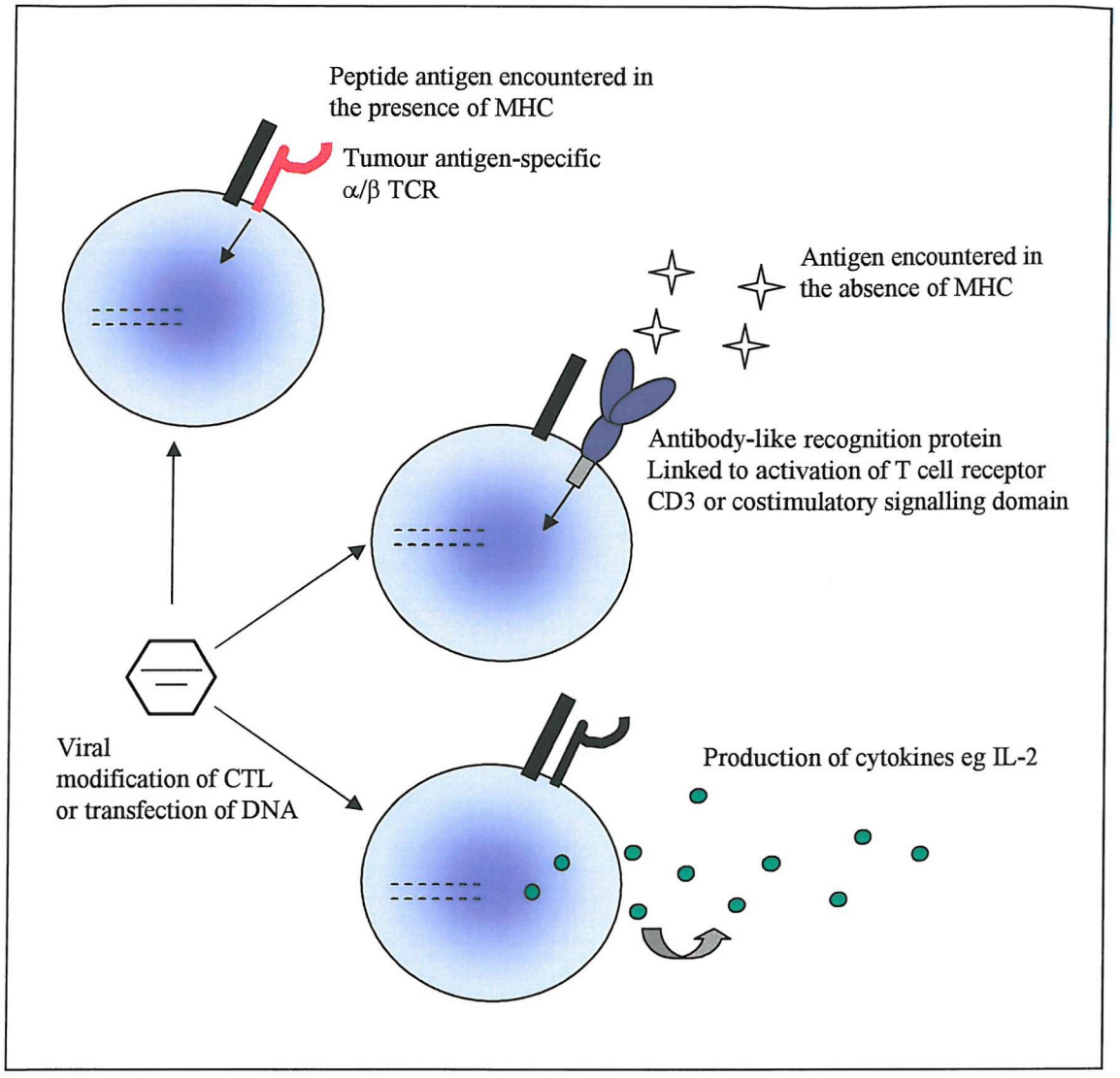


Figure 1.8 Genetic modification of T cells ***ex vivo***

Adapted from: Armstrong et al, Cellular immunotherapy for cancer *BMJ* (2001); **323**: 1289-1293

1.6.2.2.2 *Ex vivo* antigen-independent expansion of tumour specific lymphocytes

In addition to overcoming normal immune regulation, successful adoptive therapy also requires that enough cells are produced for effective tumour eradication. Generation of sufficient numbers of CTL has not routinely been accomplished and this has led to investigation of different expansion methods.

As previously explained, the expansion of CTL requires a strong signal via the TCR. This signal can be provided *in vitro* by anti-CD3 mAb (194, 195). The proliferation also requires an *in vitro* supply of exogenous IL-2 (196) or, more recently documented, IL-15 (197). Anti-CD3 and IL-2 have been shown to expand cord blood lymphocytes (198), tumour reactive vaccine primed or tumour-draining lymph node cells (195,199,200) peripheral blood mononuclear cells (PBMCs) (194) splenocytes (201) and TIL (202). The discovery that a second signal to T lymphocytes via ligation of CD28 was required for complete activation (60), coupled with the ability of CD28 to induce the expression of cell survival gene Bcl-X_L (203) lead to anti-CD28 being used in *ex vivo* expansion studies (204). These studies include the use of plate bound and soluble anti-CD3 and anti-CD28. For example Riddell and Greenberg cloned CMV-specific CTL and expanded these clones on anti-CD3 and anti-CD28 whilst maintaining their antigen-specificity (205).

More recently, artificial APC (aAPC) have been utilised to achieve T cell proliferation. In a method described by Levine *et a.*, human CD4+ T cells were expanded on anti-CD3/anti-CD28 coated magnetic dynal beads (illustrated in Figure 1.9a). The beads acted rather like APC but without the expression of negative signals such as CTLA-4 on their surface(206, 207).

Unfortunately, many groups have found that the mAb coated beads do not support the long term growth of purified CD8+ T cells in both mouse and human studies (208,209).

Another aAPC strategy for expanding cells is the loading of Fcγ Receptor transfected cell lines with anti-CD3 and anti-CD28 mAb. The rationale behind which is that the membrane of the transfected cell, being fluid in nature, would be more compliant with T cell stimulation than a rigidly coupled bead (the minimal aAPC as described by Latouche and Sadelain is illustrated in figure 1.9a) (210-212).

The cell based aAPC system has since evolved to encompass additional co-stimulatory signals, for example the TNF-R family member 41BB.

The aAPC system engineered by Maus *et al* seems superior to its predecessors. Figure 1.9b shows the multiple receptor- ligand interactions which Maus *et al* report are sufficient for CTL proliferation. Their aAPC system involved the expression of 41BBL on K562 cells which were decorated with anti-CD3 and anti-CD28 mAb via the Fcγ R (K562/41BBL/3/28). These aAPC promoted the rapid and importantly, long term (more than 2 months) growth of polyclonal antigen (influenza)-specific CD8+ T cells which were purified on MHC tetramers from human blood. The advantages reported over bead-based and earlier cell-based aAPC were that the K562/41BBL/3/28 aAPC do not possess MHC molecules and are thus more conducive to transfer to the clinic than beads and the technique is "off the shelf" meaning that any patient's CTL could be expanded on them quickly. The implications from these studies are also that the cells expanded retain a substantial replicative capacity after *in vitro* culture, importantly after they reach therapeutic numbers. Therefore they may effectively engraft in to the recipient. This is of importance as previously levels of engraftment have been low (213-215).

This technique also appears to be effective for expanding CTL which have previously been cultured on DC pulsed with apoptotic bodies of autologous tumour thus has implications for use where TAA are not known or MHC tetramers are not available.

It was thought that these aAPC increased CTL survival due to the expression of both IL-2 and importantly Bcl-xL, an anti-apoptotic factor, upon ligation of 41BB via 41BB-L. Indeed, the 41BB stimulation in this system overcomes activation-induced non responsiveness (AINR) (208), (216)(outlined in (217)).

In the previously mentioned studies by Brentjens *et al*, using CAR transfected CTL the effect of IL-15 in their *ex vivo* culture system with tumour was established. In this system of *ex vivo* expansion the transfected cells antigen (CD19 on the surface of aAPC), co-stimulation (B7-1 on the surface of aAPC) and IL-15. Again the success of this system seems to correlate with increased survival of the CTL even after many rounds of T cell stimulation indicated by the increase in Bcl-xL induced by the IL-15.

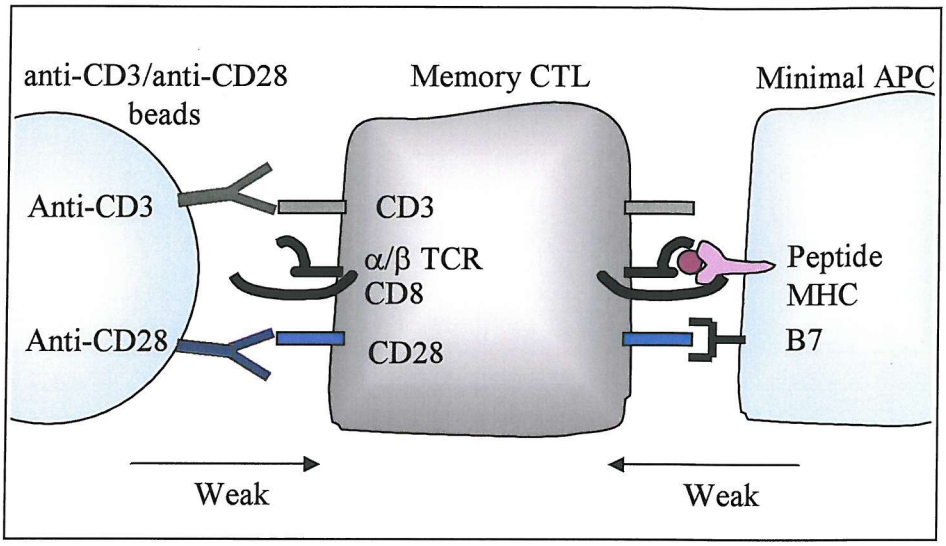


Figure 1.9a The minimal aAPC and anti-body conjugated bead expansion systems

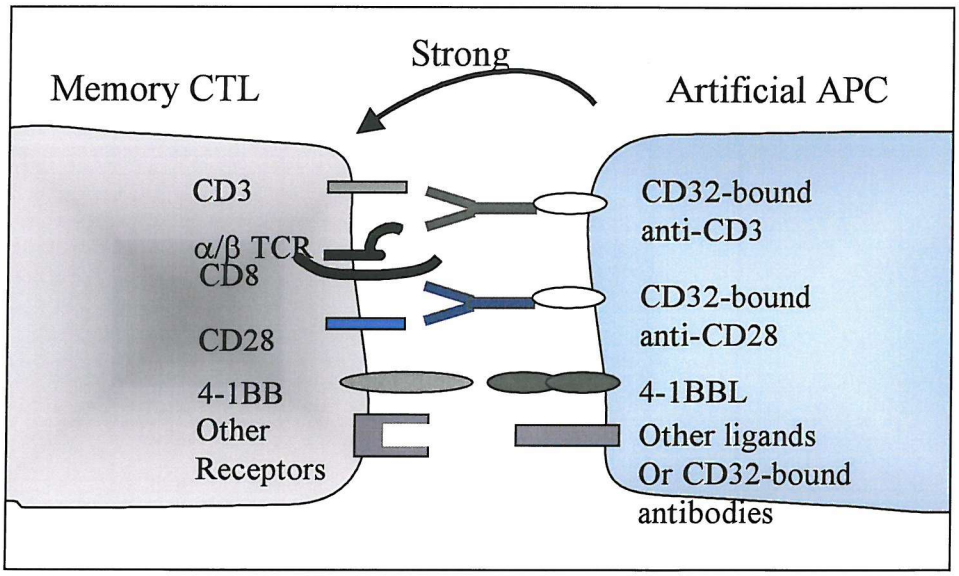


Figure 1.9b The aAPC system proposed by Maus et el.

Adapted from Dudley ME, A Stimulating Presentation: *Nature Biotchnology* (2002) **20**; 125-126

Interestingly, if IL-15 is replaced with IL-2 in this system the result is massive T cell death after three cycles of stimulation (218).

1.6.2.2.3 Manipulation of host environment in Adoptive therapies

Despite the crucial role for CTL in the immune response against cancer and the recent progress in their *ex vivo* manipulation and expansion for adoptive therapy regimes, their poor survival and homing to tumour sites *in vivo* and lack of engraftment into the host are major obstacles to successful adoptive CTL therapy.

Another method by which the balance of effector CTL to target tumour cells can be addressed and the survival and/or tracking of effector cells can be improved is by the manipulation of the host environment in to which the cells are being adoptively transferred.

As previously mentioned, the effect of IL-2 in tumour regression has been demonstrated in both metastatic kidney cancer and melanoma trials (219), (137), thus adding IL-2 along with transferred CTL is a viable strategy (162), (220),(reviewed in (140)).

Depleting lymphocytes has also been performed in an attempt to encourage engraftment of adoptively transferred T cells in the host. Murine studies have shown that lymphodepletion by sub-lethal total body irradiation may be beneficial for the treatment of tumours. Memory T cells transferred in to $Rag^{-/-}$, CD3ε-deficient and irradiated normal recipient mice can restore the memory cell compartment of the host (221,222) and lymphopenia has also been found to be critical in the specific homing of radiolabelled immune long-lived cells to syngeneic tumours (223). Mechanisms suggested for the enhancement of adoptive therapy by lymphodepletion include that of simply making space for transferred T lymphocytes (the "Lebenstraum effect") or the elimination of regulatory CD4+CD25+ T cells in the host, a mechanism which is supported by evidence that depletion of regulatory cells in vaccination protocols increases efficacy of tumour eradication (reviewed in (224)).

Importantly, the clinical application of lymphodepletion has been shown recently (225).In this work thirteen patients with metastatic melanoma were treated with adoptively transferred tumour-reactive T cells (derived from TIL) and a high IL-2 dose with prior immunodepleting chemotherapy. Six patients exhibited significant regression in this study and importantly, the persistence of individual tumour-specific

T cell clones was observed in patients' blood a result which had not previously been seen in the absence of lymphodepletion.

It is possible that, in this study, lymphodepletion was not the only factor influencing the successful engraftment of tumour specific CD8+ T cells into the host. CD4+ T cells may have been transferred with the *ex vivo* expanded TIL populations and a potential role for transferred CD4+ T cells in extending survival of CD8+ T cells has been reported.

1.6.2.2.4 The role of CD4+ T cells in adoptive therapy.

The co-operation between CD4+ and CD8+ T cells has been demonstrated in vaccination studies against virus (226, 227). CD4+ T cells are required for the maintenance of effector function of CD8+ T cells during chronic viral infections such as LCMV (228) and are also important in the perpetuation of a memory CTL response (229). A number of studies have indicated a link between CD4+ T cell help and the magnitude and persistence of CTL activity as well as increased trafficking of CTL to tumour sites (230). Previously described MHC class II restricted TAA epitopes have been isolated and studies using peptide co-immunisation with both MHC class I and class II peptides have shown that both appear to be required for tumour eradication (127, 231). Moreover, studies using transgenic CTL have shown improved expansion and differentiation of transferred cells in the presence of CD4+ T cells, especially with limiting numbers of effector CTL (232,233).

In studies where CD4+ T cells were not required for the induction of a CTL response, they were still needed to sustain or generate an optimal response (234-236). There is no doubt that CD4+ T cells are important in the provision of IL-2 at the priming stage (discussed in section 1.4.4) (144). The provision of IL-2 to CTL expressed by CD4+ T cells may indeed overcome AINR) which has been observed by CTL in culture after an initial proliferation period (208). Therefore, IL-2 provided either by CD4+ T cells or injections of exogenous IL-2 may have implications in adoptive therapy regimes as complete tumour regression presumably would require a prolonged anti-tumour immunity (231, 237).

In addition to their role in providing help for CTL, CD4+ T cells have also been observed to have direct effector functions themselves, through direct anti-tumour effects (118). These effect may be due to the production of various cytokines which can orchestrate downstream effector function. For example, there is evidence that

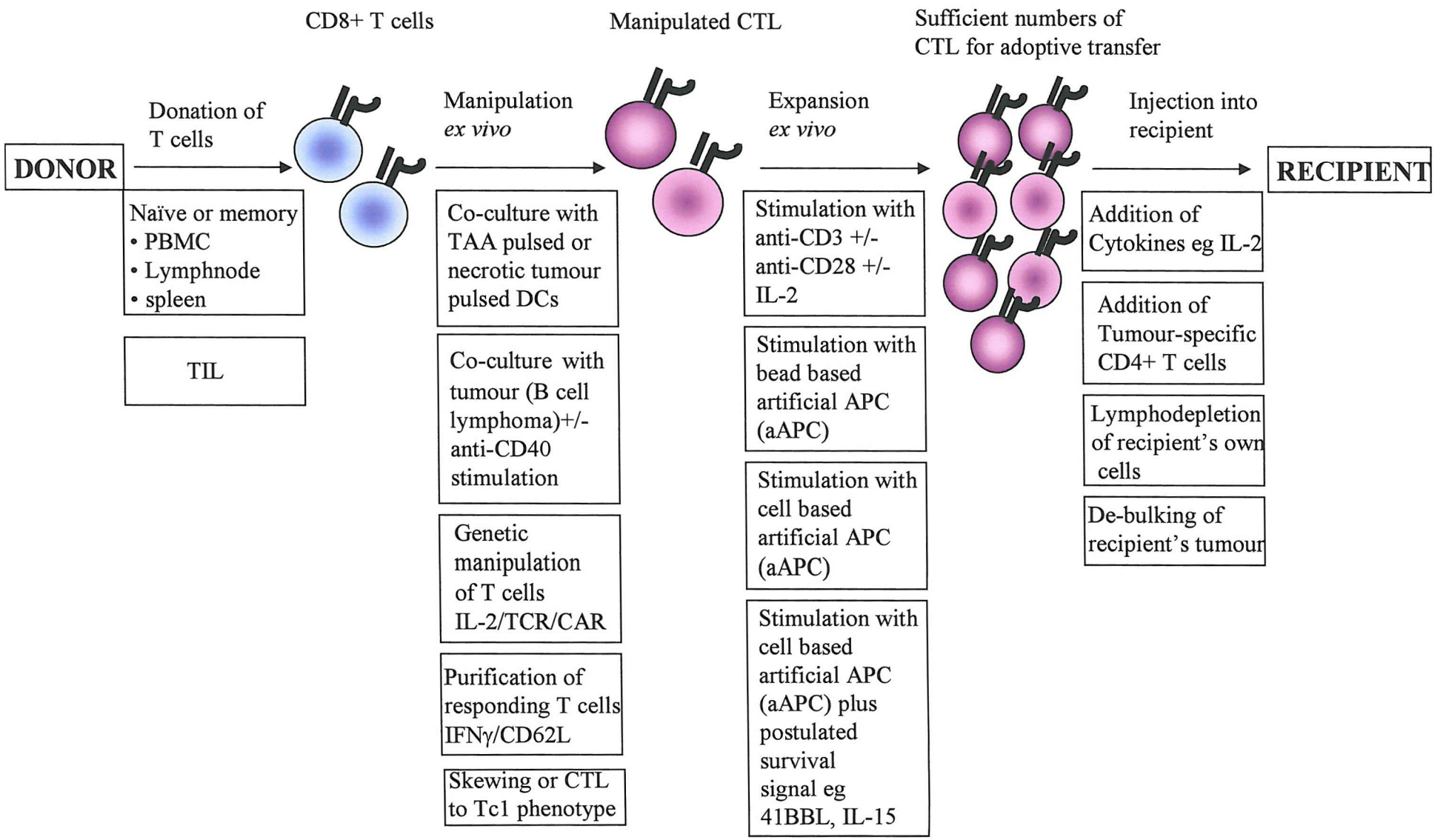


Figure 1.10 An overview of Adoptive therapy

antigen specific CD4+ T cells produce substantial amounts of IFN-γ which can up-regulate Fas on colon carcinoma cells which then renders the tumour susceptible to Fas induced apoptosis(238). Studies have shown that both T_H1 and T_H2 cytokines are needed in certain models for the induction of maximal systemic tumour immunity (239). Indeed, a potential role for eosinophils as the T_H2 activated effector cell has been implicated. T_H2 cells also activate B cells to secrete tumour–specific antibodies which may contribute to anti-tumour immunity (158).

In summary the effect of CD4+ T cells in immunotherapy regimes could be due to the maintenance of specific CTL numbers, the maintenance of CTL function, the increased infiltration into tumour sites or direct effects by CD4+ T cells themselves.

Thus, there are many methods in which to improve current adoptive therapy regimes an overview of which is illustrated in Figure 1.10.

1.6.3 Immunotherapy of B cell Lymphoma

B cell malignancies are special case for immunotherapy because most are characterised by the clonal expansion of a single B cell. B cells express a B cell receptor (BCR) which has a variable region unique to the individual B cell called the idiotype (Id) which is not only a clonal marker but also a tumour-specific antigen (240). Thus, active vaccination strategies (such as those detailed in section1.6.1) have been employed to develop T cell mediated immunity against B cell lymphomas *in vivo*. Vaccination regimes include the injection of Id protein with adjuvant (241), the injection of naked DNA encoding for Id (242) or the delivery of Id epitopes via DC(147). Lymphoma cells have also been transfected with GM-CSF and used for vaccination (reviewed in (243) and (244)).

Furthermore, B cells can act as APC. So, unlike other tumours, they can present their own antigens in the context of both MHC class II and class I. Thus there is a potential for a T cell response to B cell lymphoma initiated by the lymphoma itself. This route requires the efficient presentation of the TAA and expression of co-stimulatory molecules such as B7-1 and B7-2 and adhesion molecules on the B cell lymphoma. *In vitro* studies have shown that up-regulation of B7-1/-2 and adhesion molecules on B

cells and B cell lymphomas can be achieved via CD40 stimulation by both CD40L and anti-CD40 mAb (245). In addition, CD40 ligation has been utilised on B cell lymphomas to help them survive and proliferate *in vitro*. It has been argued that sufficient B cell numbers are able to present antigen as effectively as DC *in vitro* studies (246) thus high CD40 induced B cell numbers are advantageous in co-culture. The expansion and upregulation of co-stimulatory molecules on B cell lymphomas has been shown to allow the efficient presentation of TAA by B cell lymphomas and priming of their own T cell mediated response.
This has implications for passive immunotherapy namely adoptive therapy strategies by which patients' own T cells can be primed and grown on their own B cell lymphoma without the requirement for purification of TAA.

1.7 Aims of project

The aim of this study was to explore whether we could establish a model for adoptive therapy for the murine B cell lymphoma BCL_1.

- Investigation in to the efficacy of different T cell precursor cells for expansion *ex vivo.*

We wished to study means of expanding lymphoma specific CTL *ex vivo* for use in adoptive therapy strategies in the murine BCL_1 model of lymphoma.
It has been shown that the CD8+ T cells produced at the peak of the anti-CD40 therapy response are able to elicit cytotoxic activity against BCL_1 cells *ex vivo* (109). Thus, these cells could potentially be expanded and used for adoptive therapy in a recipient mouse. Naïve T cells from BALB/c mice could potentially be used. This approach would involve co-culture with lymphoma in an attempt to mimic the *in vivo* T cell activation as previously discussed in section 1.6.2. The third set of T cells, which could be used to create CTL *in vitro* are cells taken from mice cured of BCL_1 by anti-CD40. The existence of memory T cells specific to BCL_1 is suggested by the persistence of splenomegaly six weeks post anti-CD40 treatment and the fact that previously anti-CD40 treated mice do not succumb to re-challenge.

- Optimisation of conditions for growth and expansion of T cells.

We wished to use a co-culture system *in vitro* to attempt to expand tumour-specific T cells using irradiated lymphoma as previously discussed. Both BCL_1 tumour cells and the *in vitro* immortal cell line πBCL_1 can be considered as competent APC as they both express co-stimulatory molecules on their surface and both are available in large numbers in the mouse model allowing them to be used as an antigen source in the co-culture system this is of importance as TAA in this model have yet to be isolated. Furthermore we wished to assess the requirement for cytokines such as IL-2 and IL-7 in culture.

- Investigation in to the potential for antigen independent expansion of T cells.

For generation of large enough numbers of CTL and T_H cells for use in adoptive therapy we wished to attempt antigen-independent expansion of these cells on mAb in methods described in section 1.6.2.2.2.

- Investigation in to the role of CD4+ T cells in the BCL_1 model of lymphoma.

Due to the evidence that CD4+ T_H cells may be required for effective adoptive therapy of tumours (as previously discussed in section 1.6.2.2.4) we also wished to investigate whether there was a CD4+ T cell response to tumour in this model. Furthermore, we wished to determine whether in *vitro* co-culture systems could be used to generate BCL_1-specific T_H cells for use in conjunction with *in vitro* generated CTL in adoptive therapy strategies.

- Investigation into the the efficacy of T cell lines in adoptive therapy of BCL_1.

We aimed to establish whether any CTL lines generated were effective in adoptive therapys against BCL_1. Furthermore we wished to establish whether any T_H lines were able to improve therapy. We also aimed to investigate whether any "help" observed was due to the provision of IL-2 *in vivo*.

Chapter 2 Materials and Methods

2.1 Mouse Models, Tumour Passáge and Cell lines

2.1.1 Mouse Models

Wild type BALB/c (H-2^d) male and female mice were supplied by Harlan (Blackthorn, Oxon) and the OT-1 mouse strain on a C57BL/6 background (H-2^b) was a kind gift from Gitta Stokinger, (NIMR, National Institute for Medical Research, Mill Hill, London). Both strains were subsequently bred in house

All animals used for experimentation were both sex- and age-matched (between 6 and 12 weeks of age). BCL_1 (247) murine B-cell lymphoma line was maintained by *in vivo* intraperitoneal (i.p.) passage in BALB/c mice. Spleens were removed at the terminal stage of disease and single cell suspensions made as described below (section 2.2.2).

2.1.2 Cell Lines

πBCL_1(generated in house (248) and A20, P815, WEHI 231 and YAC cell lines (American Type Culture Collection- ATCC, Manassas, VA) were maintained in supplemented RPMI (complete media) as described below (section 2.2). X63-mIL-2 transfectant was a kind gift from Dr. Karasuyama and was routinely grown in complete media supplemented with 1 mg/ml G418 (Sigma Aldrich) and was transferred to G418-free complete media 24 hours prior to harvesting the supernatant; the concentration of IL-2 was subsequently estimated by ELISA (see section 2.11).

2.2 Cell Culture

In vitro tumour cell lines were routinely cultured in complete media in T-25cm^2 or T-75cm^2 flasks and incubated at 37°C in 5%CO_2 100% humidity. Cells were reseeded at a concentration of 1/5, when they had reached approximately 80% confluency.

2.2.1 Cell Culture Media

Routine cell culture was carried out in complete media:- RPMI 1640 medium (Gibco/ Life Technologies), supplemented with 2 mM L-glutamine (Gibco), 1mM pyruvate (Gibco), 50µg/ml 2-mercaptoethanol (Sigma) and 10% foetal calf serum (FCS) (Myoclone Plus; Gibco).

Ex vivo T cells (section 2.8) were cultured in T cell media- RPMI 1640 medium (Gibco/ Life Technologies), supplemented with 100 Units (U)/ ml penicillin (Glaxo), 100 µg/ml streptomycin (Evans), 2 mM L-glutamine (Gibco), 1mM pyruvate (Gibco), 50µg/ml 2-mercaptoethanol (Sigma) and 10% FCS (First Link (UK) Ltd). *Ex vivo* T cells were re-stimulated weekly.

2.2.2 Cell Preparation

Single cell suspensions were routinely prepared by sieving whole spleens in 10 ml of phosphate buffered saline (PBS; 1.37 mM NaCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4) through a cell mesh (Becton Dickinson (BD) Falcon). For tumour passage, splenocyte suspensions were layered onto Lymphoprep (Apogent Discoveries Ltd.), centrifuged for 20 minutes at 2500 rpm, and the interface collected.

2.2.3 Cell Quantitation

Cell concentrations were determined either by using a Coulter Industrial D cell counter (Coulter Electronics, Bedfordshire) or by using a Hemocytometer (400µl cells: 100µl trypan blue. Cells were counted in five squares and total number cells = (total cells counted / 4) x 10,000. Non viable cells, which stained blue, were excluded)

2.3 Antibodies

Antibodies, except those stated, were routinely produced from cloned hybridomas in house. Anti-CD4 (YTA 3.1.2), anti-CD8 (YTS169), anti-CD3 (KT3 or 145C211) anti-BCL_1 idiotype (Mc106A5), anti-Class I K^d (K9-18), anti-D^d (19-191), anti-L^d (HB31), anti-CD19 (1D3), anti-CD40 (3/23), anti-LFA1 (TIB237), anti-Vβ10 (21.5), anti-Vβ6 (44-22-1), anti-ICAM1 (YN1.4.7), anti-41BB (LOB12), anti IFNγ (HB170), anti B7.1(1610A1) and anti-B7.2(GL-1) and anti-FcR (2.4G2) were all prepared and FITC or PE labelled in house. Anti-CD44-FITC, anti-5-Bromo-2-deoxyuridine-PE and anti-CD49d-FITC were purchased from Serotec. Anti-CD62L-FITC was purchased from Caltag Laboratories. Anti-Vβ3 (KJ25), anti-Vβ4, anti-Vβ6 (RR4-1), anti-Vβ9, anti-Vβ10b (B21.5) and anti-Vβ14 (14-2) were purchased from Becton Dickinson Co.

Clones 1610A1, GL-1, TIB237, 2.4G2, YN1.4.7, HB170 were purchased from ATCC, YTA 3.1.2, and YTS169 were a kind gift from S. Cobbold, William Dunn School of Pathology, Oxford, K9-18, 19-191 and 3/23 were a kind gift from National Institute of Medical Research, London. 1D3 was a kind gift from D. Fearon, University of Cambridge. Mc106A5 and LOB12 hybridomas were made in house

2.3.1 Antibody Preparation

Hybridoma cells were maintained in stationary culture using DMEM culture media, supplemented with 5 % FCS

2.3.1.1 Selection of Secreting Hybridoma Clones

Hybridoma cells were cloned out by doubling dilution across a 96 well plate on freshly prepared BALB/c thymocytes in culture media. Wells were observed for the emergence of colonies grown from single cells. Screening of mouse or rat antibody secretion was carried out by ELISA (section 2.11) Briefly, coating antibody was bound on to the ELISA plate, 50µl of the hybridoma supernatant was added and a secondary, detection antibody HRP conjugate was used in order to detect presence of bound mAb from the supernatant.

Positive clones were grown up and transferred into 24 well plates and up to T-25cm^2 flasks.

2.3.1.2 Preparation of Antibody from Supernatant

Secreting clones were grown up in T-175cm^2 flasks and the supernatant and excess cells collected twice weekly upon re-feeding. The cells were centrifuged out (Mistral 3000i, at 350g for10 minutes) and the supernatant frozen and kept until a volume of 1 litre had been collected. The supernatant was then concentrated (Amicon, 4°C) until 1/10 of the original volume (100mls) Antibody was dialysed into PBS. Protein concentration was assessed by absorbance at 280nm in a 2mm cuvette
(Concentration (mg/ml) = (OD @ 280nm x 5 (for 1cm path) / 1.45(for Rat IgG).

2.3.1.3 Dialysis

Dialysis of antibodies into PBS was carried out in visking tubing (Medicell). Samples were dialysed 1:1000 with at least 3 changes of buffer for at least 2hours/change.

2.3.1.4 Fluorescein conjugation of mAbs (carried out by M. Powers)

Antibodies were fluorescein labelled by the method of Holborrow and Johnson (1967). Unconjugated FITC was then removed by passage through a G-25 column (Pharmacia), equilibrated with 0.0175M phosphate buffer, pH 6.3), followed by passage through a DEAE-cellulose column.

2.4 Measurement of surface Antigens by Immunoflurescence

Measurement of surface antigens was carried out in the following ways:
Cells at 1 x 10^6 / ml were incubated at 4° C for 30 minutes with fluorescein (FITC), phyco-erythrin (PE) or allophycocyanin (APC) conjugated (direct method) or unlabelled (indirect method) antibody of interest (10μg/ml final concentration). Cells were washed once (direct method) or twice (indirect method) in PBS-BSA-Azide (PBS, 1% Bovine Serum Albumin fraction V (Sigma), 20mM NaN_3) and re-suspended at approximately 1 x 10^6/ml. For indirect immunofluorescence, cells were further incubated for 30 minutes at 4 °C with a FITC-conjugated secondary antibody directed to the first antibody, washed once in PBS-BSA-Azide before re-suspension at 1 x 10^6/ml and subsequent analysis.

2.4.1 Measurement of intracellular IFNγ

Measurement of intracellular IFNγ was carried out as follows: CD8+ T cell lines were stimulated with πBCL_1 and IL-2, at an appropriate ratio of CTL:πBCL_1, or with IL2 alone. As a positive control CD8+ T cell lines were stimulated with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma) and 1μM ionomycin (Sigma). All stimulations were performed in the presence 10 ug/ml brefeldin A (Sigma). Cells were harvested, after an overnight incubation. Staining with mAb to surface antigens was as described section 2.4. In this method, triple fluorescence was used to detect CD8+ T cells in FL-4 using anti CD8-APC. Primary staining was performed in the presence of 10 μg/ml 2.4G2 (to block Fc:FcR interactions). Samples were washed twice in 200 μl of PBS/0.5 % BSA and fixed for 20 minutes in 1% formaldehyde at room temperature. After fixation, samples were washed twice in 200 μl of PBS/BSA and resuspended in PBS/BSA/0.5 % saponin for 10 minutes at room temperature. 100 μl of PE-labelled anti-IFNγ or isotype matched mAb in PBS/BSA/saponin were added to the required samples, mixed, and incubated for 30 minutes at 4 °C. Samples

were then washed in PBS/BSA/0.1 % Azide and assessed by flow cytometry on the FACScalibur. Samples were gated on live scatter and FL4 positive (CD8+) cells and analysed as FL2 vs FL1 dot plots.

Analysis was performed on a FACScan or FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Unless otherwise stated, 10,000 total events were collected per sample for analysis. Samples were analysed using either CellQuest software (Becton Dickinson) or WinMDI.

Fluorescence intensities were assessed in comparison with negative control samples. FSC (forward scatter) vs SSC (side scatter) analysis was used to gate for a viable population of cells as assessed by PI staining. For dual-staining, gating was also carried out on PE labelled cells (FL2) or APC labelled cells (FL4) (positive for cell marker of interest). Fluorescence intensities were expressed as histograms of fluorescence intensity (FL1) versus cell number, or as dot plots e.g. FL-1 vs FL-2 for dual fluorescence.

2.5 Carboxy Fluorescein Succinimidyl Ester (CFSE) labelling

T cell proliferation was assessed by staining with 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (CFSE;Molecular Probes, Leiden, the Netherlands)

Stock CFSE (5mM) was prepared by dissolving in DMSO and was stored frozen. *In vitro* T cells were resuspended in serum-free RPMI 1640 pre-warmed to 37°C (Life Technologies) at a concentration of 2 x 10^7/ml. Stock CFSE was added to a final concentration of 5µg/ml (1:1000 dilution) and was mixed by inversion and incubated for 10 minutes at 37°C. Cells were then washed twice in ice cold RPMI 1640 and then resuspended at an appropriate concentration in appropriate media for further *in vitro* culture.

Analysis of CFSE intensity and thus proliferation of the cells was performed on a FACScan or FACSCalibur flow cytometer as above. Typically 10,000 positive events were collected. Positive events were classed as cells which were both CFSE positive (FL-1) and positive for the antigen of interest (FL-2).

2.6 Assessment of Apoptosis

FSC vs SSC Analysis

Apoptotic cells undergo cytoplasmic shrinkage and condensation as well as nuclear condensation, such that cells which display both reduced FSC (smaller) and increased SSC (more granular) characteristics can be distinguished as apoptotic. FSC vs SSC analysis was carried out on T cell lines incubated in a humidified CO_2 incubator at 37°C for the required time under appropriate conditions. Cell samples (100 µl) were then harvested and washed once in PBS-BSA-Azide, agitated just prior to analysis which was performed via flow cytometry using the FACSCalibur. FSC and SSC parameters were adjusted to maximise separation of viable and apoptotic populations. FSC versus SSC dot plots were generated for analysis. Gating of viable and apoptotic regions, based on positive and negative controls, was used to quantify apoptosis.

2.6.1 Annexin V Binding

Following incubation with the given treatment, $1x10^5$ cells were washed in PBS and re-suspended in 200 µl of binding buffer (10mM HEPES, pH 7.4, 140 mM NaCl, 2.5mM $CaCl_2$), containing 1µg/ml FITC-annexin V (BD Biosciences), and subsequently assessed by FACS, as detailed by Vermes *et al* (249).

2.6.2 Propridium Iodide Assay

Cell viability was assessed by adding 50µg/ml Propridium Iodide (PI) to cells at a density of 1×10^5 / ml directly before analysis. Propridium iodide staining was carried out in conjunction with annexin V staining when both apoptosing and non-viable cell numbers were required.

2.7 Magnetic cell sorting (MACS)

Magnetic cell sorting using MACS beads (Miltenyi Biotec, anti FITC and anti PE micro beads) was used to isolate cell subsets of interest according to their cell surface antigens. MACS was carried out in accordance with manufacturers instructions Briefly an appropriate number of cells were labeled with either FITC or PE-conjugated antibodies specific for the surface marker. A magnetic bead coupled secondary antibody directed to either FITC or PE was used to isolate cells required on

MACS columns placed in a magnet. Unlabelled cells were collected as a negative fraction, labeled cells as the positive elutate upon removal of the column from the magnet.

2.7.1 Negative selection of CD8+ T cells (CD8+ enrichment) with MACS indirect microbeads

Negative selection or removal of unwanted cells from the splenocyte mix was required where cells with antibodies attached to the cell surface would hinder the assay, for example cytolytic activity of T cells could not be effectively assayed for when anti-CD8 was bound to its surface antigen

Single cell suspensions of splenocytes were washed in RPMI supplemented with 10% FCS only (detailed in section 2.2.1). Cells were counted and FACS was used to identify the percentage of cells with the antigens of interest as previously detailed. Cells were labelled with either FITC conjugated or PE conjugated antibodies specific for the surface markers on cells to be depleted for 10 minutes. For the enrichment of CD8+ T cells, splenocytes were labelled with anti-CD4 FITC, anti CD11c-FITC and anti CD19 FITC or PE. Antibody concentrations were equivalent to those used for FACSCalibur analysis (section 2.4). Cells were then washed in 10% RPMI twice and re-suspended in 90µl 10% RPMI per 10^7 total cells. Anti-FITC antibody conjugated microbeads were then added at a concentration of 5µl/10^7 cells and incubated on ice for 15 minutes. Cells were then washed twice in PBS, 0.5%BSA, 2mM EDTA. Separation of cells was carried out on pre equilibrated (with PBS, 0.5%BSA, 2mM EDTA) LS columns (provided by Miltenyi Biotech) in a final volume of 3 mls. The negative, unbound fraction contained the enriched CD8+ population. Negative fractions were analysed on FACS to ensure enrichment had been carried out.

2.7.2 Positive selection of T cells with MACS indirect Microbeads

Positive selection was used when there was a requirement for a pure population of cells. The general protocol used was the same as that detailed in section 2.7.1. Cells were labelled with either FITC or PE-conjugated antibodies specific for the surface markers on the cells to be selected. Columns were pre-equlibrated with MACS buffer (PBS, 0.5%BSA 2mM EDTA) and cells were placed on the column and allowed to soak in. Elutate was collected upon adding 2x 500µl MACS buffer to the column.

2.8 *In vitro* culture of CD8+ and CD4+ T cells.

CD8+ and CD4+ T cells were cultured both in the presence of irradiated lymphoma (πBCL_1) and typically, as a control, irradiated B cells separated from naïve BALB/c spleen by positive selection of CD19+ splenocytes by MACS (as detailed above). T cells were re-stimulated weekly. CD8+ and CD4+ T cells where stated either isolated from naïve (untreated), effector (BCL_1 immunised and anti-CD40 treated, cells taken at respective CD8 or CD4 peak) or memory T cells (BCL_1 immunised and anti-CD40 treated, cells taken after cure was achieved).

2.8.1 CD8+ and CD4+ T cell culture

For CTL and CD4+ T cell long term culture, T cell cultures were set up as follows: Immunised and anti-CD40 treated mice were sacrificed and spleens collected on D20-65 of therapy. A single cell suspension of splenocytes was obtained by passing the material through a cell strainer (Becton Dickinson (BD) Falcon) in 10% RPMI. CD8+ or CD4+ T cells were purified by positive selection on MACS beads under sterile conditions. T cells were counted (Coulter counter), centrifuged (350g for 5 minutes) and re-suspended in T cell medium. For routine culture, T cells were diluted at 2-4 x 10^6/ml in T cell medium along with irradiated (50Gy) πBCL_1 at a ratio of 2:1 or 4:1 T cells : πBCL_1. For routine culture 24 well plates were used . For 96 well assays final cell number was 1-2 x 10^5/well T cells: 5 x 10^4/well πBCL_1. For routine cultures anti-CD4 (YTA3.1.2) was added to CD8 culture and anti-CD8 (YTS169) to CD4 cultures at a final concentration of 10µg/ml and IL7 (Peprotech EC Ltd) was added to a final concentration of 10ng/ml. Cultures were incubated typically for 7 days before re-stimulation at 37°C, 5% CO_2. After this time T cells were examined microscopically under high and low power. If confluent, cells were counted and re-suspended to 2-4x10^6 and stimulated with πBCL_1 as previously described. (πBCL_1 irradiated at 50Gy as for set up and re-suspended to 1 x 10^6 /ml in T cell media). If not confluent, ½ ml of supernatant was removed and replaced with irradiated πBCL_1 (re-suspended to 1 x 10^6 /ml in T cell media). In the second week of routine culture IL2 (from X63-IL2 hybridoma) was added to all wells at a final concentration of 10 units/ml. Anti-CD4 (YTA3.1.2) or anti-CD8(YTS169) was added upon weekly re-stimulation and IL-7 was added as previously described if the viability of the cells (assessed by PI (Propidium Iodide) incorporation on the FACSCalibur) was below 20%)

2.9 Assay for cytolytic activity of T cells (^{51}Cr release assay)

Cytolytic T cell activity was determined by standard ^{51}Cr release assay. 10-20 x 10^6 πBCL_1, YAC, A20, P815 or WEHI target cells were washed twice in RPMI (Gibco/ Life Technologies) supplemented with 2 mM L-glutamine (Gibco), 1mM pyruvate (Gibco). Pelleted targets were re-suspended in 100-200µl ^{51}Cr (1mCi/ml, Amersham) and labellled at 37°C for 1 hour with agitation half way through incubation. Target cells were washed 3 times with RPMI (2 mM L-glutamine 1mM pyruvate) and re-suspended in 1ml complete media. Viable target cells were counted using a haemocytometer and diluted to a concentration of a minimum of $5x10^3$ per well (in 50µl). 50µl target cells were counted on the γ counter to check incorporation of ^{51}Cr.

Effector T cells were harvested and washed once in RPMI (2mM L-glutamine 1mM pyruvate) and re-suspended in full media. Cells were counted and adjusted to relevant concentrations in full media as shown below:-

5 x 10^5/ml (100µl/well = E: T 10:1)

1 x 10^6/ml (100µl/well = E: T 20:1)

2.5 x 10^6/ml (100µl/well = E: T 50:1)

50µl labelled targets were added to each well of a U-bottomed 96 well plate (Nunc). Controls were as follows: -

a) 50µl targets with 150µl media (background)

b) 50µl targets (for total counts)

Effectors were added at appropriate ratios to the targets in 100µl volumes and wells were made up to a final volume of 200µl with media.

In assays where blocking antibodies or bispecific antibodies were used, appropriate concentrations were added to the well in 50µl before the addition of targets. Plates were centrifuged at low speed (800 rpm) for 5 minutes room temperature and incubated for 4 hours at 37°C in 5%CO_2 after which 150µl NP40 was added to the total wells (a). Plates were centrifuged for 5 minutes at 1400 rpm and 100µl of the supernatants harvested into LP2 tubes and counted on a γ counter (wallac, program3, 5 minutes/tube). The % ^{51}Cr release for each sample was calculated in the following way

$$\% \ ^{51}Cr \text{ release} = \frac{\text{sample CPM} - \text{background CPM}}{\text{NP40 total CPM} - \text{background CPM}} \times 100$$

2.10 [^{3}H] Thymidine incorporation assay

Radiolabelled thymidine incorporation into cells was used to assess their proliferation status. Thymidine is only incorporated into cells in the replicating S-phase. Cells were cultured in U-bottom 96 well plates (Nunc). [^{3}H]Thymidine (Amersham) was added (0.5μCi/well in 20μl) at varying times depending on the length of culture required. Cells were harvested between 4 and 16 hours later onto glass fibres (Packard) with an automated harvester.

[^{3}H]Thymidine incorporation was determined by liquid scintillation counting. A minimum of triplicates was counted for each sample in all assays.

2.11 Enzyme-linked Immunosorbant Assay (ELISA)

Enzyme-linked immunosorbant assays were used to detect the presence of proteins within culture supernatants. Primary antibody was diluted in coating buffer (15mM Na_2CO_3, 28.5mM $NaHCO_3$ pH 9.6) and 100μl/well was added to 96 well plates (Nunc Maxisorp). Plates were incubated either over night at 4°C or for 1hour at 37°C. Excess unbound coating antibody was removed by flicking and non-specific binding sites were blocked with blocking buffer (1% (w/v) BSA in PBS) for 1 hour at 37°C. Plates were then washed three times with wash buffer (PBS-0.05% tween-20). The protein for quantification or standard was then added at a volume of 100μl/well, all dilutions were made in blocking buffer, and the plates were incubated for 90 minutes at 37°C followed by a wash step (x 5 in wash buffer).

An HRP-conjugated antibody (or biotinylated antibody in which case an extra step for the addition of streptavidin-HRP conjugate was carried out before addition of substrate) specific for the protein was diluted to its appropriate concentration in blocking buffer and added (100μl/well) for a further 90 minutes at 37°C. After washing (x 5 in washing buffer) an HRP substrate was added (100μl/well) (substrate; 20mg O-Phenylenediamine free base (O-PD) (1 tablet) in 100ml phospho-buffered citrate pH 5.0 + 100μl (30 %w/v) H_2O_2) and incubated in the dark at room temperature. The reaction was stopped by the addition of 50μl/well 2.5N H_2SO_4.

Colour change was quantified by the measurement of absorbance at 490nm on a plate reader (Dynatec 400, Dynatec). Unknowns were determined by comparison to a standard calibration curve performed, on the day of each ELISA.

2.12 cDNA Preparation and Reverse Transcript-Polymerase Chain Reaction (RT-PCR)

cDNA was prepared from $1x10^6$-$1x10^7$ cells using Quickprep micro mRNA purification kit and first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) as per manufacture's instructions. DNA amplification was performed using the technique of PCR. The basic protocol involved adding 1µl of each primer (100ng/ml) to 1µl cDNA with 2.5µl optimised reaction buffer and 0.5µl dNTP mix (10mM each dNTP/100µl) in a total volume of 24.5 µl (made up in dH2O). The mixture was pulse spun and 0.5µl polymerase (taq/ pfu) (Dynazyme) was added at a concentration of 2U/ml) and the mixture was pulse spun again. The DNA was denatured with a 5 minutes incubation at 94°C and PCR was performed using the appropriate annealing temperatures for the primers chosen for 1 minute, elongation for 2 minutes at 72°C and melting at 94°C for 30s. 30 cycles were routinely performed with a final elongation incubation of 30 minutes to ensure formation of full-length transcripts and poly (A) tails when using taq polymerase. PCR products were loaded on to agarose gels (0.7%) containing ethidium bromide and separated for 40 minutes – 1 hour at 100V along with relevant standard ladder. DNA was visualised under UV light and a photograph was taken. A detailed method and list of primers used can been seen in chapter 4 section 4.2.4.

2.13 Purification of Fas-hFc Fusion Protein

CHO-Fas-hFc cells were grown in 10% RPMI culture media (detailed in section 2.2.1) containing 1mg/ml G418. Supernatants were collected and run through a 10 ml protein A column. Columns were subsequently washed in approximately 2 litres of 40 µM Tris.HCl/ 2mM EDTA (pH 8.0), prior to eluting the fusion protein using 1 ml 0.1 M glycine/2mM EDTA (pH 3) and protein estimation by ELISA against hFc (see standard protocol above).

3 *In vivo* response to BCL_1.

3.1 Introduction

In studies attempting to eradicate B cell lymphoma, mAb against Id, CD19 and CD40 were shown to be therapeutically active in both BCL_1 and A31 mouse models (Figure 3.1 shows therapeutic effect of anti-CD40 against BCL_1). The most promising of the mAb was anti-CD40, which cured pre-existing B cell lymphoma and induced long-term protection against subsequent rechallenge, implying the mice had been immunised.

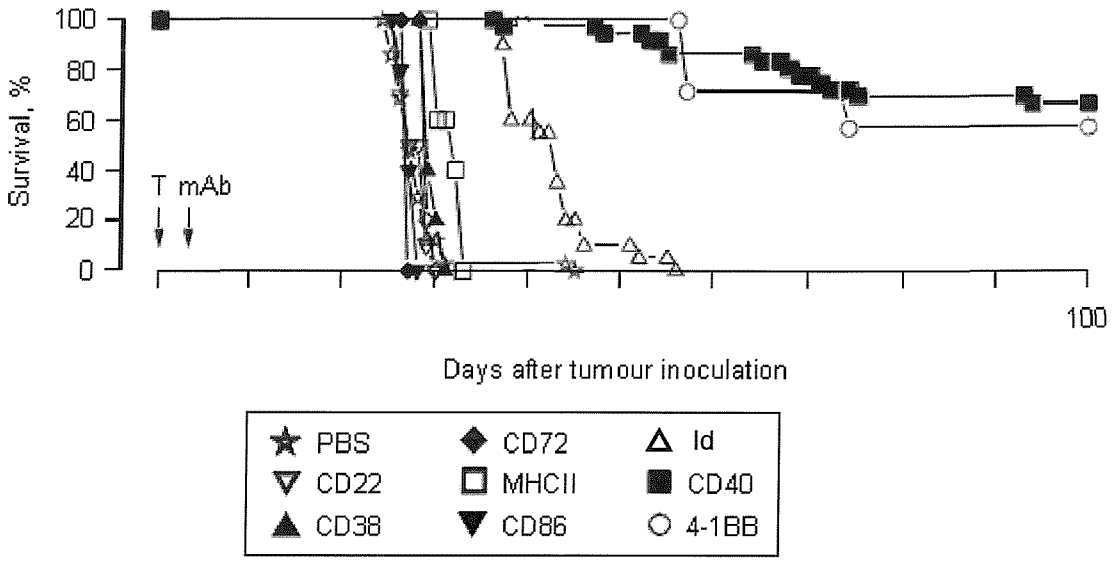


Figure 3.1. Immunotherapy of BCL_1-Bearing Mice with Rat mAbs.
Groups of five age-matched BALB/c mice received 5 $x10^7$ BCL1 cells by i.v. injection on day 0 and, 4 days later, 1mg of rat mAb directed against the antigens shown (250)

Figure 3.1 also shows the more recent observation that other mAbs such as anti-4-1BB have been able to affect tumour regression via the initiation or bolstering of an anti-tumour immune response (251, 252).

The therapeutic effect of anti-CD40 does not result from the direct cytotoxic activity of the anti CD40 monoclonal antibody (3/23), ADCC or complement activation. Anti-CD40 protection is almost complete with a large tumour burden (mice treated day 2-5 after 5 x 10^7 tumour cells injected i.v. typically, at this time 10 % of splenic

lymphocytes are tumour cells) indicating the immune system is responding to a weak or limited tumour associated antigen which may have to be present at a threshold level to ensure protection. Confirmation of the involvement of the role of the immune system was carried out, firstly by re-challenge of anti-CD40 cured mice, which did not succumb to B cell lymphoma (BCL_1). Secondly, minimal therapeutic effect was seen when anti-CD40 was used in SCID mouse models, until the mice were lymphocyte re-constituted.

CD8+ T cell involvement in anti-CD40 therapy was confirmed with depletion mAb against CD8 and CD4. The removal of CD8+ T cells resulted in the loss of therapy while CD4 depletion had no effect. The anti-tumour response was seen to have rapid kinetics which were typically as follows. In mice inoculated with BCL_1 (D0) and treated with anti-CD40 (D4), tumour increase was the same in both CD8+ T cell depleted mice and control mice for 4-5 days post anti-CD40 treatment (D8-9) followed by a rapid decrease over the following 48 hours in control mice (shown in Figure 3.2).

Tumour depletion was blockable, not only by CD8+ depleting antibodies, but also antibodies directed to co-stimulatory molecules B7.1 and B7.2. Treatment with anti-CD40 also profoundly changed the lymphocyte compartment. CD8+ T cells in particular were seen to increase ten fold, decreasing once tumour had been cleared. However, even up to six weeks after cure was achieved, splenomegaly was seen to persist with CD8+ T cells in a higher proportion than in naïve mice although never seen at the level of the peak of response D8-9 of therapy (109).

By way of introduction this chapter will outline a typical CD8+ CTL response to tumour in the BCL_1 model with the administration of anti-CD40.

For adoptive therapy a large number of cytotoxic T cells are required. Various precursor T cells could be employed to generate these CTL and this chapter also aims to determine which would be optimal. Essentially there are three types of T cell precursors which could be used. Firstly, it has been shown that the CD8+ T cells produced at the peak of the anti-CD40 therapy response (typically D8-9 of therapy as detailed above) show cytotoxic activity against BCL_1 *ex vivo*. Thus, these cells could potentially be expanded and used for adoptive therapy in a recipient mouse. Secondly, naïve T cells from Balb/c mice could potentially be used. This approach would involve co-culture with lymphoma in an attempt to mimic the *in vivo* T cell activation. As previously discussed in section 1.6.2. B cells in large enough quantities

could be used to effectively present tumour associated antigen to naïve T cells. Both the BCL_1 tumour and its *in vitro* immortal cell line πBCL_1 may be competent APC as both express co-stimulatory molecules on their surface and both are available in large numbers in the mouse model allowing them to be used as an antigen source.
The third set of T cells, which could be used to create CTL *in vitro* are CD8+ T cells taken from the spleen of anti-CD40 cured mice D20-D60 of therapy. The existence of memory T cells specific to BCL_1 is suggested at this time by the persistence of splenomegaly post anti-CD40 treatment and, more importantly, the fact that previously anti-CD40 treated mice resist re-challenge.

Lastly, this chapter aims to investigate the role of CD4+ T cells within this model. There is evidence that CD4+ T cells may be required for anti-tumour immunity in many cases (253) importantly in adoptive therapy regimes (234) Furthermore, their role in adoptive therapy using non-transgenic models where the precursor frequency of antigen specific CD8+ T cells in low is being established (254) (233). We wished to investigate whether, in the BCL_1 model of lymphoma, there were class II - restricted tumour antigens capable of eliciting a CD4+ T cell response, and therefore a potential of creating CD4+ lines *in vitro* for adoptive therapy.

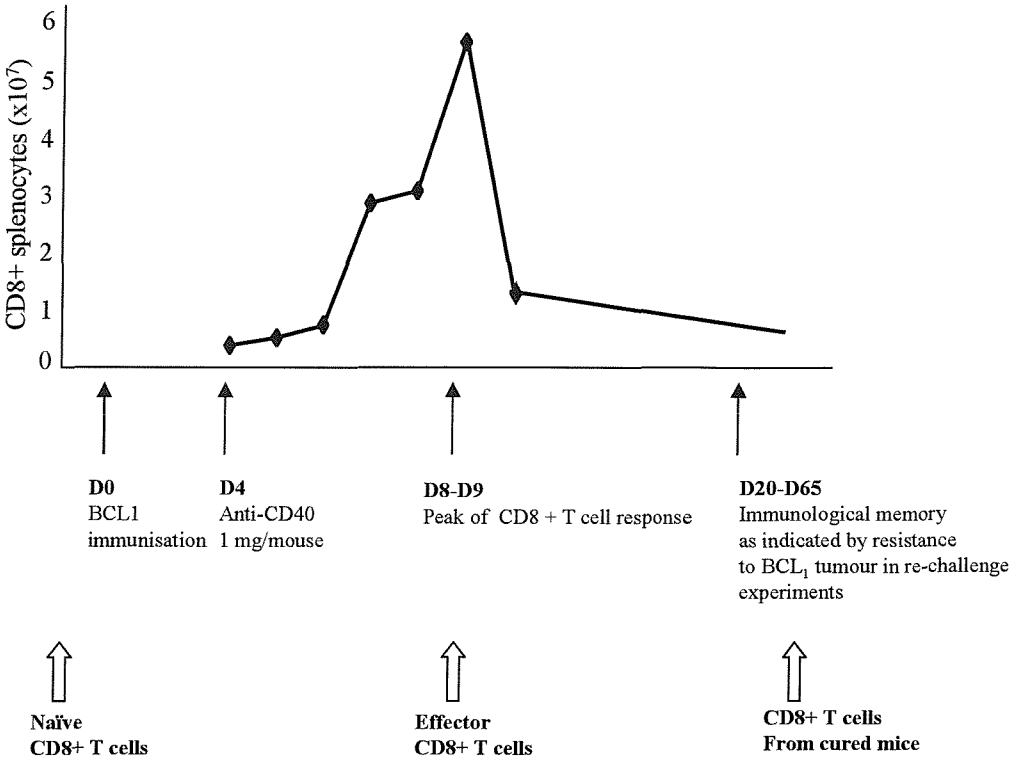


Figure 3.2 Diagram of a typical therapy of BCL_1 via administration of anti-CD40 (3/23).Mice were immunised day 0 (DO) with $5x10^7$ BCL_1 and treated D4 with 1 mg/mouse anti-CD40 i.v. The peak of the response was normally seen on D8-D9 of therapy. Mice were resistant to tumour re-challenge up to at least D65.The figure also shows the time points at which the three T cell populations discussed in this chapter were purified from the spleen of mice undergoing anti-CD40 therapy.

3.2 Materials and methods

3.2.1 Anti-CD40 treatment of BCL_1

BCL_1 lymphoma cells were isolated from splenocytes of animals at the terminal stage of disease as described in materials and methods. 5 $x10^7$ lymphoma cells were injected i.v. in to BALB/c mice day 0 (D0) followed by 1 mg of anti-CD40 when tumour constituted between 4 and 10 % of the splenic lymphocytes. Typically BCL_1-bearing animals were treated four days after primary tumour challenge on day 4 (D4) (as shown in Figure 3.2).

3.2.2 Analysis of BCL_1 lymphoma specific CD8+ T cells *in vivo*

Spleens were collected after the administration of tumour on D0 and anti-CD40 on D4, tumour alone or anti-CD40 alone and surface expression of antigens by lymphocytes during immunotherapy was assessed by flow cytometry as detailed in materials and methods Chapter 2 section 2.5.

For *ex vivo* culture of CTL from the peak of the anti-CD40 response CD8+ T cells were purified by positive selection from splenocytes using MACS (detailed in materials and methods) typically D8 or 9 of therapy where lymphoma levels were approximately 1% and CD8+ levels were between 20-30% (assessed by CD8-PE stain) these cells are called effector cells for the purposes of this thesis. Purification of CD8+ T cells from cured mice occurred day 20-60 of therapy. Naïve CD8+ T cells were purified from the splenocytes of unimmunised, untreated BALB/c mice.

For the assessment of cytotoxicity ^{51}Cr release assay was routinely used (as detailed in materials and methods). Splenocytes used for cytotoxicity assays were assessed to ensure they contained no residual tumour. If tumour was present it was removed using MACS to prevent *in vitro* quenching of CTL activity.

3.2.3 Analysis of CD4+ T cells *in vivo*

CD4+ T cells were analysed after the administration on the days stated above of tumour alone, tumour and anti-CD40 or anti-CD40 only control. Flow cytometry was carried out as detailed in materials and methods section 2.4. Briefly, CD4+ T cells were visualised using anti-CD4-PE conjugate (YTA3.1.2-PE) and FITC-conjugated mAb against antigens of interest were used to visualise the expression of activation markers.

3.2.4 Analysis of *in vivo* proliferation by lymphocytes.

5-Bromo-2-deoxyuridine incorporation was used to assess CD4+ lymphocyte proliferation *in vivo* by administering 2 mg of BrdU i.p. 1 hour before cell preparation and surface antigen staining. $1x10^6$ stained cells were then fixed overnight in 1 ml of 1 % formaldehyde and subsequently exposed to 50 Kunitz units of DNAse I for 1 hour at 37 °C before being washed and re-suspended in 0.2 % Tween-20. Each sample was then stained (4°C for 30 minutes) for BrdU incorporation using a PE-conjugated anti-BrdU mAb, washed in PBS-BSA-Azide and analysed by flow cytometry

3.3 Results

3.3.1 The role of CD8+ T cells in the eradication of BCL_1.

The therapeutic activity of 3/23 (anti-CD40 mAb) depends on CD8+ T cells. Figure 3.3.1 depicts the typical kinetics of lymphoma eradication and CTL expansion following administration of anti-CD40 (originally performed by Dr Ruth French). Kinetic studies were regularly repeated to determine the time course of the CD8+ T cell response, thereby confirming at which day to harvest effector CD8+ T cells in subsequent studies.

Dual colour FACS was used to show that eradication of tumour occurred five days post anti-CD40 treatment on D9 (Figure 3.3.1a). In conjunction with the eradication of tumour there was a large expansion of CD8+ T cells as can be seen in Figure 3.3.1b. Peak cells subsequently used were taken post anti-CD40 treatment when CD8+ levels were between 20% and 30% and the level of lymphoma was below 1%. The timing of the peak of response was seen to vary between D8 and D10 of therapy.

This data supports previous published observations that anti-CD40 induces tumour eradication by initiating or boosting an existing anti-tumour $CD8^+$ T-cell response resulting in the generation of class-I-restricted anti-tumour CTL whose numbers fall again following tumour eradication.

3.3.2 Phenotypic analysis of naïve, effector and memory T cells in anti-CD40 therapy of BCL_1.

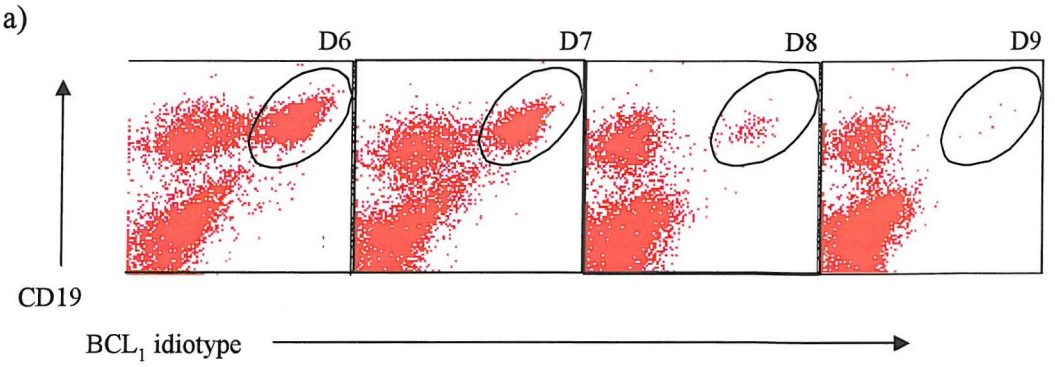
To determine the phenotype and activation status of naïve, effector CD8+ cells from cured mice, splenocytes were double-stained for CD8 and surface marker of interest and analysed by flow cytometry.

The markers used in the determination of phenotype and activation status of the three types of CD8+ T cells were adhesion molecules CD62L (L-selectin), CD44, CD49d, ICAM-1, LFA-1, and the activation markers, 4-1BB and the IL-2-Rα chain, CD25.

Figure 3.2.2 shows the phenotype of the three CD8+ T cell types during therapy in BALB/c mice. The comparative phenotypes are shown in Table 3.1.

Figure 3.3.1) The therapeutic activity of 3/23 mAb against CD40 depends on CD8+ T cells

a)



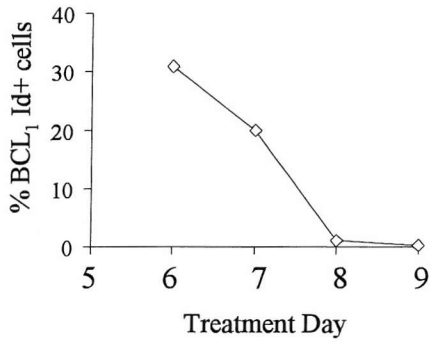
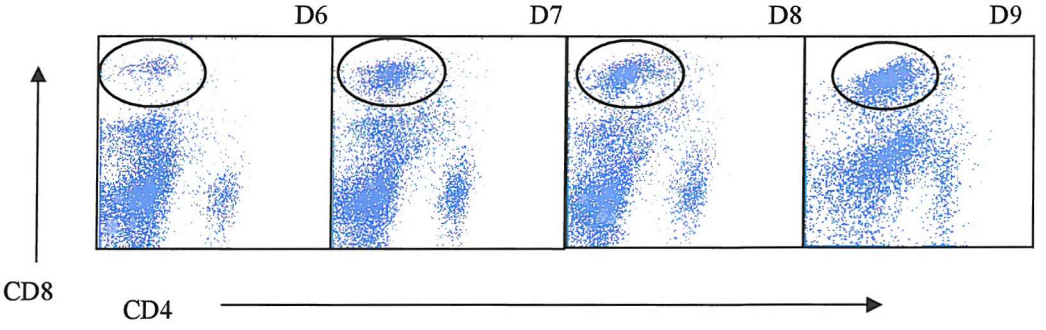


Figure 3.3.1a Depletion of BCL_1 idiotype positive tumour cells post anti-CD40 Treatment. Mice were innoculated with 5 x 10^7 BCL_1 cells i.v. D0 and treated with 1 mg 3/23 (anti-CD40) D4. BCL_1 cells in the spleen were visualised by Immunofluorescence D6-D9

b)



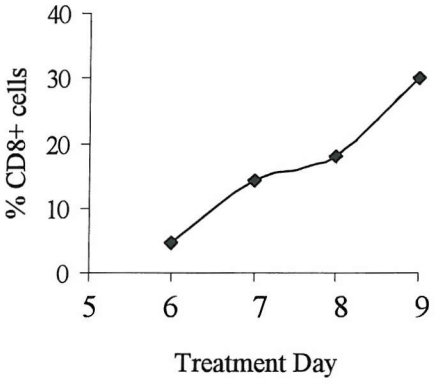


Figure 3.3.1b Expansion of CD8+ T cells post anti-CD40 treatment. Mice were innoculated with 5 x 10^7 BCL_1 cells i.v. on D0 and treated with 1 mg 3/23 (anti-CD40) D4. CD8+ cells in the spleen were visualised by Immunofluorescence D6-D9.

Figure 3.3.2) Phenotypic analysis of naïve, effector and memory T cells in anti-CD40 therapy of BCL_1

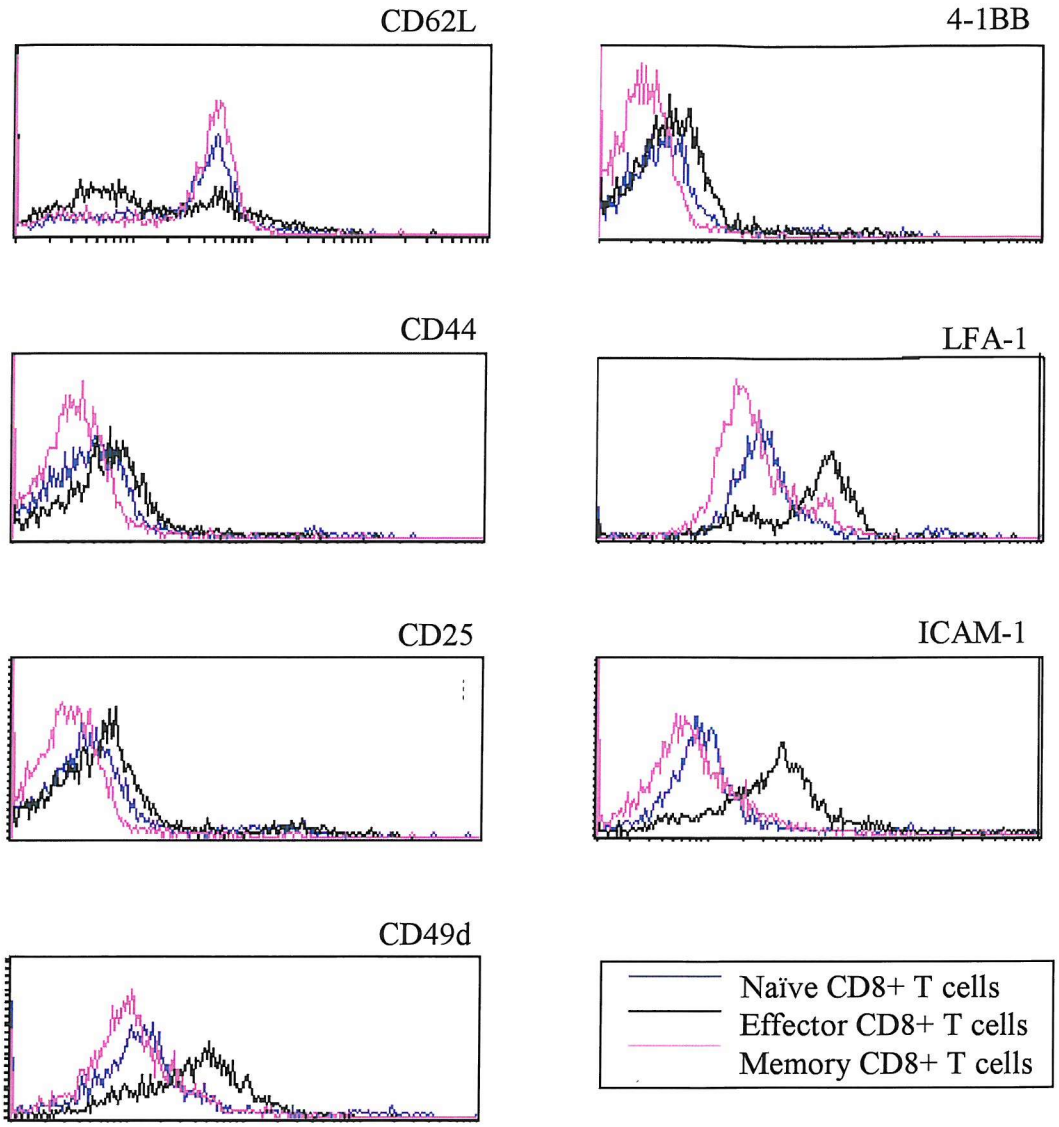


Fig 3.3.2) Assessment of the levels of activation markers on naïve CD8+ T cells, effector CD8+ T cells (taken D8) and those taken from cured mice (D34). Cells were gated on CD8 expression using YTS169-PE.The level of the second marker was determned by immunofluorescence using FITC conjugated antibodies directed against the marker of interest.

	Naïve	Effector	Cured
CD62L	High	Low	High
CD44	Low	High	Low
CD25	Low	High	Low
CD49d	Low	High	Low
4-1BB	Low	High	Low
LFA-1	Low	High	Low
ICAM-1	Low	High	Low

Table 3.1. The relative expression of markers of activation on the three CD8+ T cell types examined.

The effector population of CD8+ T cells from the peak of the anti-CD40 response could, therefore, be distinguished from both naïve and CD8+ T cells from cured mice by its unique phenotype. Naïve CD8+ T cells and those taken form cured mice, however, were not distinguishable from each other by these phenotypic markers.

3.3.3 *In vivo* TCR skewing of CD8+ CTL at the peak of response

In addition to the activation status of effector, naïve and CD8+ T cells taken from cured mice, TCR receptor skewing was investigated in all three T cell groups. This was to further characterise the *in vivo* peak response and also to investigate whether a skewed TCR repertoire could distinguish the postulated memory T cell population from the naïve population. TCR skewing was assessed by immunofluorescence after labelling CD8+ T cells with anti-CD8-PE and the Vβ chain of interest with anti Vβ-FITC conjugates. CD8+ T cells taken from spleen of naive, D10 (effector), D40 (postulated memory), terminal spleen (inoculated with BCL_1 and left untreated), and control mice (given anti-CD40 only) were analysed .

Pooled data from three separate experiments (Figure 3.3.3) shows that the effector peak cells were slightly skewed towards Vβ6 and Vβ8 TCR usage when compared to

Figure 3.3.3) *In vivo* skewing of CD8+ T cells during anti-CD40 therapy.

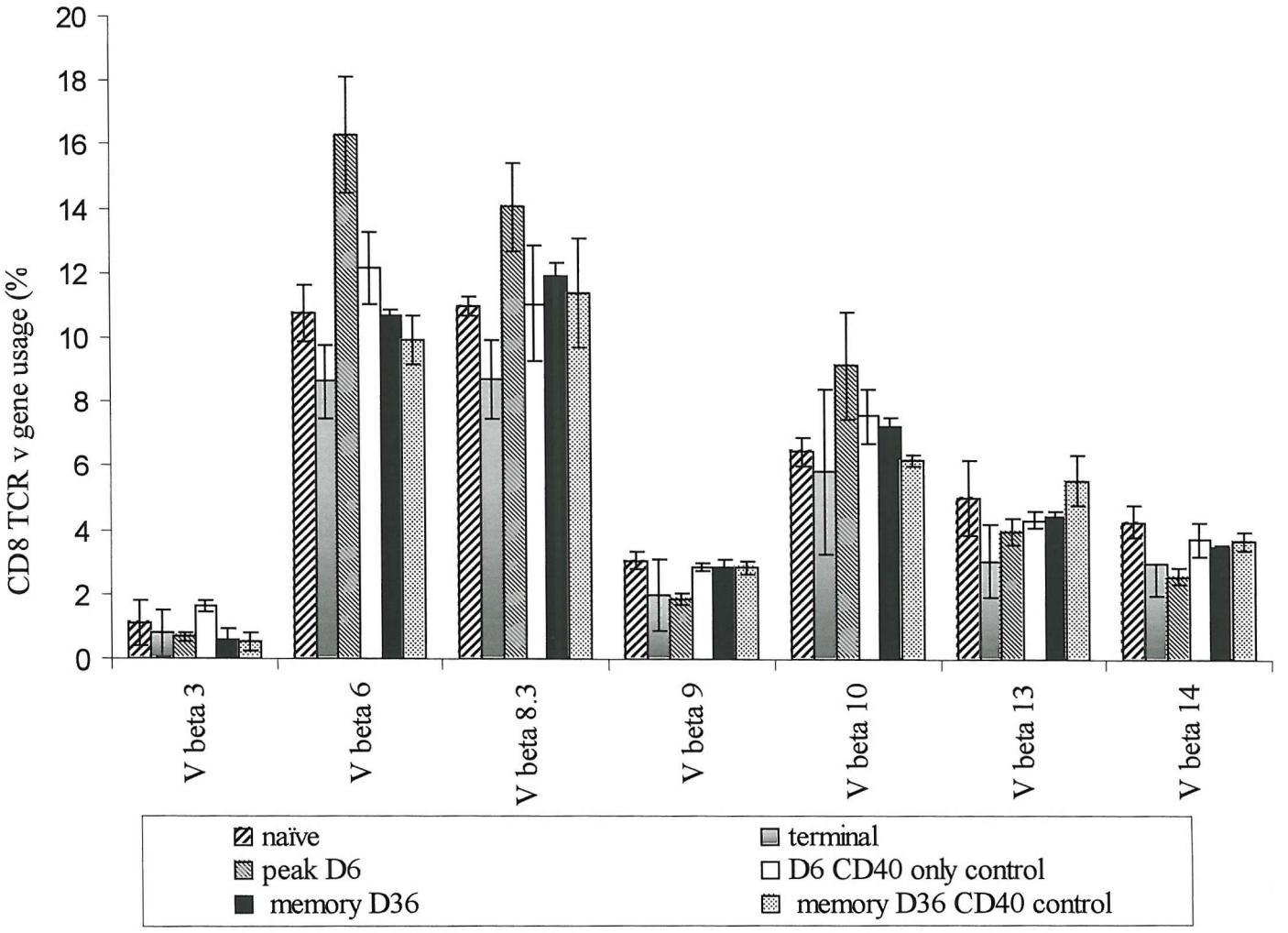


Figure 3.3.3) *in vivo* TCR skewing was assessed by immunofluorescence after labelling CD8+ T cells with anti-CD8-PE and the Vβ chain of interest with anti Vβ-FITC conjugates. CD8+ T cells taken from spleen of naive, day 6 post anti-CD40 treatment (peak), day 36 post anti-CD40 treatment of BCL_1 (memory), terminal spleen (inoculated with $BCL_1$and left untreated), and control mice (given just anti-CD40) were analysed .

naïve, memory and control CD8+ T cell populations. However the response at peak was still polyclonal. A CD8+ T cell TCR receptor skewing was not seen in cured mice and again naïve CD8+ T cells and those from cured mice were not distinguishable from one another.

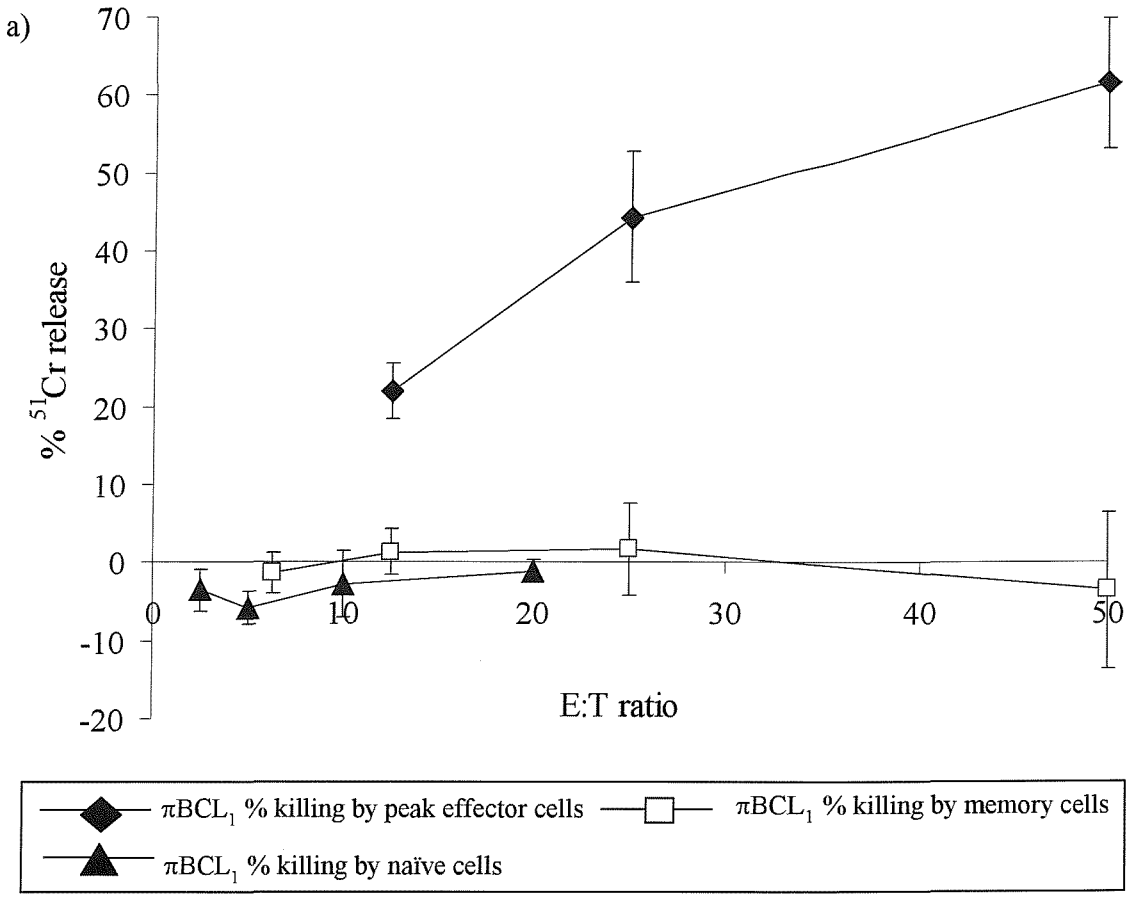
3.3.4 *Ex vivo* killing of lymphoma by enriched splenocytes

To determine the cytotoxic capacity of all three pools of CD8+ T cells, we used a standard ^{51}Cr release assay directly *ex vivo*. T cell populations were enriched (by removal of CD19+, CD4+ and CD11c+ cells as detailed in materials and methods) from splenocytes taken at all three T cell time points as shown in Figure 3.2. Figure 3.3.4a shows that only T cells removed at the peak of response (D8) were significantly cytotoxic against πBCL_1 in the ^{51}Cr-release assay. Neither enriched naïve T cells nor enriched T cells from anti-CD40 cured mice were able to kill target lymphoma. The extent of natural killer (NK) cell mediated killing in each of these cell preparations was assessed by performing a ^{51}Cr release assay against Yac targets (shown at a 25:1 E:T ratio on Figure 3.3.). All pools of CD8+ enriched cells showed NK dependent killing between 15 and 20 % killing at all E:T ratios used to assess the naïve pool and (5:1-20:1) and at higher E:T ratios (25:1-50:1) for the effector and anti-CD40 cured T cell enriched cell pools (data not shown). Thus the effector cells taken from the peak of response were the only CD8+ T cell pool able to kill target tumour in a CD8+ dependent manner directly *ex vivo*.

3.3.5 Therapeutic activity of CD8+ T cells in BCL_1 re-challenge mice.

As outlined previously depletion of CD8 and CD4 T cells in the primary response during anti-CD40 therapy of tumour showed that CD8+ T cells and not CD4+ T cells were required for tumour eradication. We decided to investigate whether CD8+ T cells were also the effector cell which cleared tumour upon rechallenge. The reasons for this were two-fold. Firstly, the presence of memory CD8+ T cells had been suggested in cured mice and we wished to confirm this. Secondly, we wanted to establish whether CD4+ T cells were important in the secondary response. If CD4+ T cells were needed for protection from re-challenge it might be that tumour specific

Figure 3.3.4) *Ex vivo* cytotoxic activity of enriched splenocytes



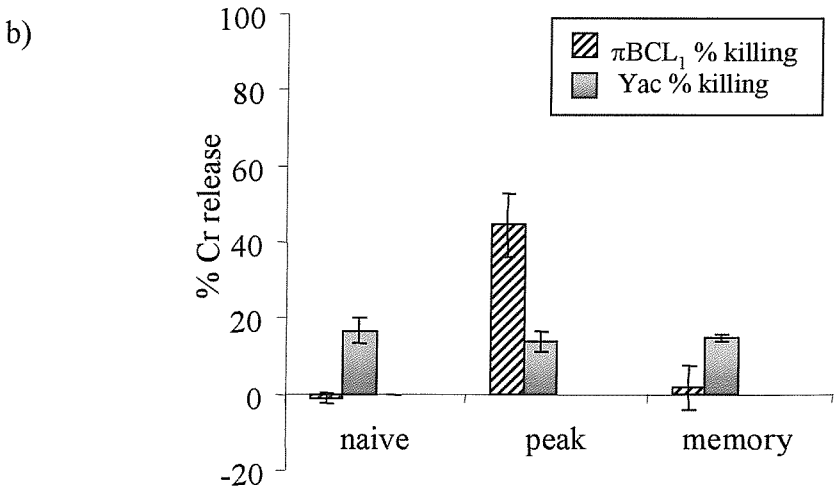


Figure 3.3.4) *Ex vivo* killing of πBCL_1 by naive, peak effector and memory CD8+ T cells was assessed by standard ^{51}Cr release assay using πBCL_1 targets (a). NK dependent killing was shown at an E:T ratio of 25:1using Yac targets (b)

Figure 3.3.5 Therapeutic activity of CD8+ T cells in BCL_1 re-challenge mice

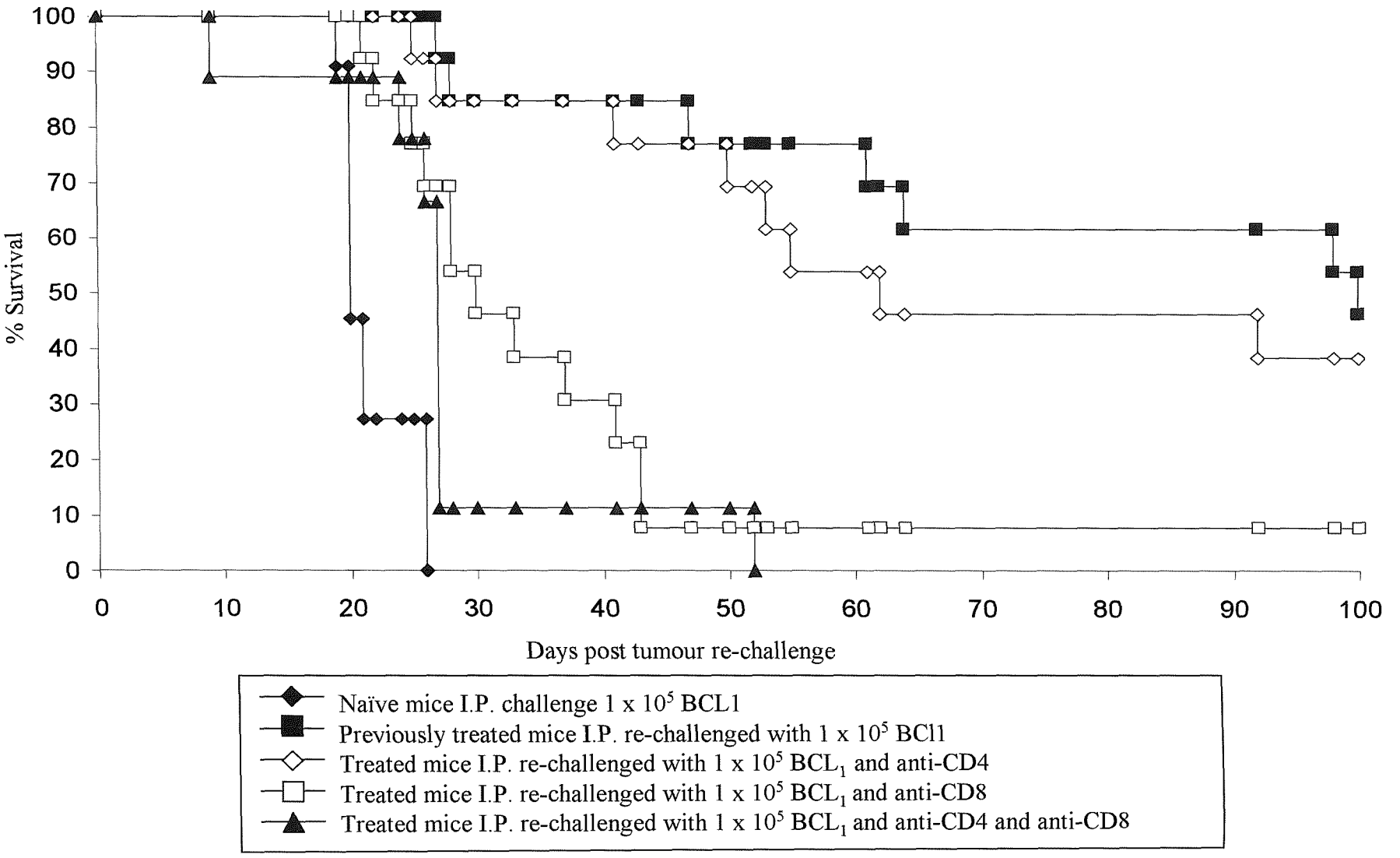


Figure 3.3.5) Contribution to tumour immunity upon re-challenge by CD4+ and CD8+ T cells. Mice were cured of BCL_1 with anti-CD40 (3/23) in the usual manner. Greater than 30 days later mice were re-challenged with 1 x 10^5 BCL_1 I.P. In the groups where anti-CD4(YTA3.1.2) or anti-CD8(YTS169) 1mg and 0.5mg respectively were injected I.P. every 3 to 4 days throughout the experiment. BCL_1 was also injected in to control naïve Balb/C recipient mice.

CD4+ T cells exist in this system which could be employed in adoptive therapy strategies.

Mice which had previously been cured of BCL_1 by anti-CD40 treatment were exposed to BCL_1 again ($1x10^5$ tumour cells i.p), no less than 30 days after the initial therapy. CD4+ or CD8+ T cells or both were depleted throughout the therapy. Control groups consisted, firstly, of mice which were re-challenged but not given either anti-CD4 or anti-CD8 or both and secondly, a group of naïve BALB/c mice challenged with tumour for the first time. This experiment was carried out by Alison Tutt a number of times and results shown are pooled.

Figure 3.3.5 shows that naïve mice which were given $1x10^5$ BCL_1 i.p. did not survive more than 27 days, whereas over 40% of cured mice re-challenged with the same number of BCL_1 cells survived to more than 100 days. In the experimental groups where anti- CD8 was administered (either with or without anti-CD4) survival was significantly reduced almost back to the level of the naïve control. However, in the group where anti-CD4 alone was administered, 40% of mice were still alive at 100 days post challenge; this was not significantly different from the group where no T cells had been depleted. These results indicate that CD8+ T cells were still the effector cells in the rechallenge response. This data also suggests that CD4+ T cells were not required to clear tumour in the rechallenge response.

Naïve CD8+ T cells, effector CD8+ T cells and CD8+ T cells from cured mice were subsequently examined for their ability to survive, proliferate and generate tumour-specific, cytotoxic CTL lines when stimulated with irradiated πBCL_1. CD8+ T cells from cured mice were able to respond to πBCL_1 stimulation by proliferating and producing cytokines (generation of CTL lines is discussed in chapter 4) whilst maintaining >20% viability in culture. However, viability of both effector and naïve CD8+ T cells could not be maintained *in vitro* and thus these cells were not used to generate CTL lines (data not shown).

3.3.6 CD4+ T cell expansion in the primary response to BCL_1

We wished to look for evidence of an MHC class II restricted response to BCL_1. To establish evidence of this, the CD4+ T cell response *in vivo* was studied after administration of BCL_1 with and without anti-CD40 therapy.

The numbers of CD4+ T cells in the spleen of mice during typical anti-CD40 therapies were examined. From preliminary experiments (data not shown) the "peak" of the CD4+ T cell response was seen to be at day 2-3 post anti-CD40 treatment (D6-D7) and the following data shows results obtained at this time point.

The three experimental groups in the following experiments were: 1) mice injected with anti-CD40 (3/23 1 mg) only on D4, 2) mice challenged with $5x10^7$ BCL_1 on D0 and treated with anti-CD40 (3/23 1mg) on D4 and 3) mice challenged with BCL_1 on D0 and injected with PBS control on D4. Thus we were able to investigate the response of CD4+ T cells to tumour (BCL_1) alone, to BCL_1 during its treatment with anti-CD40 and the response to anti-CD40 alone.

Figure 3.3.6a shows Facs profiles comparing percentage lymphoma and T cells in typical spleens from BCL_1 immunised mice 1, 2, and 3 days after anti-CD40 treatment or PBS injection (untreated) (D5,6,and 7 of therapy).

Absolute numbers of CD4+ T cells in the spleen of two mice/day (Figure 3.3.6b) were calculated all experimental groups. In order to calculate absolute CD4 + T cell numbers, the percentage CD4+ T cells in the spleen (Figure 3.3.6a) and the total cell numbers of the same spleen (data not shown) were examined.

In the anti-CD40 only injected mice the total CD4+ T cell number in the spleen remained constant from D5-7 of therapy. In mice immunised with BCL_1 and left untreated total CD4+ T cell numbers increased between D5-6 of therapy, with 1 mouse showing a two-fold increase in splenic CD4+ T cell numbers between D4 and 5. By D6 in the untreated group numbers of CD4+ T cells had fallen but were significantly higher than the numbers found in the anti-CD40 control group. The BCL_1 treated mice showed increased splenic CD4+ T cell numbers between D5 and 7 compared to the other two experimental groups. In the treated mice CD4+ T cell numbers were seen to rise from 2.5-3 x 10^7 on day 1 to 4.5-5.5 x 10^7 on D7.

Figure 3.3.6) CD4+ T cell responses to BCL_1 and BCL_1 treated with anti-CD40.

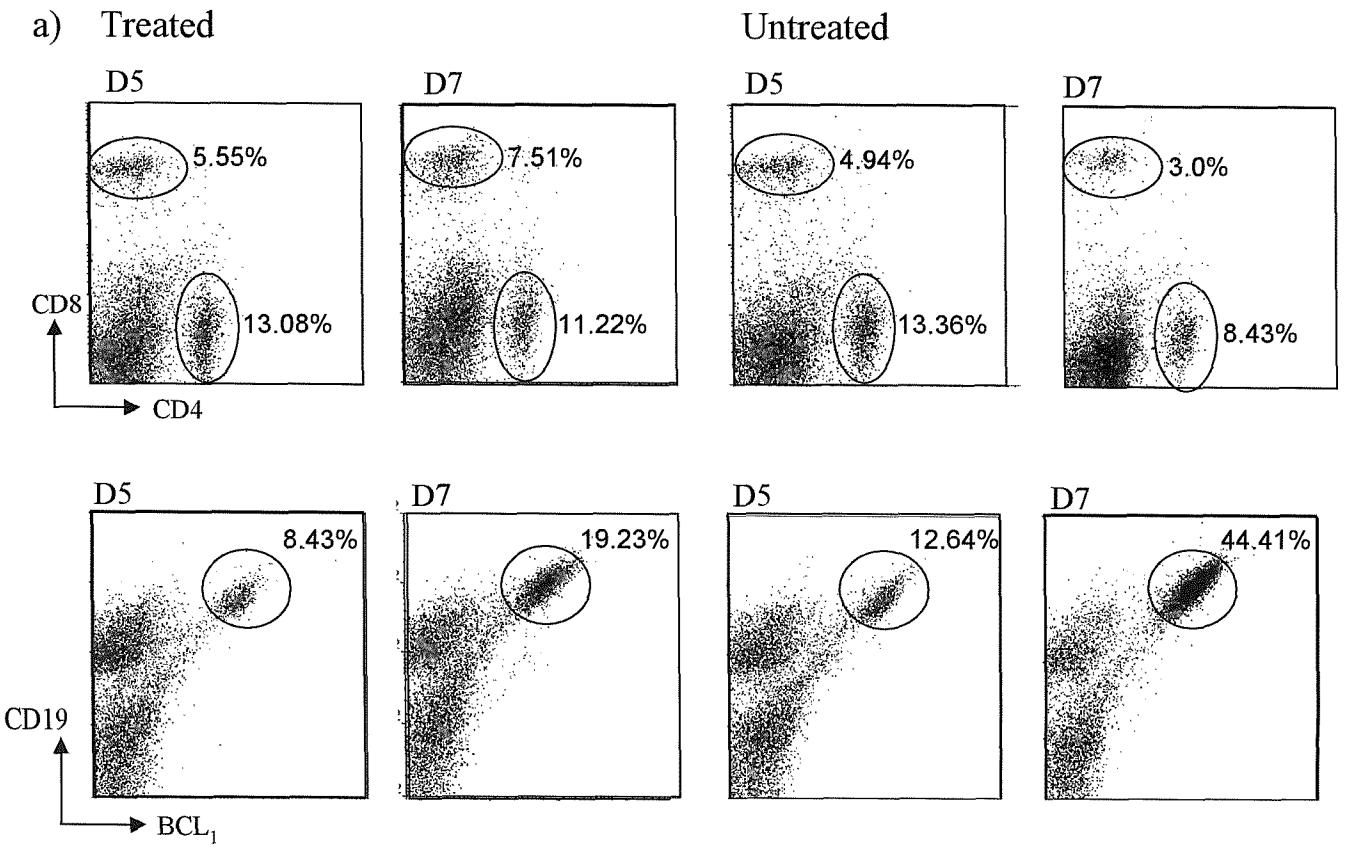


Figure 3.3.6a Flow cytometry was used to compare percentage lymphoma and T cells in typical spleens from BCL_1 immunised mice both anti-CD40 treated and untreated. Mice were culled on D5,6and 7. D5 and D7 are shown. Splenocytes were stained for CD19 and BCL_1 idiotype (bottom row) and CD8 and CD4 (top row) and percentage cell numbers are shown.

Figure 3.3.6) CD4+ T cell responses to BCL_1

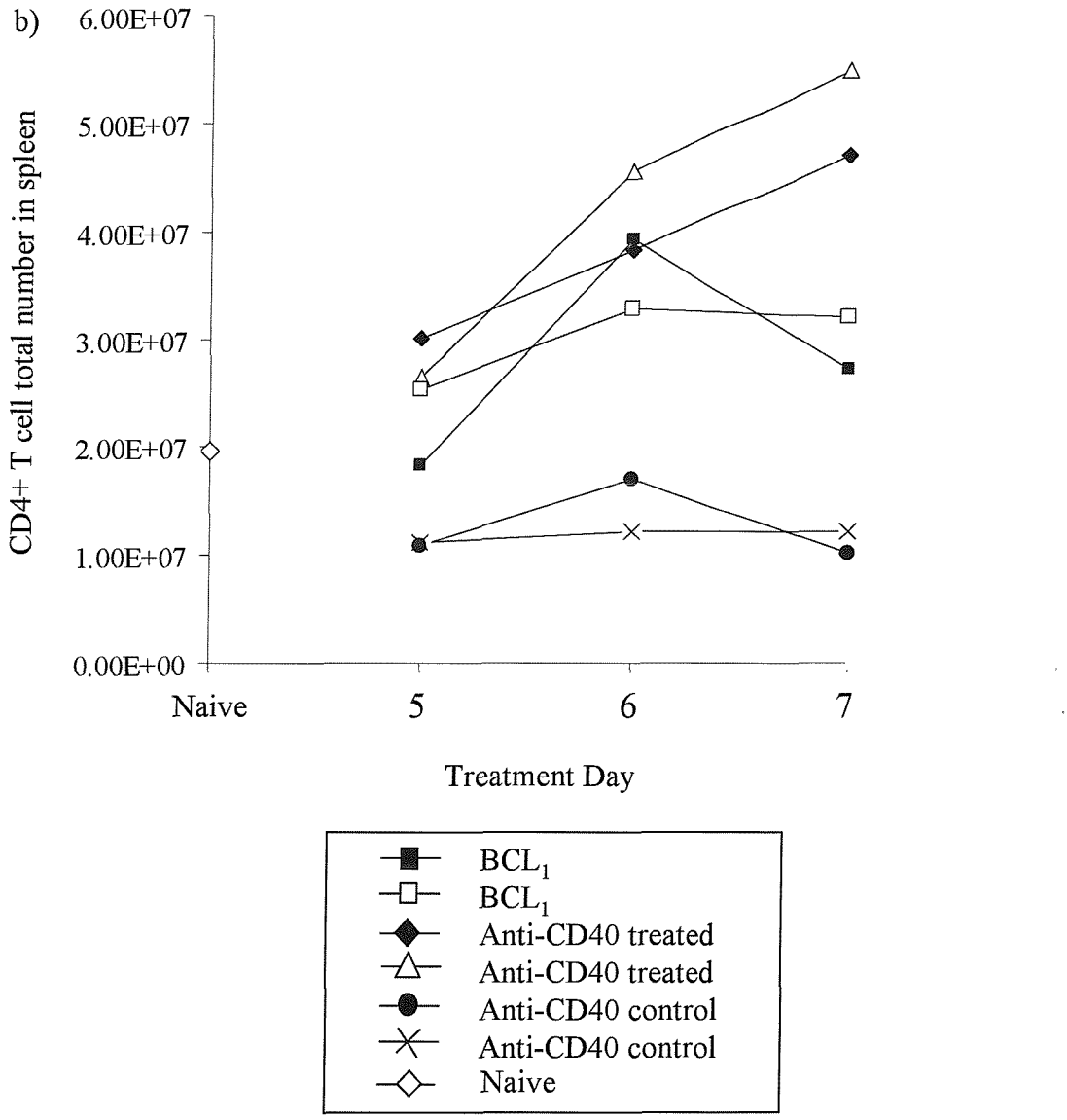


Figure 3.3.6b) Total number of CD4+ T cells in the duplicate spleens from all experimental groups calculated from the percentage CD4+ T cells (figure 3.3.6a) and the total cell numbers of the equivalent spleen.

On D7 the treated group had more splenic CD4+ T cells than splenic CD8+ T cells (CD8+ T cells not shown).

3.3.7 BrdU (5-Bromo-2-deoxyuridine) incorporation into CD4+ T cells during anti-CD40 treatment of BCL_1.

To investigate proliferation of CD4+ T cells, *in vivo* BrdU (5-Bromo-2-deoxyuridine) administration was carried on either D5 or D7 of therapy to compare the proliferative response of CD4+ T cells in the three experimental groups. BrdU is incorporated into the DNA of proliferating cells during S phase and can be detected by intracellular staining using an anti-BrdU mAb (PE conjugated). BrdU was administered on the day of culling and flow cytometry was used to visualise CD4+ T cells which had taken up BrdU. Figure 3.3.7a shows the flow cytometric analysis on D5 and D7 (controls for this experiment are shown in figure 3.3.7b and are of particular importance since the distinction between the CD4 and positive and negative groups needs defining as staining of CD4+ T cells in FL1 was not bright). Figure 3.3.7c is a positive control for this experiment showing BrdU incorporation into CD8+ T cells on D5 and D9 of a typical therapy.

Figure 3.3.7a shows that between D5 and D7 of anti-CD40 treatment there was an increase of BrdU+ CD4+ T cells in the spleen of both mice examined. One mouse showing an increase of 3% to 9% BrdU+CD4+ T cells over the two day period. This is shown in graphical from in figure 3.3.7d. One untreated mouse showed a decrease in splenic BrdU+CD4+ T cells, the other a slight increase between D5 and D7. In the anti-CD40 control group levels of BrdU+CD4+ T cells increased from 1% to only 2% between D5 and D7.

The effect of anti-CD40 treatment of BCL_1 between D5 and D7 of therapy was to increase proliferation of CD4+ T cells to a greater extent than seen without treatment or with anti-CD40 administration alone. CD4+ T cells never proliferated to the same extent as CD8+ T cells during therapy.

3.3.8 CD4+ T cell activation. Levels of 41BB, OX40 and LFA-1 on CD4+ splenocytes.

To investigate whether the CD4+ T cells were activated at the peak of their modest proliferative response (D7) we investigated the expression of activation

Figure 3.3.7) BrdU incorporation into CD4+ T cells following immunisation with BCL_1 with and without anti-CD40 treatment.

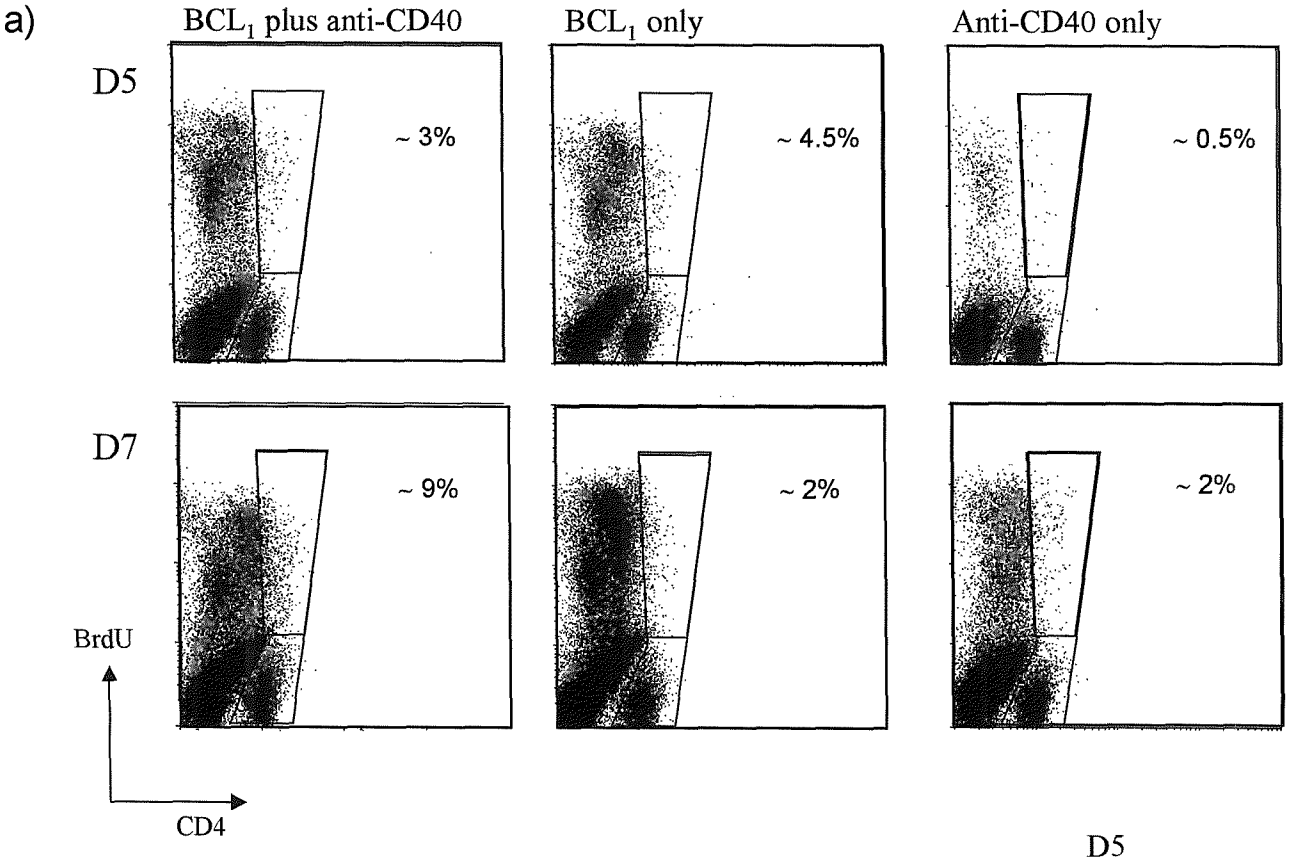


Figure 3.3.7) BrdU(5-Bromo-2-deoxyuridine) incorporation in to CD4+ T cells. Untreated, treated and control mice were administered with 2 mg BrdU 1 hour prior to harvesting of splenocytes. Flow cytometry was used to visualise proliferating cells which stained positive for BrdU using an anti-BrdU mAb conjugated to PE.CD4+ T cells were visualised on FL1 using a FITC labelled anti-CD4 mAb. Both CD4+ and CD4+BrdU+ cells are gated. Percentages shown are BrdU+CD4+ cells as a percentage of total CD4+ T cells. Day 1 and day 3 post treatment with anti-CD40 or control are shown.
Figure 3.3.7b) Control stain using either irrelevant control antibody in both FL1 and FL2 for purposes of correct gating.
Figure 3.3.7c) Positive control.BrdU incorporation in to CD8+ T cells flow cytometry performed on day 1 and day 5 post anti-CD40 treatment.

Figure 3.3.7) BrdU incorporation into CD4+ T cells following immunisation with BCL_1 with and without anti-CD40 treatment.

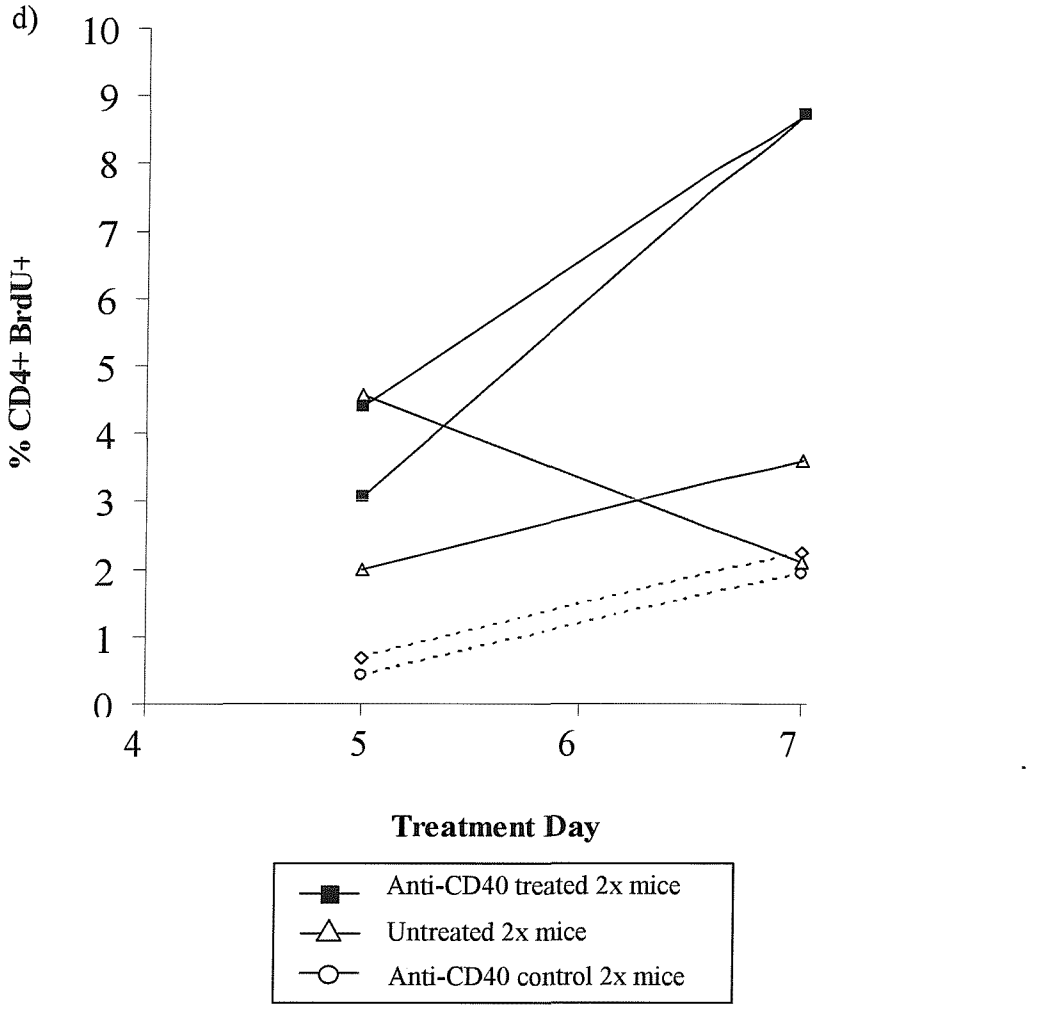


Figure 3.3.7d) BrdU(5-Bromo-2-deoxyuridine) incorporation in to CD4+ T cells. Untreated, treated and control mice were administered with 2 mg BrdU 1 hour prior to harvesting of splenocytes. Flow cytometry was used to visualise proliferating cells which stained positive for BrdU using an anti-BrdU mAb conjugated to PE.CD4+ T cells were visualised on FL1 using a FITC labelled anti-CD4 mAb.Here BrdU+CD4+ cells are shown as a percentage of total CD4+ T cells in duplicate mice for each experimental group.

and adhesion molecules on the surface of CD4+ splenocytes from mice from the three experimental groups during a typical therapy. Flow cytometry was carried out on splenocytes from three mice from each experimental group on D6,7,8,9 and 10 of therapy. Phenotyping was carried out using mAb against CD25, 4-1BB, OX40, LFA-1, ICAM-1, CD49d and CD62L. Figure 3.3.8 shows graphically the percentage of total CD4+ T cells which were CD4+41BB^{hi}, CD4+OX40^{hi} or CD4+LFA-1^{hi} cells over the five days as these were the markers which showed the most marked increase in expression. Data could not be collected for the untreated group on D10 as these mice succumbed to the lymphoma on D9.

In the anti-CD40 treated mice, levels of OX-40, 4-1BB and LFA-1 peaked at D7 of treatment. 30% of CD4+ T cells had and OX-40^{hi} phenotype, and over 40% were both 4-1BB^{hi} and LFA-1^{hi} on this day. However, neither the CD4+ T cells from untreated mice nor the anti-CD40 only control mice were as activated on D7 showing significantly lower levels of OX-40, 4-1BB and LFA-1 expression than CD4+ T cells from the treated mice on this day. CD4+ T cells from untreated mice became activated upon D9, whereas those from anti-CD40 treated mice failed to become significantly activated over the five day period.

3.3.9 Evidence of a class II restricted antigen-use of anti πBCL1 T cell hybrids

πBCL_1 specific hybrids were generated by fusion of splenocytes from mice cured of BCL_1 by anti-CD40 treatment and BWZCD8α fusion partners. Each fusion clone generated could potentially recognise a different tumour epitope (Graham Crowther personal communication). Originally this method was used to create CD8+ hybrids however, upon measurement of hybrid activation (described in materials and methods) in response to πBCL1 a class II restriction was observed in one of the hybrid clones. Figure 3.3.9 shows the response of this hybrid clone to increasing numbers of πBCL1. The response was measured by production of β-galactosidase, increased β-galactosidase production (measured at 570nm) indicating increased response to πBCL_1. Here mAbs have been used at a concentration of 50μg/ml to block the interaction between MHC on the πBCL_1 and the TCR on the hybrid clone. mAbs against a panel of MHC class I (Kd, Ld, Dd), CD8 and class II were used as well as an irrelevant control. Figure 3.3.9 shows that the only antibody capable of

Figure 3.3.8 Levels of 41BB, OX40 and LFA-1 on CD4+ splenocytes

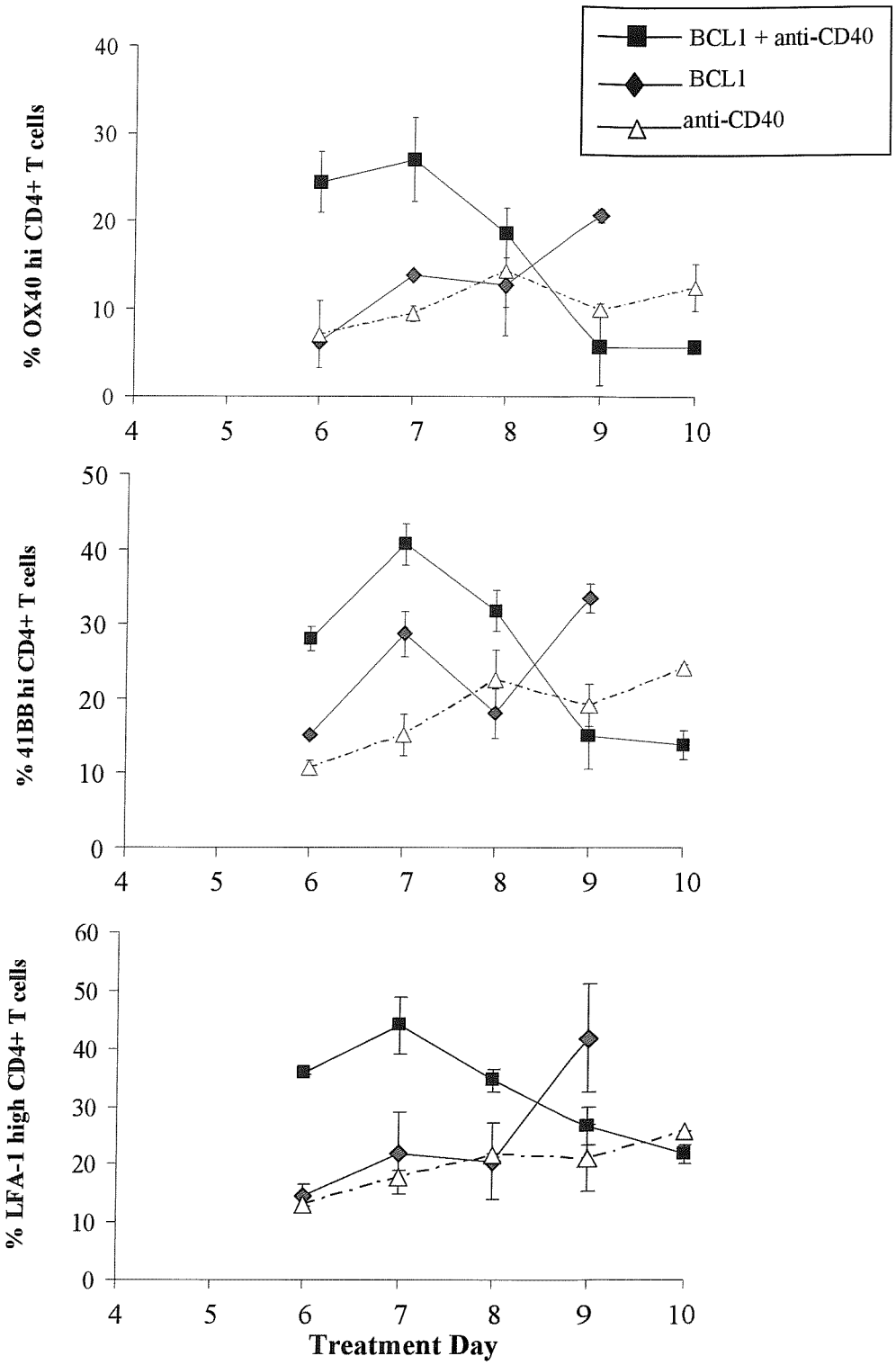


Figure 3.3.8) Levels of 41BB, OX40 and LFA-1 on CD4+ splenocytes from mice immunised with 5 x 10^7 BCL_1 on D0 and treated with anti-CD40 on D4. Control groups were left untreated or were injected with anti-CD40 only. Splenocytes were harvested D6-D10 and CD4+ T cell activation was investigated using flow cytometry. Double staining was carried out using PE conjugated anti-CD4 mAb and FITC conjugated mAbs against activation markers of interest

Figure 3.3.9) Evidence of a class II restricted antigen-use of anti πBCL_1 T cell hybrids

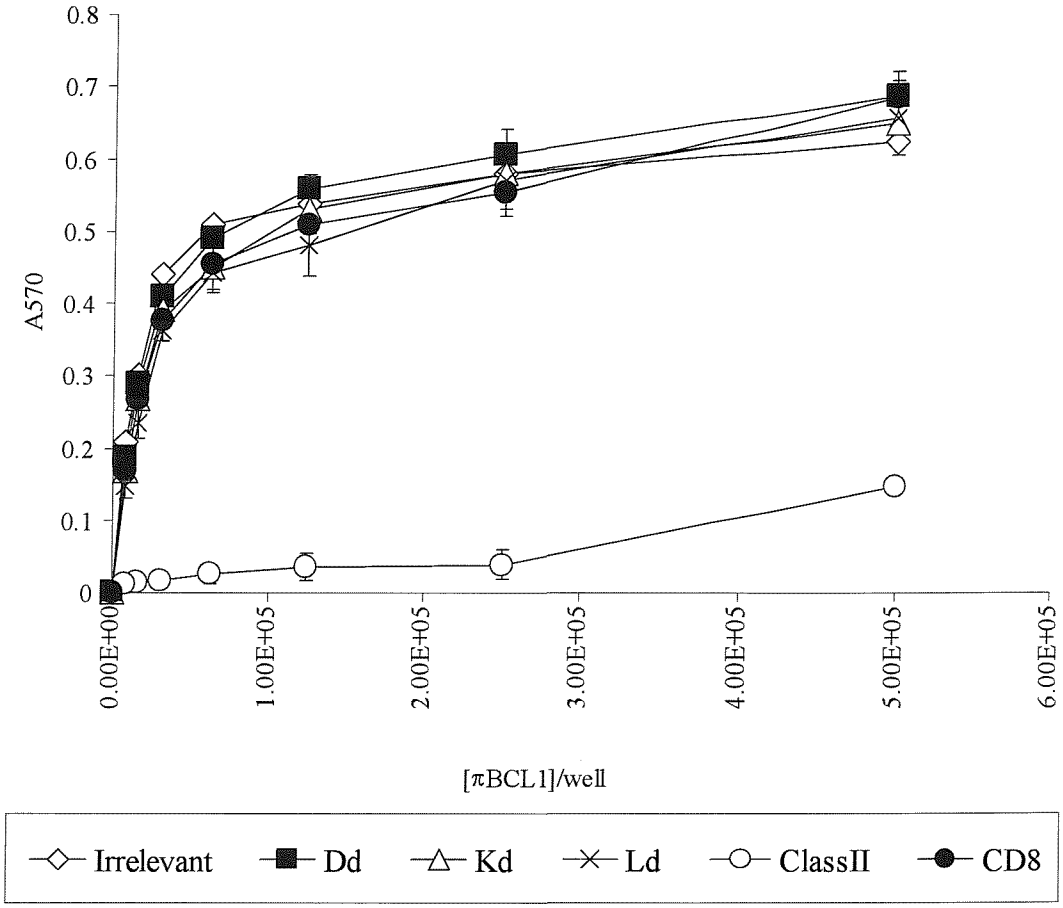


Figure 3.3.9) Evidence of a class II restricted antigen-use of anti πBCL1 T cell T-cell hybrids were stimulated overnight with πBCL_1 and relevant blocking antibodies,washed once in PBS, and fixed. After washing X-Gal substrate was added and the reaction was allowed to develop overnight and the cells examined microscopically the next day. Absorbance was read at 570nm.

blocking the interaction between πBCL_1 and this hybrid clone was the anti-class II mAb. All anti-class I, anti-CD8 and the irrelevant control mAb were unable to block the interaction between πBCL_1 and the hybrid clone shown by an increase in the production of β-galactosidase to a maximum at the highest concentration of πBCL_1.

3.4 Discussion

In order to create the large population of CTL required for effective eradication of BCL_1 by adoptive transfer three pools of CD8+ T cells could potentially be used, naïve, effector and those taken from cured mice. The therapeutic activity of anti-CD40 mAb depends on the expansion of CD8+ T cells whose numbers decline, after tumour has been eradicated level which is higher than in naïve mice. Naïve CD8+ T cells, effector CD8+ T cells (harvested from spleen D8-D10) and those from cured mice (D20-D65) were assessed in this chapter for their potential to generate CTL lines for use in adoptive therapy.

Phenotyping of cells was carried out to confirm whether this could distinguish the CD8+ T cell types. The level of cell activation and adhesion molecules such as CD62L (L-selectin) and CD44 were used to phenotype the CD8+ T cells. CD62L is involved in lymphocyte extravasation allowing the homing of naïve lymphocytes to lymph nodes and Peyer's patches. Upon activation CD62L is down-regulated and re-circulation prevented. Thus an activated pool will show low levels of CD62L and the non-activated naïve cells show a $CD62L^{hi}$ phenotype. However, memory cells cannot always be distinguished from effector CD8+ T cells since a $CD62L^{low}$ phenotype may be retained after the period of activation is complete and in the BALB/c the pattern is not always as clear as in other strains(255). Likewise the naïve $CD44^{low}$ and memory $CD44^{hi}$ phenotypes are not always clear-cut. Lymphocytes, upon activation also acquire a $CD49d^{hi}$ phenotype. CD49d is involved in the rolling and tethering of lymphocytes against endothelium. Memory cells normally express low levels of CD25 (IL2-R α chain) and the activation marker 4-1BB. The effector population harvested from spleens at the peak of response was distinguishable from both the naïve CD8+ T cell pool and those taken from cured mice with a $CD62L^{low}$, $CD49d^{hi}$, phenotype and increased levels of CD44, CD25, 41BB, ICAM-1 and LFA-1. However, naïve and postulated memory T cell phenotypes were very similar and the existence of memory CD8+ cells could not be confirmed in this way. Similarly the

TCR Vβ usage was did not reliably distinguish between memory and naïve CD8+ T cells.

The assessment of both NK dependent and tumour specific cytotoxic activity using naïve, effector and CD8+ T cells from cured mice showed that *ex vivo*, tumour specific, cytotoxic activity was only seen in the peak cell group. All CD8+ T cell enriched pools showed some NK dependent killing indicative of the contamination in the CD8+ T cell enriched splenocytes by NK cells or in fact NKT cells.

Memory CD8+ T cells subsequently proved the only CD8+ T cell pool suitable for co-culture with irradiated tumour (as detailed in chapter 4). In the treatment of BCL_1 *in vivo* by anti-CD40 mAb 3/23, help provided through CD40 in the primary response and contact with antigen will cause naïve CD8+ T cells (with the relevant TCR recognising that antigen in the context of MHC) to proliferate and differentiate into effector cells. On removal of the tumour most effector CTL are eliminated to preserve the primary T cell repertoire. However immune responses usually culminate in a state of specific T cell memory. Thus the secondary response to the same antigen(s) if the animal is ever exposed again, should be more effective and vigorous than the primary response. In keeping with this, the pool of CD8+ T cells from mice cured of BCL_1 by anti-CD40 were the pool of CD8+ T cells able to survive and proliferate on antigen provided by irradiated πBCL_1.

The naïve CD8+ T cells did not survive on πBCL_1 and thus were not considered in this model to create CTL *in vitro*. Even if specific naïve cells responded to antigen on πBCL_1 a primary response requiring co-stimulation would have to ensue. In the *in vivo* therapy of BCL_1 by anti-CD40, the role of DC in the uptake of tumour antigen(s) and the presentation to naïve CD8+ T cells has yet to be fully elucidated, thus it is unclear whether DC would have to be utilised in an *in vitro* culture to generate CTL from naïve pre-cursors. There would however be fewer antigen specific cells in the naïve pool so their use for adoptive therapy was ruled out.

The peak effector CD8 T cells, used in these experiments, die in the *in vivo* situation once tumour is cleared (data not shown) and thus are assumed to have succumbed to activation induced cell death (AICD). *In vitro*, AICD also seemed to occur as we were unable to establish long term cultures using these cells.

We also sought to determine whether there was a role for CD4+ T cells in the BCL_1 model of lymphoma.

There is increasing evidence that, even if CD8+CTL are the main effector arm for the clearance of tumour as they are in both the primary and secondary response in the BCL_1 model of lymphoma, in cases where tumour specific CD8+ T cell precursor frequency is low or where in adoptive therapy strategies CTL numbers are limiting then there may be a role for tumour specific CD4+ T cells in boosting the response against tumour (233), (234), (226). It was therefore decided to further investigate whether in the BCL_1 model of lymphoma there was any evidence of a role for CD4+ T cells, or class II restriction for the purpose of utilising CD4+ T cells in adoptive therapy regimes where it is difficult to create enough CTLs and where CD4+ T cells may play a therapeutic role.

To investigate whether there was evidence of CD4+ T cells responding to tumour in the primary response CD4+ T cell numbers were investigated. Previously CD8+ numbers had been investigated (109) and these were used as a control to ensure that the therapies investigated were in keeping with previous published data.

Both experimental groups which received BCL_1 (treated and untreated) showed an increase in splenic CD4+ T cell numbers reaching a peak at D7. This response was augmented by the addition of anti-CD40 in the treated group. The CD4 response to anti-CD40 alone was not equivalent to that of either the treated or untreated groups. This indicates that the expansion of CD4+ T cells was not antigen independent suggesting an MHC class II restricted response to tumour was occurring in the groups which had received tumour.

This data was further supported by the work with BrdU. This showed that BCL_1 immunised mice which had been treated with anti-CD40 had higher levels of proliferating CD4+ T cells than the other two experimental groups.

Figure 3.3.7 shows that in typical therapies between D5 and D7 post splenic CD4+ T cells from treated mice increased expression of OX40, 41BB and LFA-1 on their cell surface compared to controls. Figure 3.3.7 also shows that expression of OX40,41BB and LFA-1 on the splenic CD4+ T cells from untreated mice increases later than on the splenic CD4+ T cells of treated mice.

Evidence of at least one MHC class II restricted antigen epitope in the BCL_1 model is inferred by data shown in Figure 3.3.9. Here, splenocytes taken after D20 of therapy were fused with BWZCD8α fusion partners, which allowed the cloning of hybrids

which were grown on πBCL_1. Any πBCL_1 specific clones were tested for MHC class I and MHC class II restriction. Activation of the clones could be determined by production of β-galactosidase which could be measured optically at 570nm. Thus in blocking experiments using anti MHC class I antibodies (Dd, Kd,Ld) and an anti-MHC class II antibody (which is specific for the constant region of MHC class II) MHC class II restricted clones were discovered. Figure 3.3.9 shows the blocking experiment carried out on one such clone. This data shows that CD4+ T cell hybrids can be generated which are specific for πBCL_1. Thus at least one MHC class II restricted tumour antigen epitope must exist. Different CD4+ T cell clones could recognise the same epitope in the context of class II (perhaps a dominant epitope) or different epitopes. This would have to be established in further work. In future work it would also be of use to establish whether CD4+ T cell hybrids were also specific for BCL_1 as well as πBCL_1.

^{51}-Chromium release assays carried out using CD4+ T cells isolated from the spleens of anti-CD40 treated mice on D5-9 failed to detect any cytotoxic activity against πBCL1 by these CD4+ T cells directly *ex vivo* (data not shown). There is debate as to whether *in vivo* CD4+ T cells are able to directly kill tumour, an issue which will be addressed further in chapter 6.

The work carried out in this chapter has determined that memory CD8+ T cells appear the most suitable for the generation of CTL lines *in vitro* (chapter 4). Despite a lack of a requirement for CD4+ T cells in the primary and secondary response, there is some evidence of a class II restricted response to tumour in this model. Therefore, it was decided that CD4+ T cell lines would be generated in the same way as their CD8+ T cell counterparts, characterised (Chapter 5) and investigated along with any CTL lines generated in adoptive therapy studies (Chapter 7).

Chapter 4 Generation of CTL *in vitro* from memory CD8+ T cells.

4.1 Introduction

In the previous chapter, the three available pools of CD8+ T cells were investigated for their suitability in generating CTL for use in adoptive therapy. CD8+ T cells taken from the spleen of anti-CD40 cured mice were established as the most suitable T cell pool. In the BCL_1 model of lymphoma, TAA have not been identified. Therefore, in order to generate CTL lines for use in adoptive therapy we decided to use irradiated πBCL_1 (the *in vitro* line of BCL_1) as an APC in a co-culture system with CD8+ T cells from cured mice. This strategy requires the efficient presentation of TAA and expression of co-stimulatory molecules such as B7-1 and B7-2 and adhesion molecules on the B cell lymphoma. The levels of co-stimulatory and adhesion molecules on πBCL_1 were assessed by flow cytometry. πBCL_1 was consistently B7-1^{hi}B7-2^{hi}CD40^{hi}ICAM-1^{hi}LFA-1^{hi} and although we could not directly investigate the efficient presentation of any TAA, we did assess that expression of both MHC class I and class II were also high.

Chapter 4 aims to assess the conditions by which CTL can best be generated *in vitro* using the πBCL_1 co-culture sustem and further characterise these cells.

4.2 Materials and Methods

4.2.1 CD8+ T cell culture

Immune mice were sacrificed and spleens collected on D20-65 of therapy. A single cell suspension of splenocytes was obtained by passing the material through a cell strainer (Becton Dickinson (BD) Falcon) in 10% RPMI (as detailed in materials and methods Chapter 2). CD8+ T cells were purified by positive selection on MACS beads under sterile conditions. T cells were counted (Coulter counter), centrifuged (350g for 5 minutes) and re-suspended in T cell medium. For routine culture, T cells were diluted at 2-4 x 10^6/ml in T cell medium along with irradiated (50Gy) πBCL_1 at a ratio of 2:1 or 4:1 T cells : πBCL_1. For routine culture 24 well plates were used . For 96 well assays final cell number was 1-2 x 10^5/well T cells: 5 x 10^4/well πBCL_1. For routine cultures anti-CD4 (YTA3.1.2) was added at a final concentration of 10μg/ml and IL7 (Peprotech EC Ltd) was added to a final concentration of 10ng/ml.

Control wells were set up as described but with out irradiated πBCL_1 added. Cultures were incubated typically for 7 days before re-stimulation at 37°C, 5% CO_2. If confluent, cells were counted and re-suspended to 2-4x10^6 and stimulated with.πBCL_1 as previously described. In the second week of routine culture IL-2 (from X63-IL-2 hybridoma described in materials and methods) was added to all wells at a final concentration of 10 units/ml. Anti-CD4 (YTA3.1.2) was added upon weekly re-stimulation as previously described.

4.2.2 IFNγ, IL-5,TNFα and IL-10 ELISA

Standard ELISAs were carried out as stated in materials and methods chapter 2.oating and detection antibodies are listed in table 4.1 below.

Table 4.1 IFNγ, IL-5,TNFα and IL-10 ELISA reagents

ELISA	Coating antibody	Detection antibody
IFNγ	rat anti mouse IFNγ (HB170 from ATCC)	anti-IFNγ-biotin (XMG1.2 from Serotec)
IL-5	rat anti mouse IL-5 (TRFK-5 from Biosource Int.)	anti-IL-5 – biotin (TRFK-4 from Biosource Int.)
TNFα	rat anti mouse TNFα (MP6-XT3 from Biosource Int.)	anti-TNFα - biotin (Biosource)
IL-10	rat anti mouse IL-10 (Part 840125 from R&D DuoSet)	anti IL-10 - biotin (Part 840126 from R&D DuoSet)

4.2.3 Intracellular Staining for IFNγ

Intracellular staining was carred out as stated in materials and methods section 2.5.1. CTL lines were incubated with irradiated πBCL_1 at a 2:1 ratio CTL:lymphoma 48 hours proir to intracellular IFNγ staining being carried out.

4.2.4 Reverse Transcription PCR (RT-PCR)

To determine the CTL TCR usage (of both α and β genes) PCR was used. The basic protocol is detailed in materials and methods.

1x10^6 – 1x10^7 cells were harvested and re-suspended to a volume of 10μl in RNAse free water in a sterile DNAse. /RNAse free 1.5ml eppendorf tube. mRNA was

isolated from the cells (in accordance with manufacturers instructions: Amersham Pharmacia Biotech, mRNA purification kit) and of the total volume of 20µl, 8µl of RNA was then reverse transcribed and converted to cDNA using the Pharmacia single strand cDNA synthesis system using a single master mix for all samples. Equal concentrations were then used for PCR (detailed in materials and methods) and assessed on a 2% agarose gel. For each sample β actin or GAPDH PCR controls were run to assess the variability in quantity and quality of mRNA in the samples. Primers used for the investigation of Vα and Vβ TCR usage are detailed in Table 4.2.

Table 4.2 RT-PCR for Vα and Vβ usage, sequences of primers for TCR Vα and Vβ genes

α genes

Primer	Sequence (5'-3')
Vα1	GCA CTG ATG TCC ATC TTC TC
Vα2	AAA GGG AGA AAA AGC TCT CC
Vα3	AAG TAC TAT TCC GGA GAC CC
Vα4	CAG TAT CCC GGA GAA GGT C
Vα4.4	CAA TAT CCT GGA GAA GGT C
Vα5	CAA GAA AGA CAA ACG ACT CTC
Vα6	CAA CCA GAT TCA ATG GAA AG
Vα7	TCT GTA GTC TTC CAG AAA TC
Vα8	CAA CAA GAG GAC CGA GCA CC
Vα9/10	CAA TCC TTC TGG GAC AAA GCA TAG T
Vα11	CCC TGC ACA TCA GGG ATG CC
Vα12	TCT GTT TAT CTC TGC TGA CC
Vα13	ACC TGG GAA TCC TAA GC
Vα14	GAC AAA ACG TCA AAT GGG
Vα15	AAG CTG GAA AGG GTC TCC AC
Vα16	GGG AAT TCT CCT TAC ATA TAA CAG CTG CA
Vα17	GGG AAT TCT TCC ATC GGA CTC ATC ATC AC
Vα18	GGG AAT TCC GCA ATG GAA GAC TCA GAG C
Vα19	GGG AAT TCA GCC GCT CGA ATG GGT ACA G
Cα	CAT GTC GAC CAC AGT TTT GTC AGT

β genes

Primer	Sequence (5'-3')
Vβ1	AGT CGT TTT ATA CCT GAA TGC
Vβ2	CCG GTG CTG ATT ACC TGG CCA
Vβ3	ATG ACT GAA AAA CGA TTC TCT
Vβ4	AGT CGC TTC CAA CCT CAA AGT
Vβ5	CCA GCA GAT TCT CAG TCC AAC
Vβ6	CTC TCA CTG TGA CAT CTG CCC
Vβ7	ACA TCC CTA AAG GAT ACA GGG
Vβ8	ATA TCC CTG ATG GGT ACA AGG
Vβ9	ACT TTT GAG AAG TTC CAA TCC
Vβ10	AAG GCG CTT CTC ACC TCA GTC
Vβ11	GGA ACG ATT CTC AGC TCA GAT
Vβ12	TCA AAG CTG AGA TGC TAA ATT
Vβ13	TTT TCT GCT GTG AGG CCT AAA
Vβ14	GTG CAA CTG AAC CTC TCA GTT
Vβ15	ACC CAG GAA AAA TTT CCC ATC
Vβ16	AAG GAT CAG TTT TCA GTT GAA
Vβ17	TGG TCA AGA AGA GAT TCT CAG
Cβ	GTC TGC TCG GCC CCA GGC CTC

4.3 Results

Chapter 4 concentrates on the characterisation of three *in vitro* generated CTL lines, however many lines were set up. Lines CD8-π-1, CD8-π-2 and CD8-π-3 will be detailed here to show the characteristics of the *in vitro* generated CTL typical of many other lines.

Having established that CD8+ T cells from cured mice (as defined in Chapter 3) were the most viable candidates for the generation of CTL *in vitro*, optimal conditions for their generation were sought. Memory CD8+ T cells were first purified from the spleen of immunised mice (D20-D65) and their requirement for IL-2, IL-7 and antigen to induce viable, active CTL was addressed. Figures 4.3.1a-c show investigation into the generation of CTL lines before any long-term CTL cultures were set up.

4.3.1 Optimising the culture conditions for generating CTL from memory T lymphocytes.

Levels of IFNγ produced by the T cells 72 hours post stimulation with irradiated πBCL_1 was determined to investigate whether the *in vitro* generated cells were activated within the first and second week of co-culture (Figure 4.2.1a). To assess reproducibility, two sets of CD8+ T cells from separate cured mice were immunised at different times were investigated (Data shows results from one such experiment). The conditions for this experiment were as follows: MACS purified CD8+ T cells were stimulated in the presence or absence of irradiated πBCL_1 (at a ratio of 2:1 T cells:lymphoma) for two weeks. IL-2 or IL-7 or both were added to cultures on week one or week two as indicated in Figure 4.3.1 a-c. The supernatant taken from the cultures 72 hours post stimulation during the first and second week of culture was assayed for IFNγ.

In the first week of *in vitro* culture, supernatants contained <1ng/ml IFNγ for all conditions. CD8+ T cells incubated without πBCL_1 secreted IFNγ only in the presence of IL-2 and minimal IFNγ secretion was seen in the supernatants of CD8+ T cells incubated with no cytokines or with IL7 alone. During the second week of *in vitro* co-culture, CD8+ T cells incubated in the presence of πBCL_1 were producing significantly more IFNγ than those incubated with cytokine alone. IL-2, IL-7, or both were needed to augment the IFNγ response to substantial levels (i.e.> 2 ng/ml).

Figure 4.3.1a IFNγ produced over 2 weeks by CD8+ T cells cultured *in vitro* under varying conditions

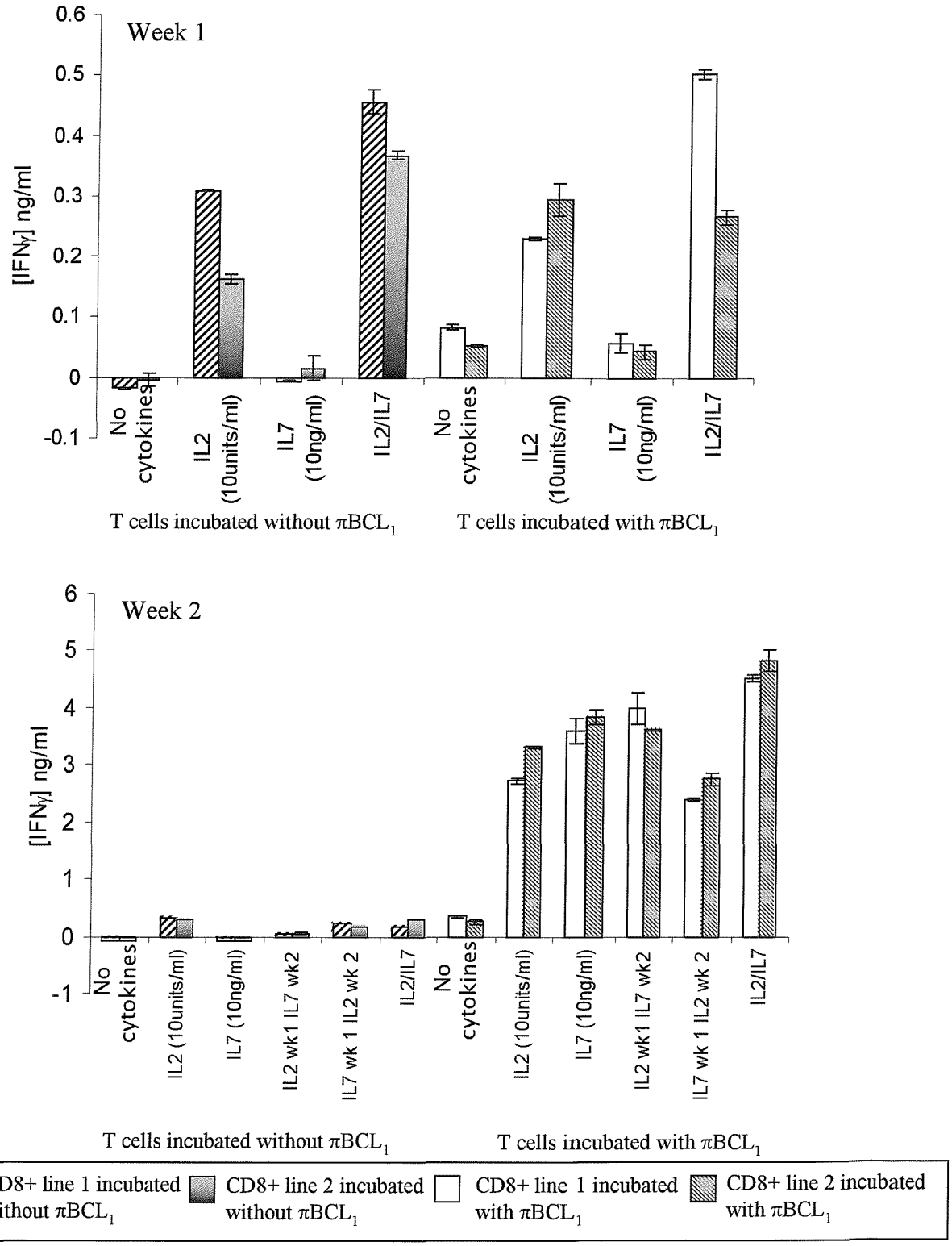


Figure 4.3.1a) IFNγ production by CD8+ T cells after during the first and second week *in vitro* culture under varying conditions.CD8+ T cells were purified by positive MACS selection and re-stimulated weekly with or without πBCL_1 (at a T: lymphoma ratio of 2:1)and under differing cytokine environments. IFNγ was assessed in the supernatants by standard ELISA 72 hours post stimulation.

Figure 4.3.1b Cytotoxic activity of CD8+ T cells assessed after 2 weeks *in vitro* culture with or without πBCL_1 in the presence of varying cytokines

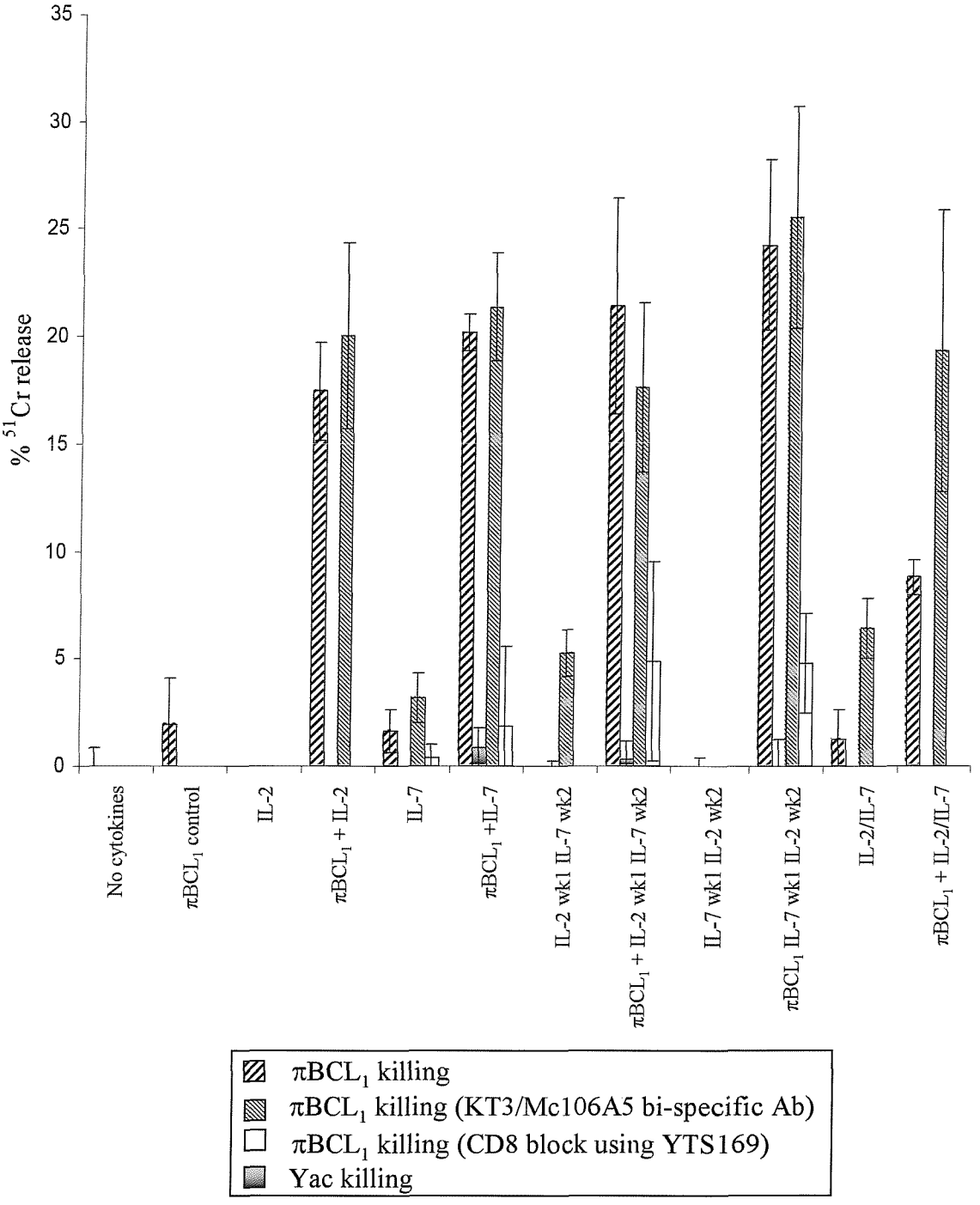


Figure 4.3.1b) Cytotoxic activity of memory CD8+ *in vitro* generated CTL as assessed by standard ^{51}Cr release assay after 2weeks *in vitro* culture with weekly re-stimulation. CD8+ T cells were positively selected using MACS and then incubated with and without πBCL_1 in varying cytokine environments.

Figure 4.3.1c) Viability of CD8+ T cells cultured with and without πBCL_1 and in varying cytokine environments

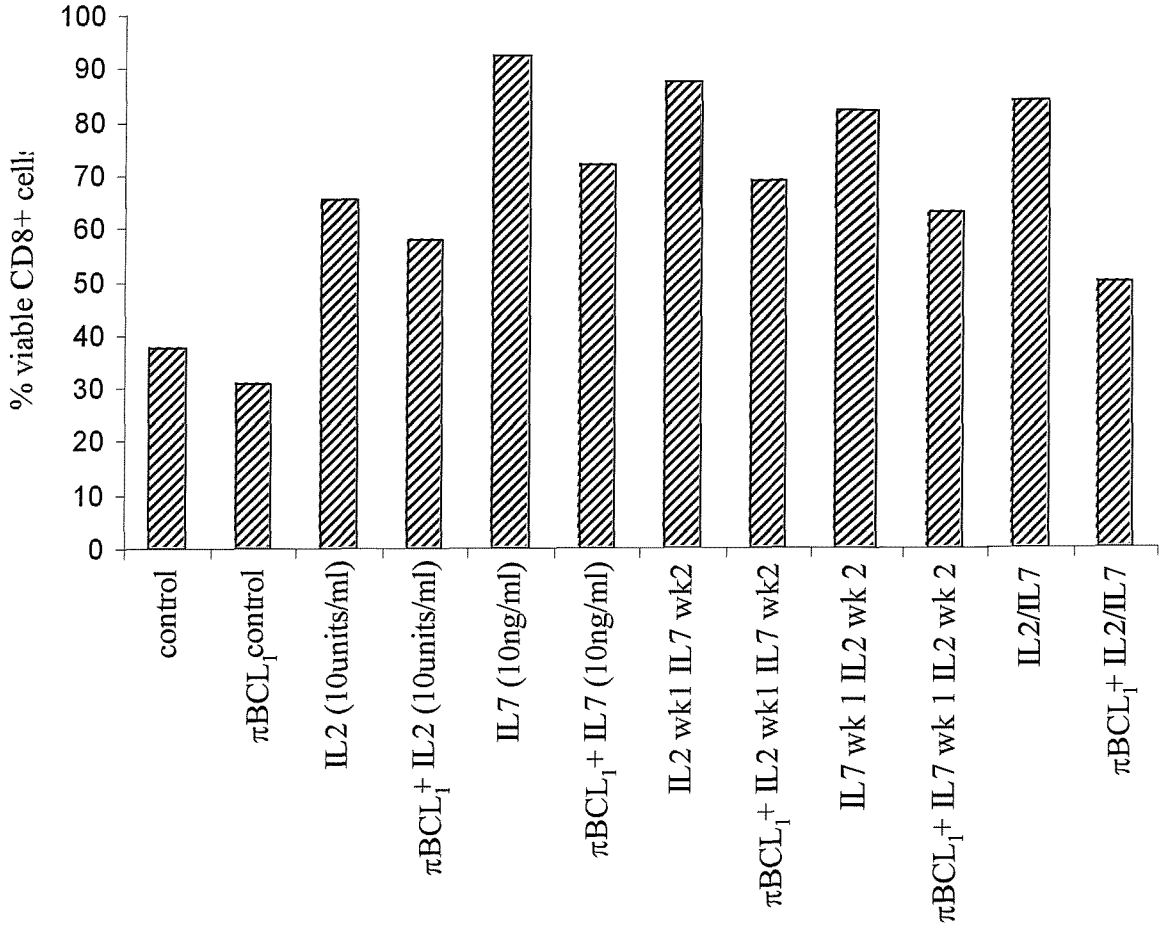


Figure 4.3.1c) Viability of *in vitro* generated CTL from memory CD8 T cells after two weeks in culture with weekly restimulation with and without πBCL_1 in differing cytokine enviroments. Viability was assessed by PI incorporation. Irradiated πBCL_1 positive cells which would increase the number of non-viable cells seen on the FACS were visualised by immunofluorescence using Mc106A5-FITC conjugate and gated out from viability calculations (anti-BCL_1 idiotype-FITC) and thus % viable cells shown on the graph are CD8+ live cells.

As a demonstration of their potential CTL activity we performed a standard ^{51}Cr assay using CD8+ T cells stimulated in the same conditions as detailed previously at a 10:1 E:T ratio (Figure 4.3.1b). πBCL_1 and Yac targets were used to assess tumour specific and NK dependent killing respectively. CD8 tumour specific killing was assessed by blocking CD8 using YTS169 (anti-CD8 mAb) and a bi-specific antibody directed to CD3 and the BCL_1 idiotype (KT3/Mc106A5) was used as a positive control of πBCL_1 killing (which works bringing the effector and target cells together). When cytokines were added in the absence of tumour cells together in various timings and combinations, effective CTL against πBCL_1 were not generated. Both πBCL_1.and either IL-2, IL-7 or both were needed for effective πBCL_1 killing which was shown to be class I restricted by blockade with anti-CD8. Killing under these conditions was as effective as when KT3/Mc106A5 was used.

Viability studies carried out on the CD8+ T cell cultures (Figure 4.3.1c) shows that cells cultured with IL-7 alone or in conjunction with IL-2 were more viable than those cultured with IL-2 alone after two weeks. The addition of either cytokine, however, showed greatly increased viability compared to cells which were incubated in the absence of any cytokine. When co-cultured with πBCL_1, CD8+ T cells were not as viable after two weeks as those with the corresponding cytokine treatment, in the absence of πBCL_1. For example, CD8+ T cells stimulated with IL-7 alone were 90% viable compared to IL-7 plus πBCL_1, which showed ~ 70% viability.

On the basis of these 3 analyses, (viability, ^{51}Cr release and IFNγ production) all cells were subsequently cultured with IL-7 @ 10ng/ml for the first week and IL-2 @ 20 units/ml from week two onwards. CTL were stimulated with πBCL_1 a ratio of 1:2 – 1:4, πBCL_1 : T cell. Using these conditions we wished to further characterise the CTL produced. The following experiments will focus on three *in vitro* generated lines CD8-π-1 and CD8-π-2 which were subsequently investigated in adoptive therapy studies (chapter 7) and CD8-π-3.

4.3.2 Investigation of the proliferative response of two *in vitro* generated CTL lines, CD8-π-1 and CD8-π-2.

In order for adoptive therapy using CTL generated *in vitro,* CD8+ T cells must be able to expand *in vitro.*

The proliferative response of CD8-π-1 and CD8-π-2 was investigated. Following CFSE labelling CD8-π-1 and CD8-π-2 were cultured in the presence of 20 units/ml IL-2 for 6 days with irradiated πBCL_1 or normal B cells as a control for non-specific CTL expansion. The proliferative response was investigated by observation of progressive dilution of CFSE intensity in FL-1 by flow cytometry. Specific CD8+ T cell proliferation was studied by co-staining with anti-CD8-PE conjugate (YTS169-PE) and gating on these cells such that only CD8+ T cells are shown (Figure 4.3.2).Over the 6 day period (days 3,4,5 and 6 shown) both CD8-π-1 and CD8-π-2 proliferated in the presence of irradiated πBCL_1 as shown by dilution of the CFSE stain. Neither line proliferated in the presence of irradiated B cells (Figure 4.3.2 shows CD8-π-2 stimulated with irradiated B cells, data not shown for CD8-π-1). CD8-π-1 showed the strongest proliferation in response to tumour as indicated by elevated numbers of cells with less CFSE stain particularly evident on days 3-6.

We wished to assess whether CD8 CTL lines showed specificity in their proliferative response and could proliferate on other irradiated mouse tumours which grow in BALB/c mice and which have the same MHC background (D). WEHI 231 and A20 which are both lymphoma and P815 which is a chemically induced mastocytoma were assessed. Figure 4.3.2b shows the proliferative response by CD8-π-2 on differing irradiated tumours 4 days post stimulation. CD8-π-2 cells incubated with irradiated WEHI 231, A20 or P815 did not proliferate as shown by the lack of CFSE dilution, CD8-π-2 proliferated in response to πBCL_1 shown by the dilution of CFSE seen here on day 4.

4.3.3 Investigation of the cytotoxic activity of *in vitro* generated CTL

The cytotoxic activity of CD8-π-1 was assessed, and the dose-dependency of the cytotoxic activity observed. CD8+ dependent cytotoxic activity of these cultures was investigated in standard ^{51}Cr release assays. Figure 4.3.3a shows the cytotoxic activity of CD8+ T cells (CD8-π-1) cultured ± πBCL_1 for 3 weeks against YAC and πBCL_1 targets. These data confirm that only CD8+ T cells cultured in the presence of irradiated lymphoma (πBCL_1) were capable of killing target lymphoma (as seen also in Figure 4.3.1b) and that CD8+ T cells cultured without πBCL_1 could not. CD8+ T cells cultured either with or without πBCL_1 did not kill YAC targets at a range of

Figure 4.3.2) πBCL_1 specific CTL show πBCL_1 dependent proliferation over 6 days *in vitro* stimulation.

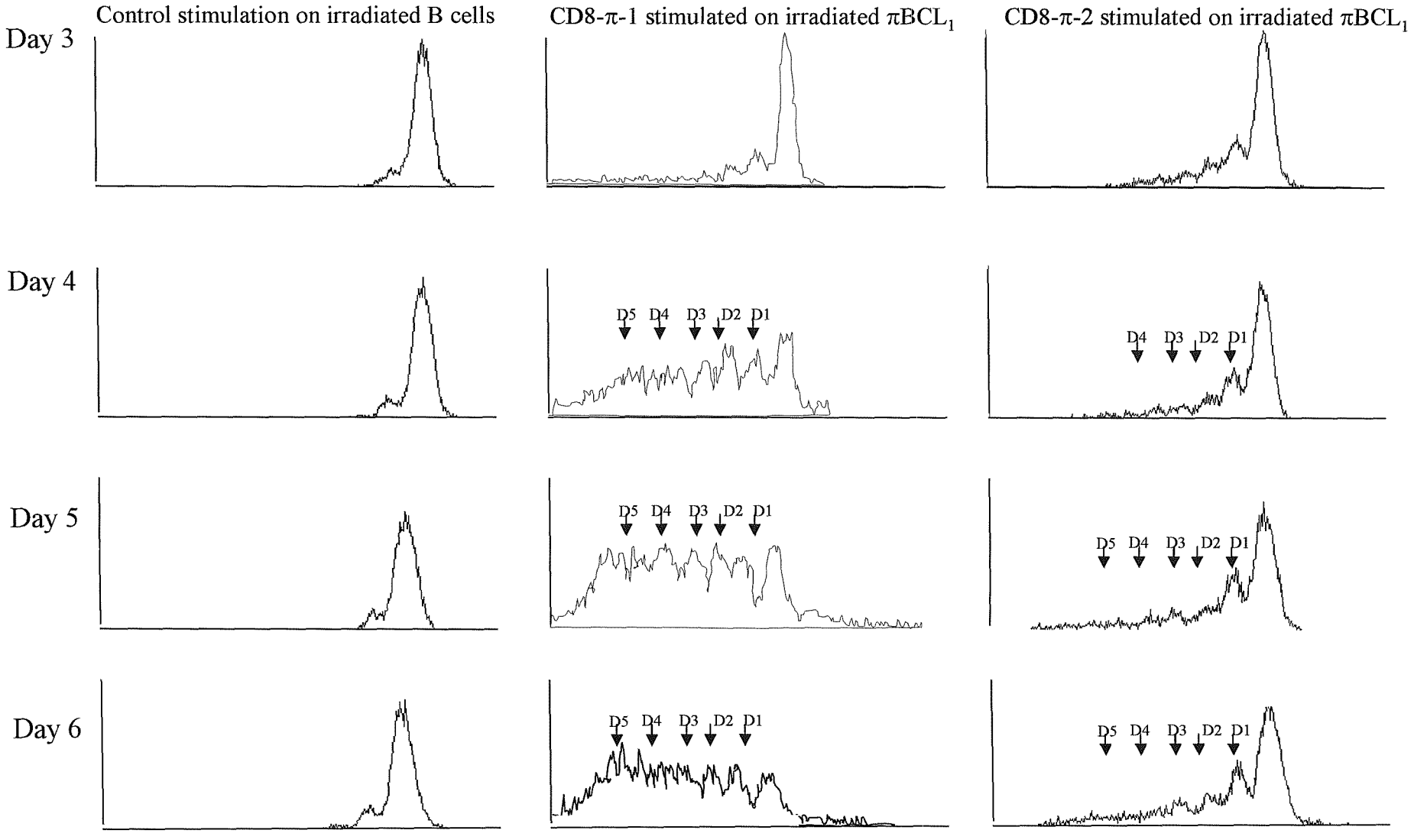


Figure 4.3.2) The proliferative response of in vitro generated CTL to cultured with πBCL_1 was assessed using CFSE labelling and incubation over 6 days with and with out irradiated πBCL_1.CTL were incubated in 96 well format at a density of 1x 10^5 cells/well πBCL_1 was used at 5 x 10^4 /well. Cells were analysed by flow cytometry day 1-6 to investigate the division of the cells (seen by progressive dilution of the CFSE staining). IL 2 was used at a final concentration of 20 units/ml, IL7 at a final concentration of 10 ng/ml.

Figure 4.3.2b) Proliferation of line CD8-π-2 on differing tumours day 4 post stimulation.

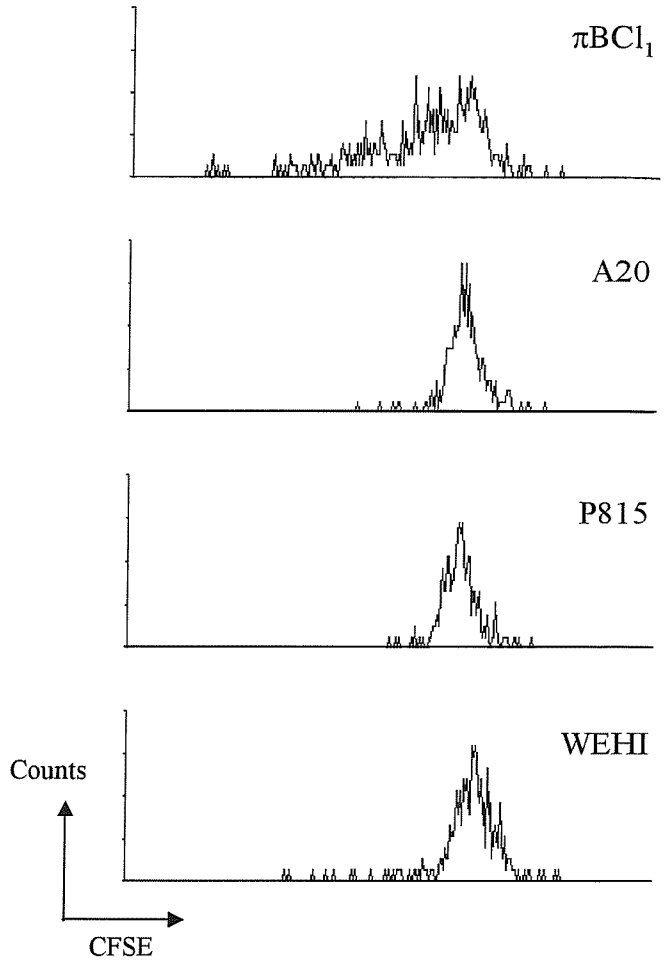


Figure 4.3. 2b) Proliferation of an established *in vitro* generated CTL (CD8-π-2) cultured with πBCL_1, WEHI, P815, A20 or B cells was assessed.CD8+ T cells cultured previously for 6 weeks under standard conditions were CFSE labelled and cultured with πBCL_1 WEHI, P815 or A20 in the presence of IL2 (20 units/ml) in 96 well format at a density of 1x 10^5 CD4+ T cells/well and5 x 10^4 tumour or B cells/well. Cells were analysed by flow cytometry day 1-6 to investigate the division of the cells (seen by progressive dilution of the CFSE staining D1-D6). Day 4 is shown.

Figure 4.3.3a) NK dependent and independent cytotoxic activity of *in vitro* T cells CD8-π-1 after 3 weeks of culture with or without πBCL_1

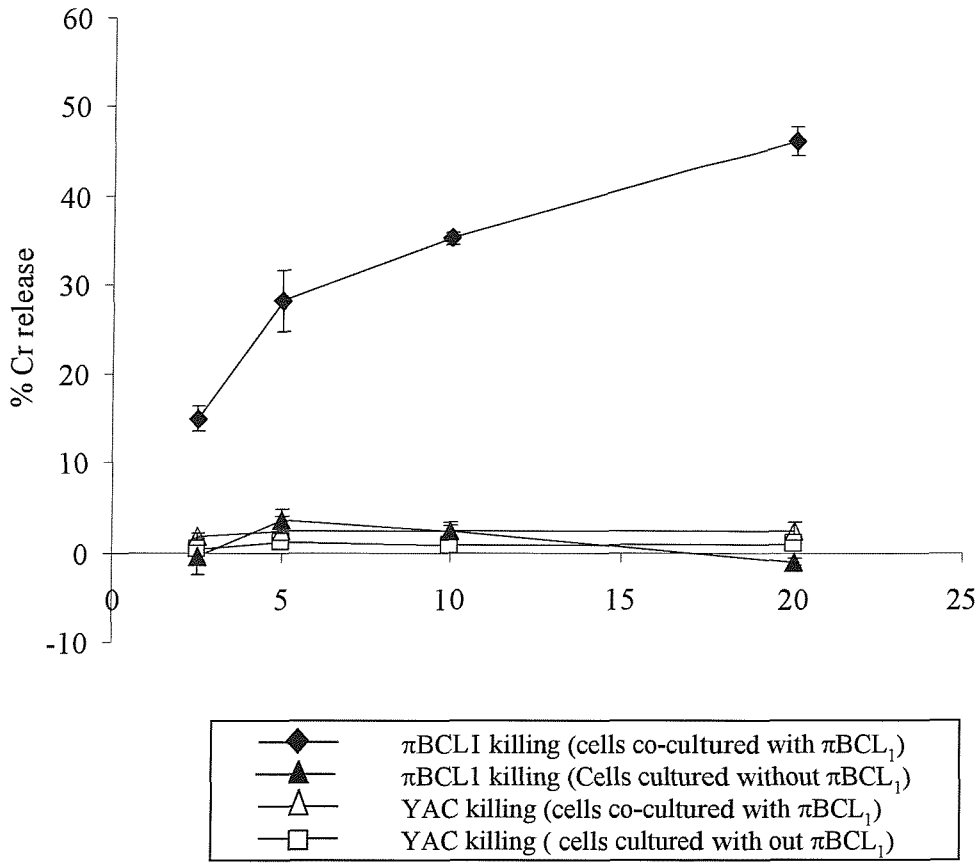


Figure 4.3.3a) Cyototoxic activity of CD8-π-1 cultured *in vitro* with and without πBCL_1 stimulation. Standard ^{51}Cr release assay was used to assess cytotoxic activity against $\pi BCL_1$and YAC cells, after CTL had been cultured for 3 weeks *in vitro* of in the presence of IL7 week 1 and IL2 thereafter. ^{51}Cr release assay was routinely performed 5 days post – stimulation

Figure 4.3.3b) Increasing cytotoxic activity of *in vitro* generated CTL over 3 weeks in culture with πBCL_1.

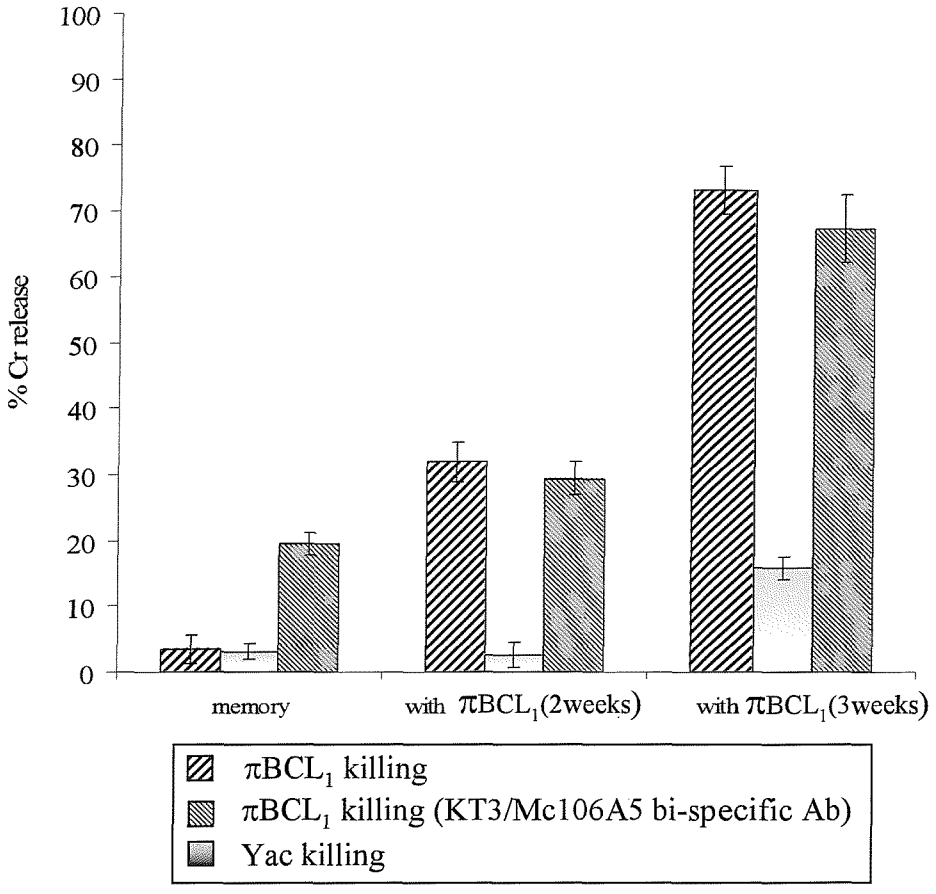


Figure 4.3.3b) Increasing cyototoxic activity of memory CD8+T cells cultured *in vitro* over time in the presence of πBCL_1. Standard ^{51}Cr release assay was used to assess cytotoxicity against $\pi BCL_1$and YAC cells after 2 and 3 weeks culture in the presence of IL7 week1 and IL2 thereafter. Bi-specific antibody CD3 x BCL_1 idiotype (KT3/Mc106A5) at 1μg/m final concentration was used as a positive control.

effector : target ratios indicating that killing of lymphoma in the group cultured with πBCL_1 was NK independent. The figure also shows that cytotoxic activity was highly dose-dependent and that even at a low effector : target ratio of 2.5 :1 lysis was achieved.

Upon further culture of CD8-π-1 T cells, CTL activity was seen to increase as shown in figure 4.3.3b from below 5% release in direct *ex vivo* memory CD8+ T cells to 70% after 3 weeks in culture.

4.3.4 Investigation into the cytotoxic specificity of CD8-π-1 and CD8-π-2

Having previously determined the dose-dependent cytotoxic activity of CD8-π-1 after co-culturing in the presence of πBCL_1, we wished to assess the cytotoxic specificity of the CTL culture generated in this way. Thus, CTL were tested for cytotoxic activity against the other mouse tumours which also grow in BALB/c and were previously used in proliferation experiments. Figure 4.3.4a shows CD8-π-1 cells grown with πBCL_1were able to effectively kill πBCL_1 in an MHC class 1 dependent manner, although these CTL were not able to kill substantial numbers of the other lymphomas tested, 10% of P815 were killed at a ratio of 20:1. CD8+ T cells grown in the absence of πBCL_1 were not able to kill πBCL_1, WEHI, A20 or P815 (data not shown).

CD8-π-2 was also investigated in the same way and despite showing πBCL_1 specificity and MHC class I restricted cytotoxicity (Figure 4.3.4b) in standard ^{51}Cr release assays the cytotoxic behaviour of this line did not increase above 25% at an E:T ratio of 20:1

4.3.5 *In vitro* generated CTL, cytotoxic against πBCL_1 can also be cytotoxic against other tumours which have the same MHC background.

The characterisation in this chapter mainly concerns two *in vitro* generated πBCL_1-specific CTL lines, however, other CTL lines were generated *in vitro* which were also able to lyse different tumours in standard ^{51}Cr release assays. Figure 4.2.5 shows data from three separate ^{51}Cr release assays on 3 lines generated as detailed in materials and methods. Figure 4.3.5 shows that CD8-π-3 was able to lyse both πBCL_1 and P815 effectively (>50% lysis at an E:T ratio of 50:1) and was cytotoxic against WEHI 231 to a lesser extent (>20% at 50:1). CD8-π-4 was cytotoxic against P815 and to a

Figure 4.3.4a) Cytotoxic activity of CD8-π-1 CTL, against πBCL_1, A20, P815 and WEHI 231

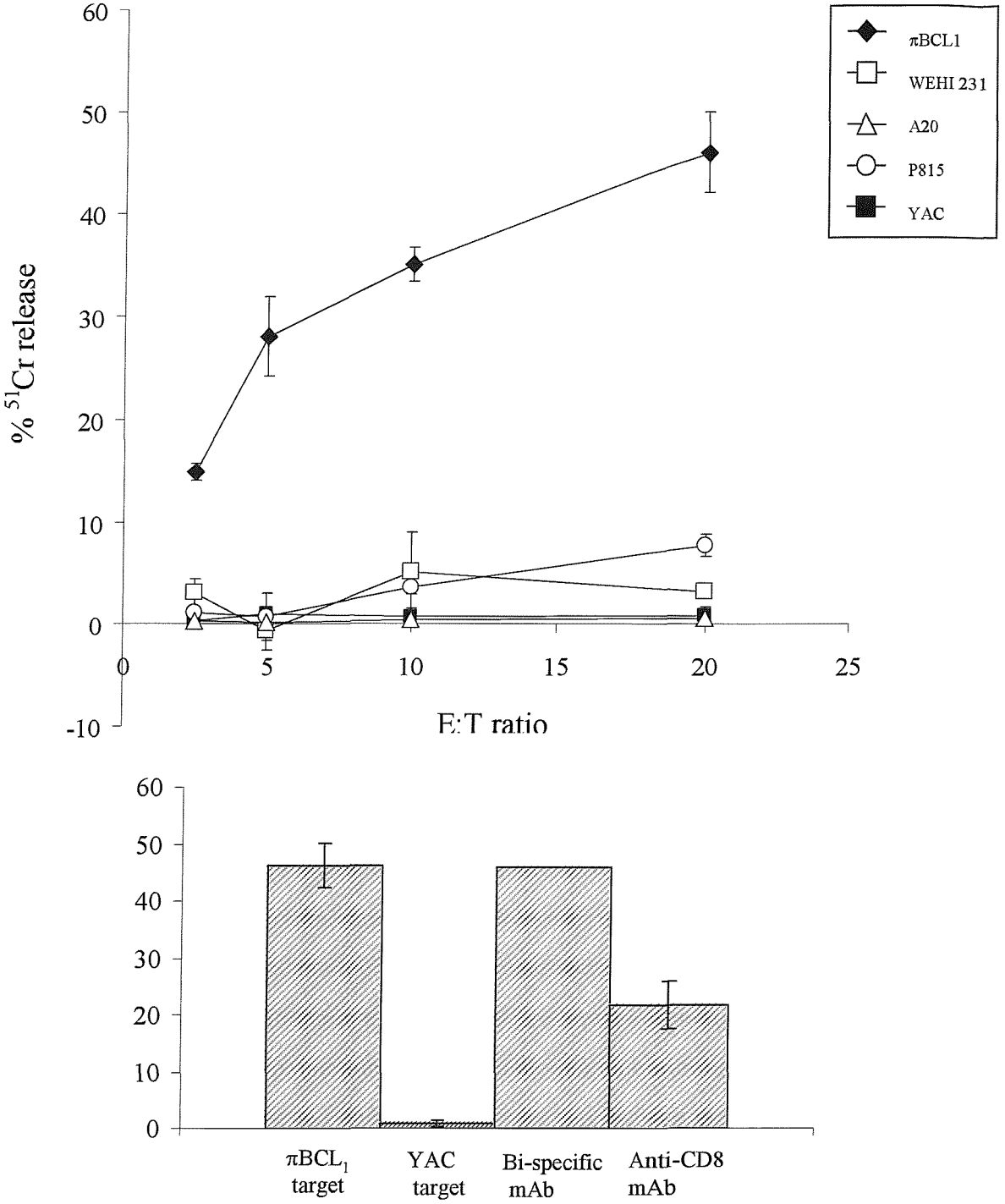


Figure 4.3.4a) CD8+ T cells (CD8-π–1) were grown in the presence of cytokines as previously described, with πBCL_1 for 3 weeks and the cytotoxic activity against πBCL_1, A20, P815 and WEHI 231was assessed. YAC killing control was performed to assess NK dependent killing. Killing against πBCL_1 (at E:T ratio of 20:1)was also carried out in the presence of bi-specific antibody KT3/Mc106A5 and a blocking antibody against CD8 (YTS169) (bottom graph).

Figure 4.3.4b) Cytotoxic activity of CD8-π-2, against πBCL_1, A20, P815 and WEHI 231

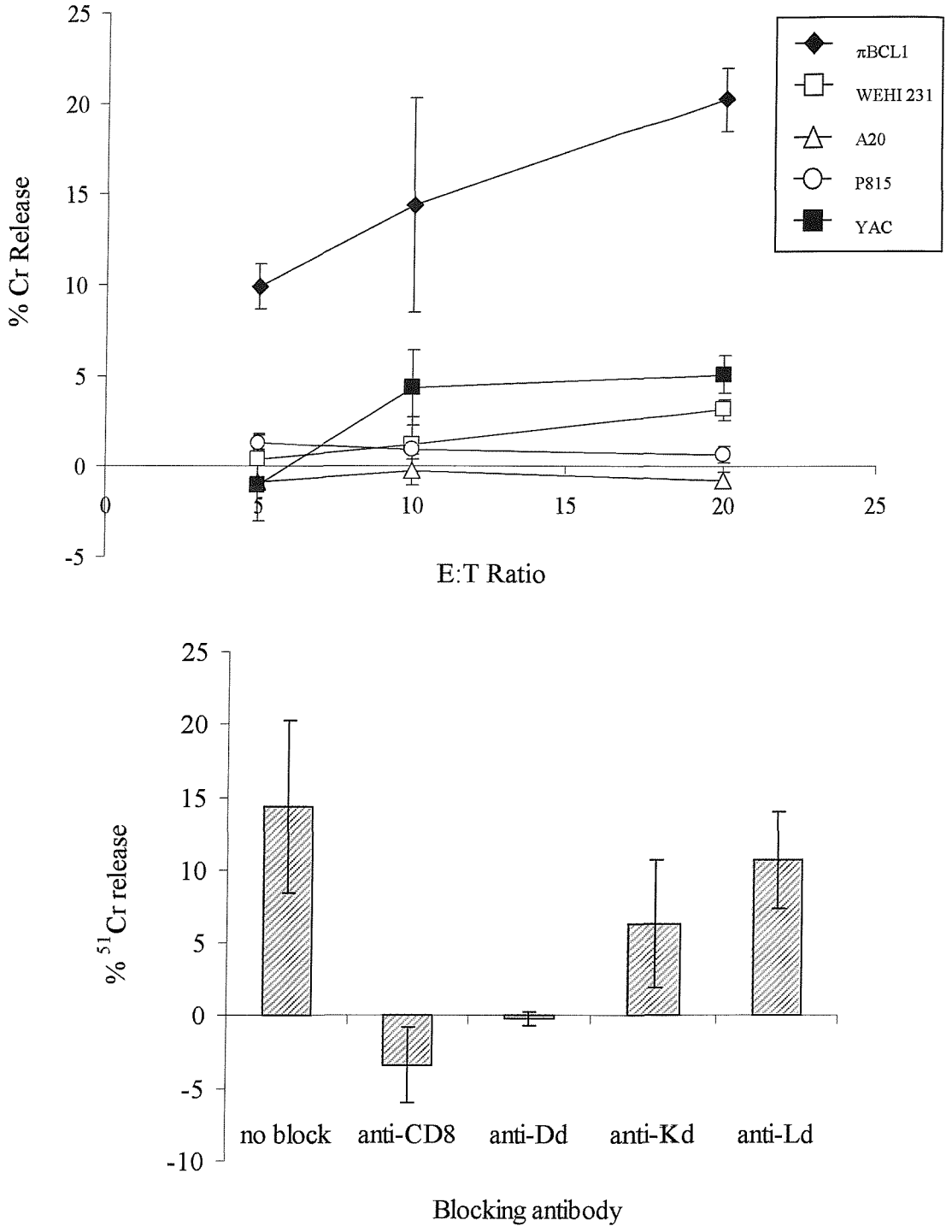


Figure 4.3.4b) CD8+ T cells (CD8-π–2) were grown in the presence of cytokines as previously described, with πBCL_1 for 2 weeks and the cytotoxic activity against πBCL_1, A20, P815 and WEHI 231was assessed. YAC killing control was performed to assess NK dependent killing. Killing against πBCL_1 (at E:T ratio of 10:1)was also carried out in the presence of blocking antibodies against CD8, and MHC class I (bottom graph).

Figure 4.3.5) Cytotoxic behaviour of *in vitro* generated CTL lines.

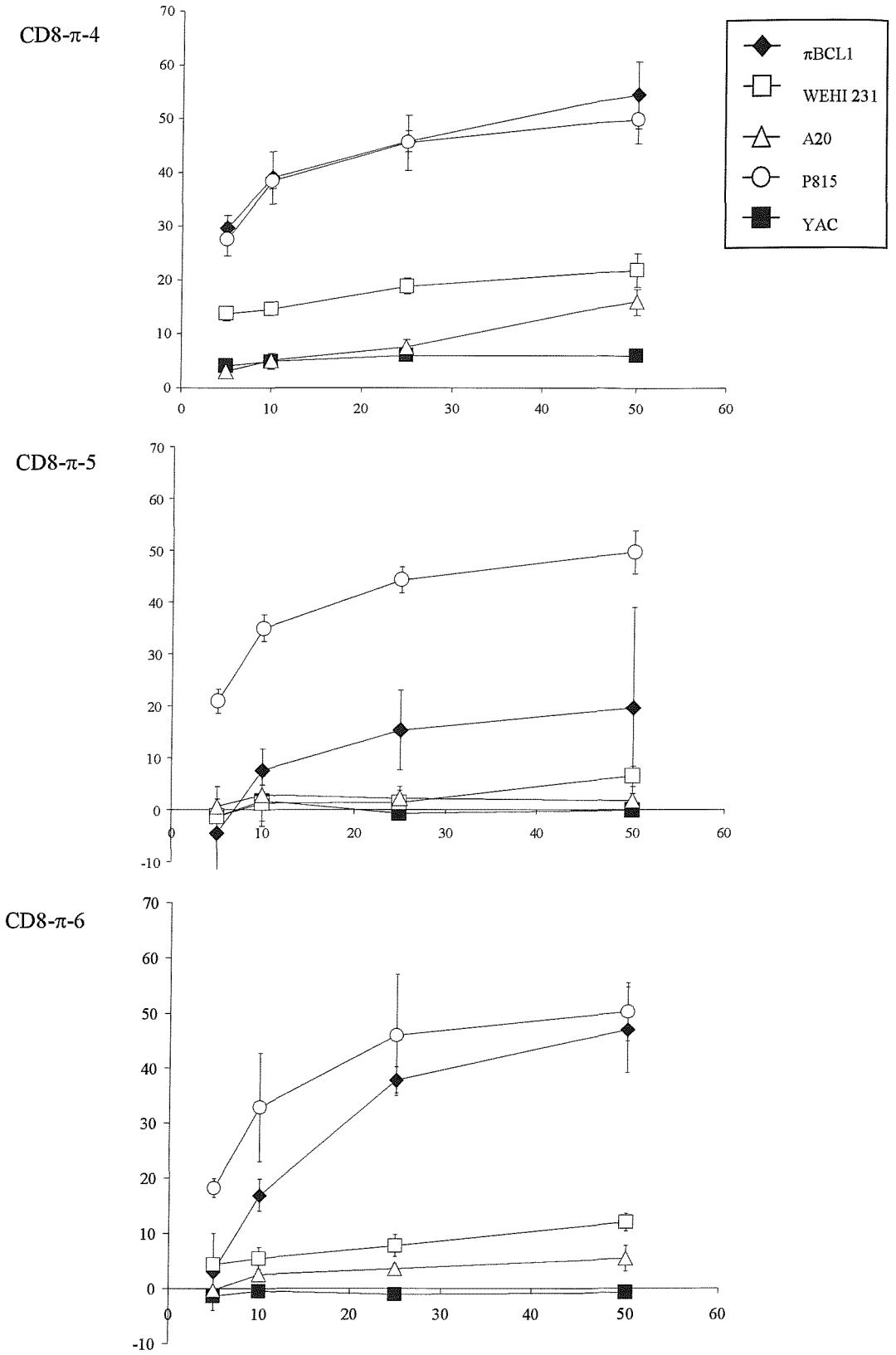


Figure 4.3.5) CD8+ T cells were grown in the presence of cytokines as previously described, with πBCL_1 for > 6 weeks and the cytotoxic activity against πBCL_1, A20, P815 and WEHI 231was assessed. YAC killing control was performed to assess NK dependent killing.

lesser extent πBCL_1 lysing 50% and 20% of target cells respectively at an E:T ratio of 50:1. CD8-π-5 showed the same cytotoxicity against P815 as CD8-π-4 and could also kill 45% of πBCL_1 targets at an E:T ratio of 50:1 but was also able to lyse 10% WEHI 231 and 5% A20 at this E:T ratio. Other *in vitro* generated CTL were effective at lysing A20 (Data not shown- personal communication G. Crowther). This data is indicative of the presence of many different TAA in this system and reiterates the point that some TAA may be shared.

4.3.6 Cytokine profiles of *in vitro* stimulated CD8+ T cells

Cytotoxic T cells can be defined as T_C1 or T_C2 rather like their CD4+ T_H1 and T_H2 counterparts, based on cytokine secretion profiles. Adoptive transfer studies have suggested that T_C1 CD8+ T cells (which are known to produce IL-2, IFNγ and TNFα) are more effective at clearing tumour *in vivo* than T_C2 cells (which secrete IL4, IL5 and IL10) (189). For this reason the cytokine profiles of CD8+ T cells produced in the *in vitro* culture system were investigated.

Figure 4.3.6 illustrates IFNγ, TNFα, IL-5 and IL-10 secretion by CD8+ T cells (CD8-π-1) incubated with or without πBCL_1 in the presence of cytokines as stated in materials and methods over the first two week period of culture. Supernatants were collected after 7 days stimulation with πBCL_1 or normal B cells as a control.

An increase in IFNγ was detected only in supernatants of CD8-π-1 co-cultured with πBCL_1 over the two week period. Levels of TNFα, IL-5 and IL10 were unaffected by co-culture with irradiated lymphoma.

4.3.7 IFNγ production by CD8-π-2, in response to culture over one week with πBCL_1, A20, P815,WEHI 231 and B cells.

The cytokine profile of an alternative CTL line, CD8-π-2, was also determined. We investigated the specificity of IFNγ production relative to the presence of πBCL_1 and other tumours, and secondly to see if that response was T_C1 or T_C2 in nature. Figure 4.3.7 shows IFNγ secretion by CD8-π-2 CD8+ T cells over a 6 day period in the presence of πBCL_1, A20, WEHI 231, P815 and normal naïve B cells. The *in vitro* generated CD8-π-2 like CD8-π-1 produced the T_C1 cytokine IFNγ when stimulated with πBCL_1. The level of IFNγ remained above 5ng/ml between days 1 and 6 after

Figure 4.3.6) Cytokine secretion by *in vitro* cultured CTL, grown in with or without πBCL_1.

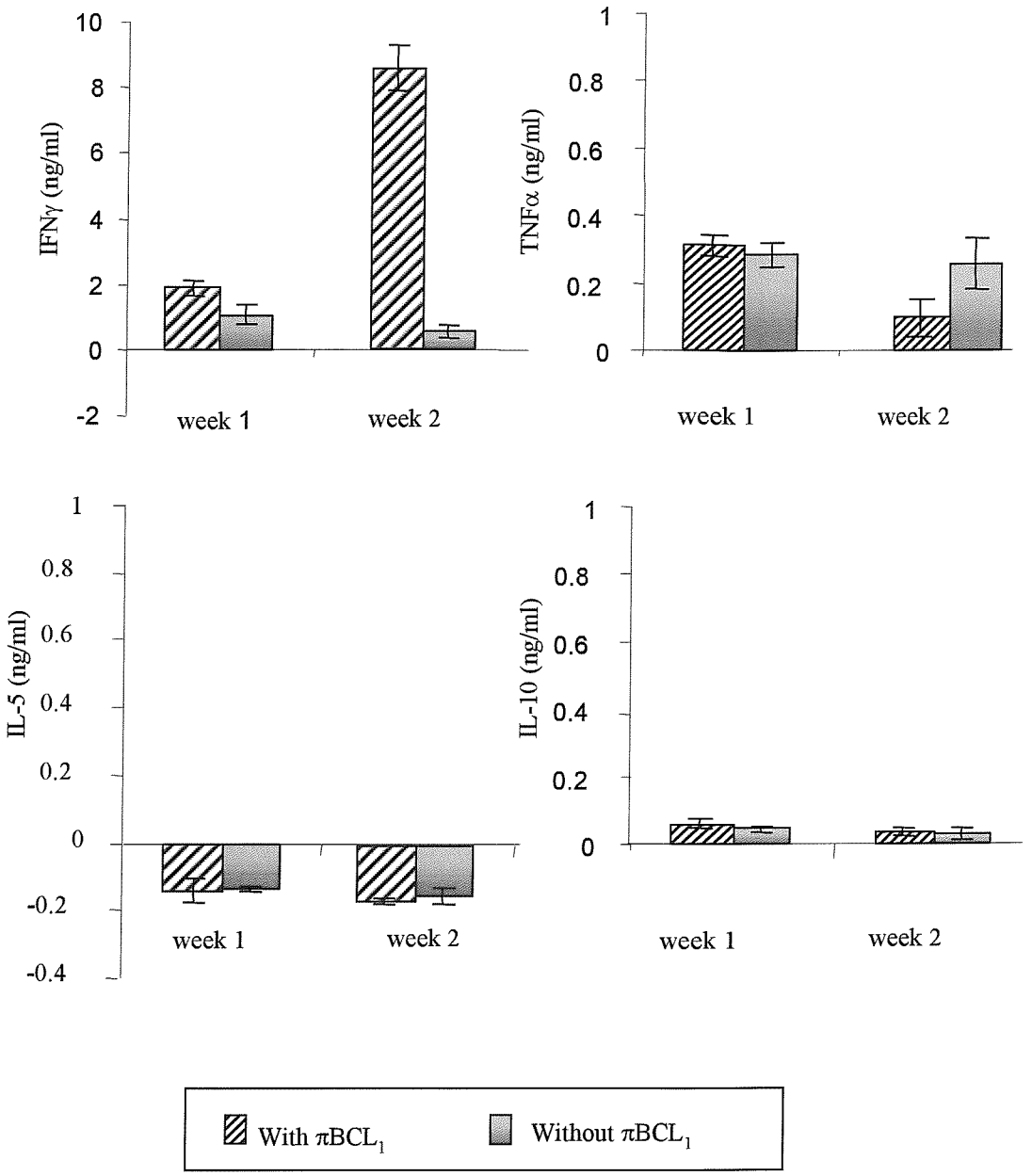


Figure 5.3.6a)CD8-π-1CD8+ T cells cultured previously for 3 weeks under standard conditions were cultured for 2 weeks with either irradiated πBCL_1, or naive B cells in the presence of IL2 (20 units/ml) in 96 well format at a density of 1x 10^5 T cells/well and 5 x 10^4 tumour or B cells/well. Supernatants were taken from triplicate wells on day 7 where CD4+ T cells were re-stimulated and day 14 and IFNγ, TNFα, IL-5 and IL-10 levels were established by standard ELISA.

Figure 4.3.7) IFNγ production by an established CD8+ T cell line (CD8-π-2) generated *in vitro* with πBCL_1 in response to culture over 1 week with πBCL_1, A20, P815, WEHI 231 and B cells.

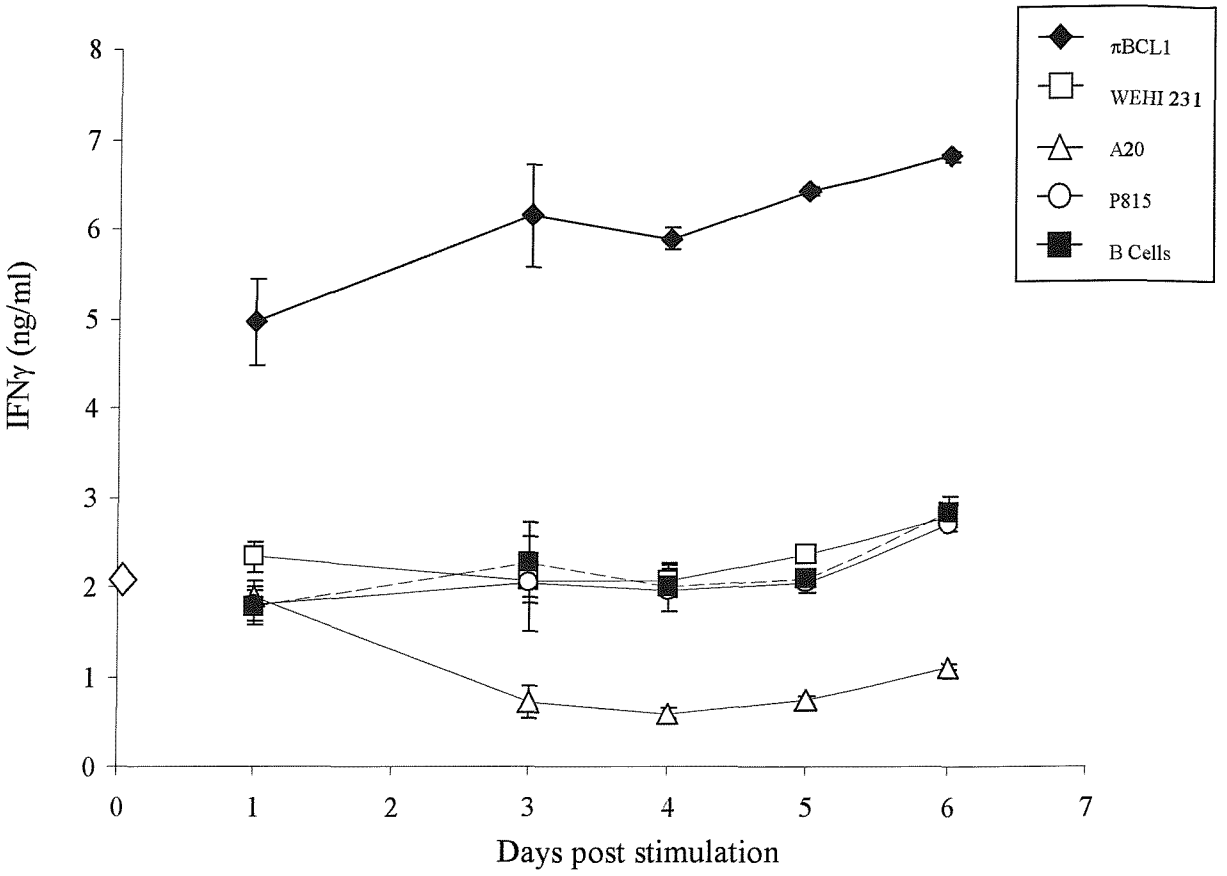


Figure 4.3.7)CD8-π-2 cells cultured previously for 6 weeks under standard conditions were cultured for 1 week with either irradiated πBCL1,A20, P815, WEHI 231, or B cells in the presence of IL2 (20 units/ml) in 96 well format at a density of 1x 10^5 T cells/well and 5 x 10^4 tumour or B cells/well. Supernatants were taken from triplicate wells on day 1-6 post re-stimulation and IFNγ levels were established by standard ELISA.

stimulation with irradiated πBCL_1. CD8-π-2 did not secrete IFNγ in response over basal level in response to the control irradiated B cells, WEHI or P815 and IFNγ levels decreased in response to irradiated A20.

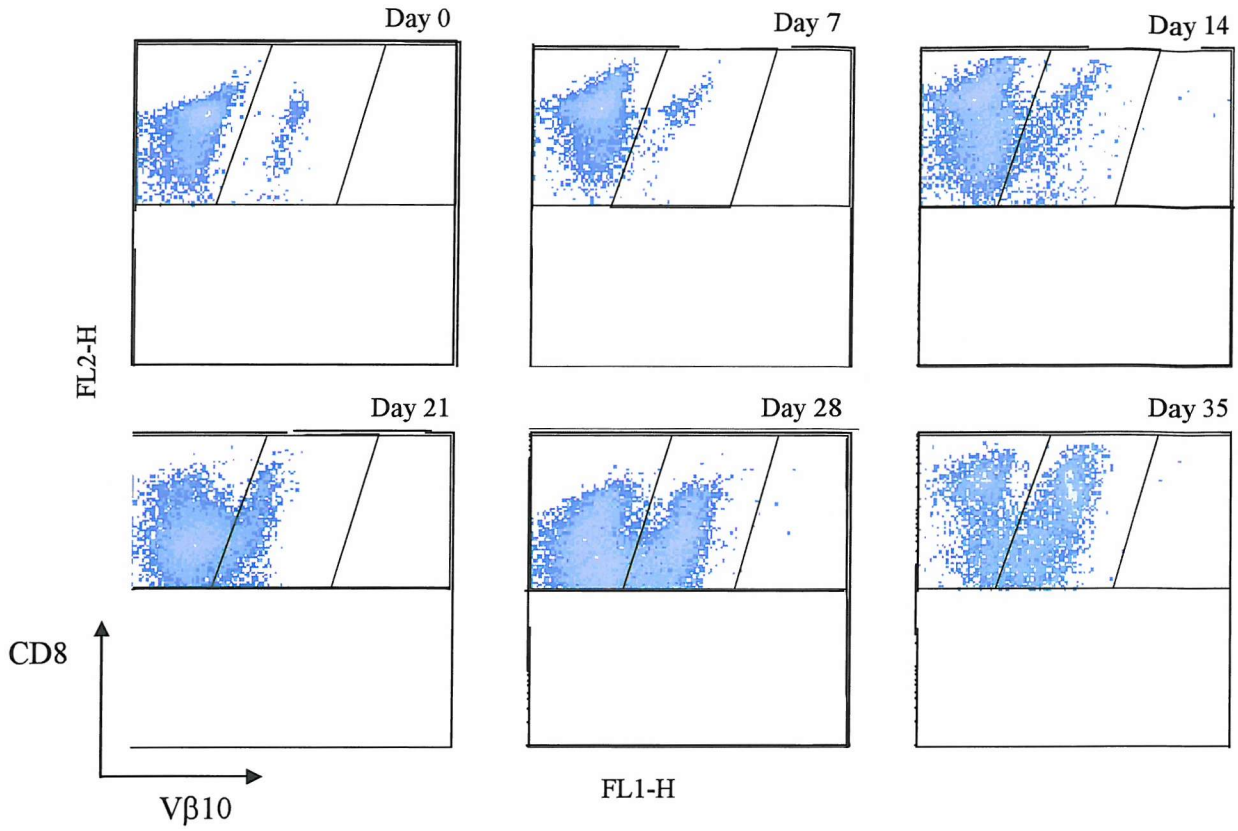
T_C2 cytokine IL-5 was not detectable in the CD8-π-2 supernatants on any of the days tested (data not shown).

4.3.8 TCR skewing of *in vitro* generated CTL – CD8-π-3.

Havign demonstrated in Chapter 3 that CTL from the peak of the anti-CD40 response in immune spleen corresponded to a skewing of the TCR as assessed by flow cytometry, we sought to determine whether skewing may occur *in vitro* in the presence of antigen .

Over five weeks in culture, the TCR Vβ usage by the CTL, was analysed by flow cytometry. This analysis revealed skewing by one particular CTL line (CD8-π-3) to Vβ10 from approximately 10% in the initial purified memory CD8+ T cells to approximately 60% after five weeks incubation with πBCL_1. Skewing was not seen when the same CTL were incubated on cytokines alone (Figure 4.3.8a). Evidence of skewing was also assessed using RT-PCR shown in Figure 4.3.8b.Vα skewing was also able to be assessed using this technique. RT-PCR was carried out on cDNA purified from *in vitro* generated CTL on day 0, day 7 and on day 35 of culture using a reverse primer to the constant region of the Vα or Vβ chain and different forward primers to the variable regions of each individual Vα or Vβ chain. Seventeen separate Vβ forward primers and nineteen separate Vα primers were used in this assay (Table 4.1 materials and methods). OT1 splenocytes which are transgenic on a $rag^{-/-}$ background and only express Vα2 Vβ5 TCR were used as control cells in this assay to ensure primers were specific and did not anneal to more than one variable region gene. Figure 4.3.8b shows the RT-PCR on OT1 splenocyte cDNA, and demonstrates that a single band is observed with the Vα and Vβ primers (Figures 4.3.8c-e show the same assay performed on cDNA taken from CTL day 0, and after 7 and 35 days in culture). Day 0 and day 7 analyses show that CTL TCR at this early time point had a polyclonal mix of T cells are present in the cultures. After 35 days however, there is a clear skew towards the Vβ10 TCR and the Vα chain is also skewed although the pattern remained polyclonal (bands were seen for Vα4, 4.4 ,8 ,9/10 and 19).

Figure 4.3.8a) Skewing of *in vitro* generated CTL CD8-π-3 to a Vβ10 TCR



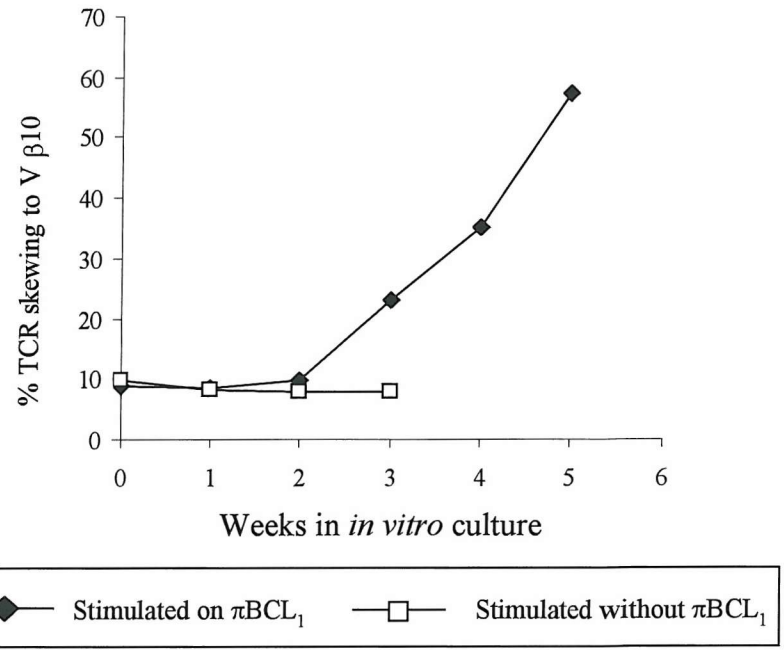


Figure 4.2.8) TCR skewing to Vβ10 was assessed by immunoflourescence. *In vitro* generated CTL were labelled with anti-Vβ10 FITC conjugate and anti-CD8-PE conjugate and the percentage of viable (as assessed by FSC vs SSC) CD8+ which expressed Vβ10 was calculated.

Figure 4.3.8 RT- PCR analysis skewing of *in vitro* generated CTL to a Vβ10 TCR

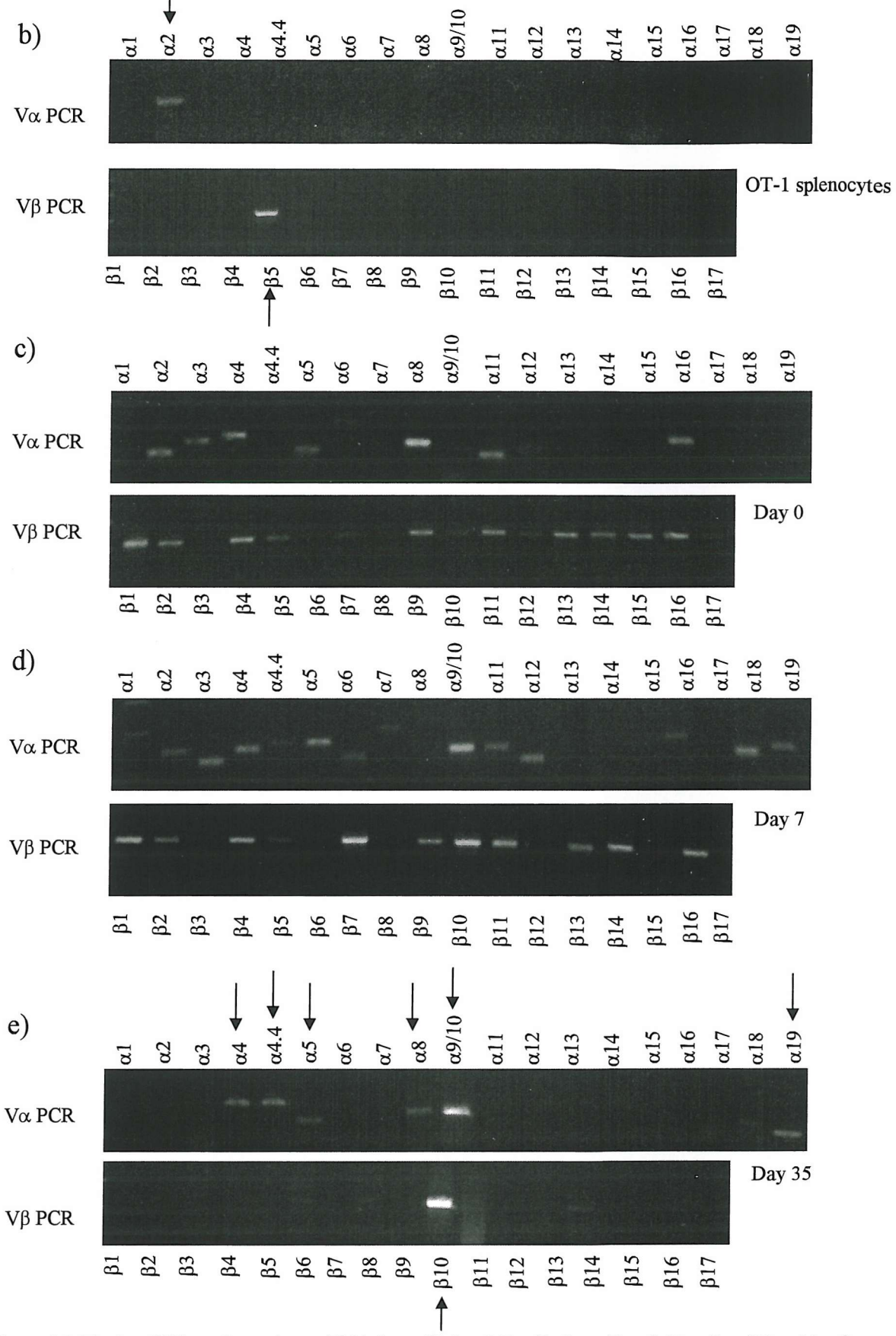


Figure 4.3.8 b-e) rt-PCR performed on mRNA from CD8-π-3 T cells from Day 0, Day 7 and Day 35 of *in vitro* culture using Va and Vb primers as detailed in materials and methods. Control rt-PCR was performed on OT-1 splenocytes to investigate primer specificity.

Figure 4.3.8f) Flow cytometric analysis showing that blasted CTL possess a vβ10 TCR and are $CD8^{hi}$

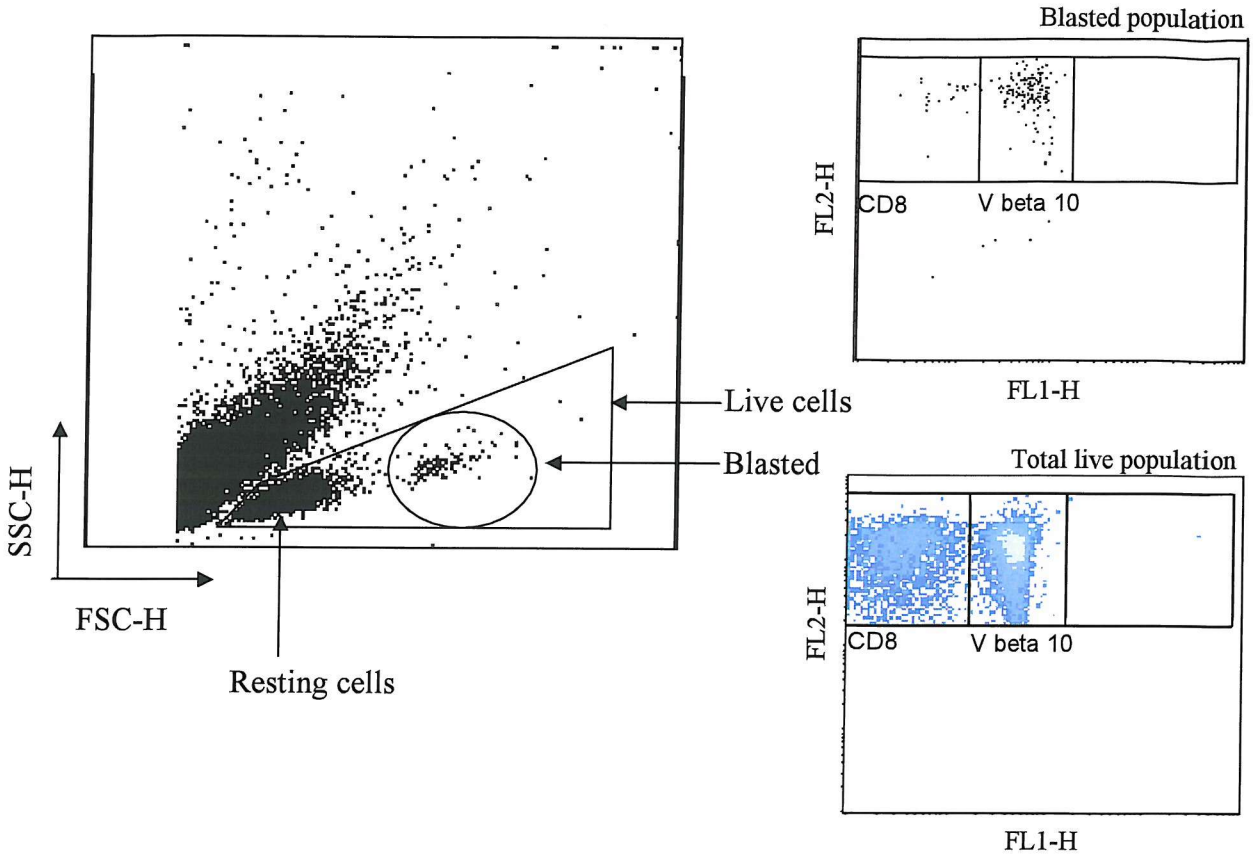


Figure 4.3.8f) Blasted cell were visualised by flow cytometry as high forward scatter. Vβ10 skewed CTL after 5 weeks *in vitro* culture were analysed to assess the phenotype of both the blasted and total cell population. Both blasted cells (high forward scatter) and the total live population were analysed for CD8+ Vβ10 positive T cell number.

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Flow cytometry using forward and side scatter analysis also showed that 48 hours after stimulation of the Vβ10 skewed cell line with πBCL_1, cells showing a blasted appearance could be detected. These cells (whose appearance indicated activation) revealed a $CD8^{hi}$Vβ10+ phenotype, shown in figure 4.3.8f.

4.3.9 Intracellular IFNγ production by CTL

Following the observation that *in vivo* and *in vitro* CTL can skew to a particular TCR, namely Vβ10 and Vβ6 *in vivo* and at least Vβ10 *in vitro* , an unskewed CTL (CD8-π-1) was used to assess whether those cells which respond to πBCL_1 (by producing IFNγ upon culture with irradiated lymphoma) preferentially expressed either Vβ6 or Vβ10 TCR.

In vitro generated CD8-π–1 showing no TCR skew after 4 weeks were incubated for 16 hours in the presence of IL-2 with or without πBCL_1. As a positive control the same CTL lines were stimulated with PMA/ionomycin as stated in materials and methods.

Figure 4.3.9 illustrates that, firstly, in an *in vitro* CTL line cultured for four weeks, IFNγ production by CD8+ T cells in response to πBCL_1 and IL-2 is greater than that produced in response to IL-2 alone. However, this experiment showed that cells responding to πBCL_1 (as determined by IFNγ production) did not preferentially possess a Vβ6 or Vβ10 TCR.

4.3.10 Skewing of CTL to alternative TCR

TCR skewing studies of tumour specific CTL *in vivo* (chapter 3) indicated that the response to tumour antigen was polyclonal, suggesting that multiple antigens could be recognised by these CD8+ T cells.

Although one line showed skewing a Vβ10 TCR. Other T cell lines were tested for skewing and results indicate that other CTL either did not skew (data not shown) and were said to be polyclonal lines or skewed to different TCR. Figure 4.3.10 shows flow cytometry performed on two CTL lines generated by G. Crowther. Data shows that over 90% of π-1 CTL possessed aVβ8 TCR, whereas π-2 was skewed to both Vβ10 (>50%) and Vβ8 (>30%).

Figure 4.3.9) Intracellular IFNγ production by CTL in response to πBCL_1 stimulation

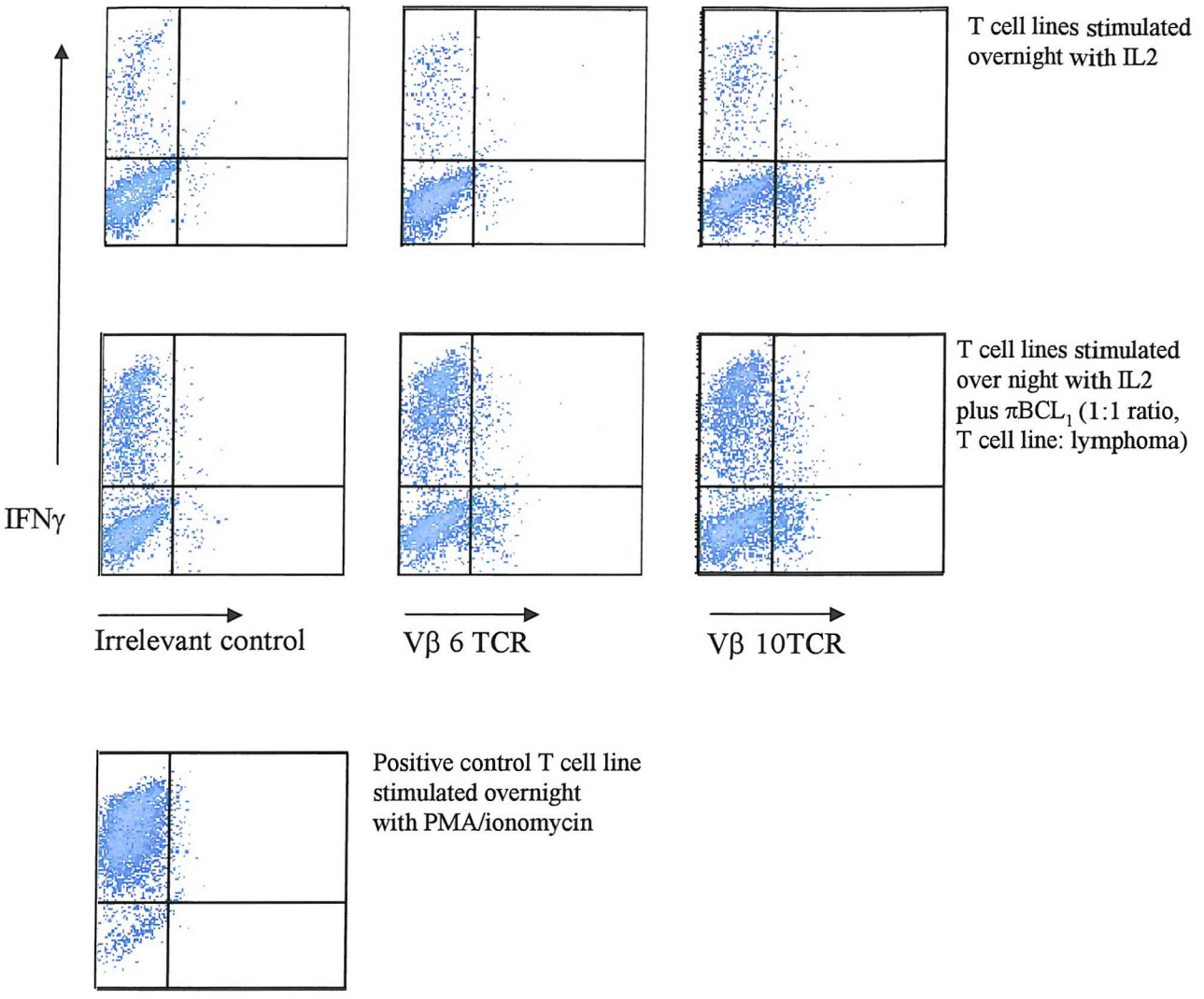
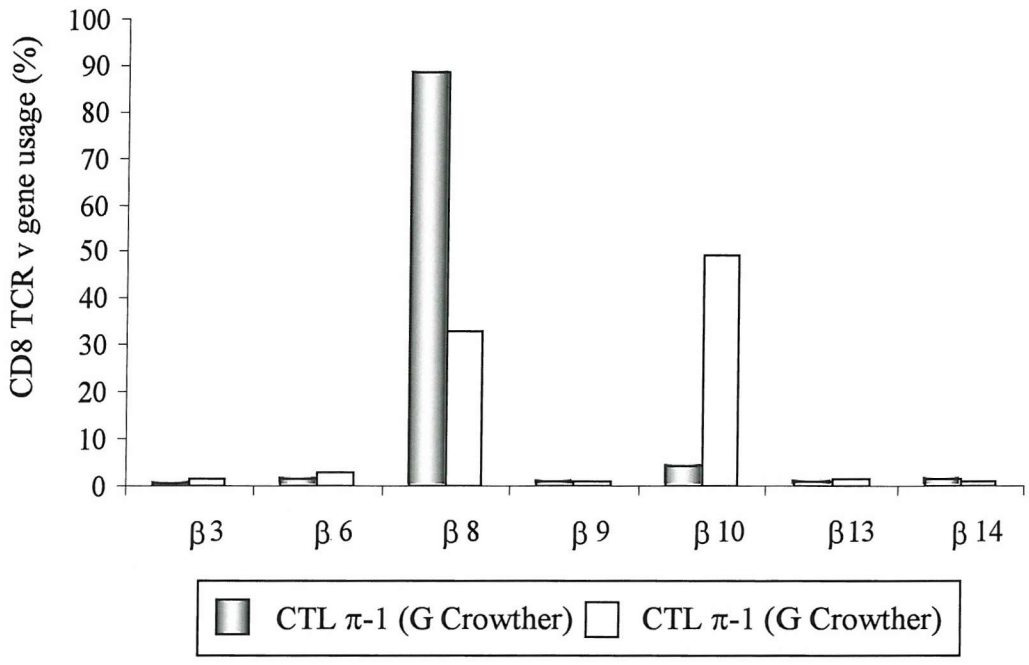


Figure 4.3.9) Intracellular Interferon γ production by polyclonal T cells line. *In vitro* generated (week 4) CTL (line 4)were incubated with or without πBCL_1 at a T cell: lymphoma ratio of 1:1 over night in the presence of IL-2 at 20 units/ml. A positive control of CTL stimulated with PMA plus ionomycin was performed.CD8+ T cells were visualised by flow cytometry using anti CD8-APC conjugate. The TCR of interest was visualised using the relevant anti TCR-FITC conjugate. Intracellular IFNγ was detected with anti-IFNγ mAb upon permeabilising the cells.

Figure 4.3.10) Skewing of 2 *in vitro* generated CTL to a Vβ8 TCR and to both a Vβ8 and Vβ10 TCR



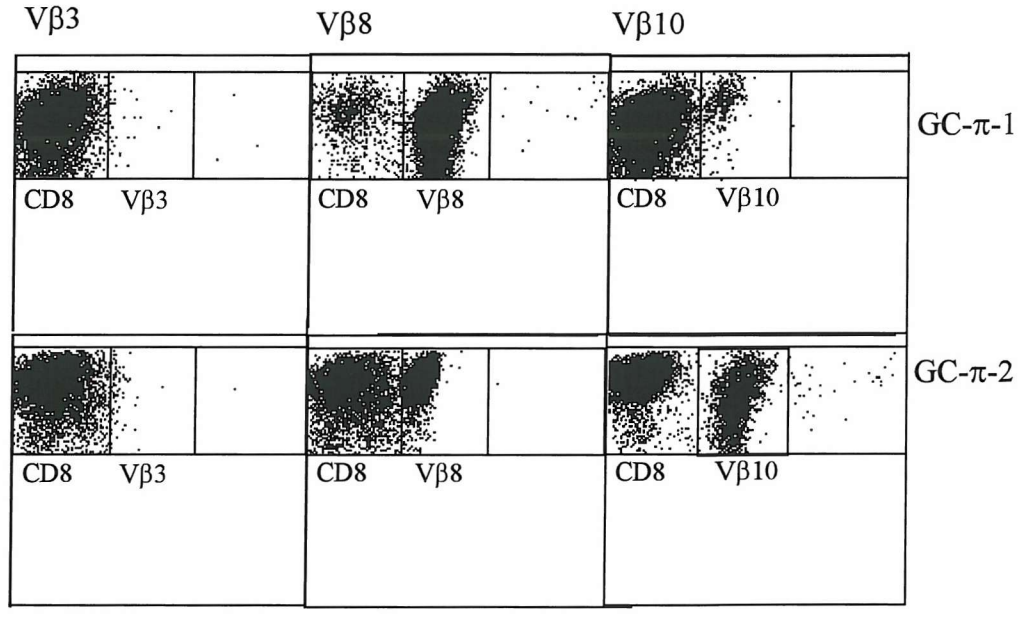


Figure 4.3.10) TCR skewing to of Vβ was assessed by immunoflourescence. *in vitro* generated CTL were labelled with either anti-Vβ3,6,8,9,10,13,or 14 FITC conjugate and anti-CD8-PE conjugate and percentage of viable (ass assessed by FSC vs SSC) CD8+ which had skewed was calculated.

4.4 Discussion

In this chapter the optimal conditions in which to generate CTL from memory CD8+ T cells were investigated and those T cells characterised. Firstly, we showed that only CD8+ T cells incubated in the presence of πBCL_1 secrete IFNγ. This indicates, that memory T cells are generated during the *in vivo* response from effector CTL, which recognise antigens presented either directly by BCL_1 or by DC. These memory T cells expand *in vitro* and respond to re-stimulation *in vitro* with the πBCL_1 lymphoma line. Our data also show that a favourable cytokine environment is needed in conjunction with antigen to elicit an IFNγ response from CD8+ T cells after two weeks *in vitro* culture. The requirement for antigen in generating πBCL_1 specific CTL is also indicated by the CTL killing experiments. Here only those CD8+ memory T cells incubated with antigen were able to show cytotoxic activity against the lymphoma. The cytokine environment required for optimal cytotoxic activity was determined to be IL-7 at 10ng/ml in the first week of culture followed by IL-2 at 20 units/ml on subsequent weeks. These cytokines were chosen because IL-7 provides survival signals (256) and IL-2 aids in proliferation (257). These cytokines augmented the production of IFNγ cytotoxicity and expansion of the CTL in culture but could not induce cytotoxic behaviour or significant amounts of IFNγ production alone.These data show that there is a requirement for antigen in the form of irradiated πBCL_1 to create CTL, which are specific for the tumour.

Two CTL lines subsequently grown in these conditions showed that they were capable of πBCL_1 dependent proliferation in CFSE dilution experiments. Memory T cells exist *in vivo* to enable the immune system to mount a faster and more efficient response to a previously encountered antigen. Memory cells can secrete a larger range of cytokines than their naïve counterparts on encounter with appropriate antigen and are able to proliferate without the need for signal 2 on APC. However once the antigen is removed and these cells are no longer needed, how do they persist? Memory T cells *in vivo* are thought to have a turnover rate which is higher than naïve T cells although memory T cells are also able to persist as non dividing cells. The idea that trace amounts of antigen are required for this division is controversial as it may be that memory T cell division is MHC independent (Reviewed in (255)). Normal turnover of memory T cells could be controlled by cytokines; indeed studies have shown that $CD8+CD44^{hi}$ cells proliferate *in vivo* after IFNα,β or γ injection. In

this case, it is thought that IFN act not directly on CD8+ memory cells but on DC, which are induced to produce an IL-2 like cytokine, IL-15. Although IL-2 stimulates any CD25+ T cell to proliferate IL-15 is able to stimulate a select group of CD8+ memory cells since one of the IL-15 receptor components CD122 (IL-2Rβ) is expressed at a higher level on the CD8+ memory T cell subset. IL-7 is thought to induce a low level signal which is sufficient to keep cells alive but not induce them to enter the cell cycle. Therefore in the *in vitro* system used here, the memory T cells have an indiscriminate survival signal through IL-7, which should keep all of the T cells alive. In this system, IL-2 will aid in the generation of effector cells only if CD25 is expressed on their cell surface, presumably when they are activated by antigen. This could account for the lack of proliferation seen with both CTL lines in the presence of IL-7 and IL-2 and B cells. CD8-π-2 was also incubated in the presence of irradiated WEHI 231, A20, P815 and did not proliferate CD8-π-2 and CD8-π-1 were thought to be πBCL_1-specific.

Both CD8-π-1 and CD8-π-2 were capable of NK independent killing against target lymphoma (πBCL_1) at differing levels of lysis. Increasing cytotoxic activity over a three week period, which also is indicative of tumour specific CTL outgrowth was seen in the CD8-π-1 line. CD8-π-1 appeared to be highly specific for πBCL_1 as after three weeks *in vitro* culture CTL activity was almost exclusively πBCL_1 specific. This suggests that CTL, specific for unique antigen(s) presented in the context of MHC I on πBCL_1 can be grown on πBCL_1 with variable efficiency in cytotoxicity assays.

The generation of CTL lines, CD8-π-4, 5, and 6 which, in addition to killing target πBCL_1 were also cytotoxic against P815, A20 and WEHI 231 suggests the presence of shared antigens in this system. *In vivo* re-challenge experiments indicate the presence of a πBCL_1/A20-shared antigen, since re-challenging BCL_1 cured mice. with A20 (i.p. $1x10^5$) results in survival (258). Future work would aim to investigate the proliferative response of these lines in response to different tumours.

To further characterise the CTL, their cytokine secretion profiles were assessed. It has been reported that CD8+ T cells can be divided into T_C1 and T_C2 subsets based on cytokine secretion (188). Following tumour antigen encounter, T_C1 or type 1 CD8+ cells secrete IFNγ, IL-2 and TNFα, whereas T_C2 or type 2 CD8+ cells secrete IL-4, IL-5 and IL-10. Both sets kill predominantly via the perforin pathway, however T_C2

cells also exhibit some Fas-mediated killing. It is likely that T_C1 and T_C2 cells differ in their modes of effecting tumour regression. In one study, relative effects of adoptively transferred OVA specific T_C1 and T_C2 populations were assessed against ova-transfected B16 melanoma. Both T_C1 and T_C2 mediated a decrease in lung tumour growth with subsequent prolonged survival times, and both T_C1 and T_C2 cells accumulated in the site of tumour, however T_C1 cells persisted for up to three weeks at the tumour site after it had been cleared whereas T_C2 numbers rapidly diminished and protection was shown to be five times more effective with T_C1 cells. Interestingly the efficacy of T_C1 and not T_C2 cells was eradicated when CD8+ T cells from $IFN^{-/-}$ mice were used, suggesting that efficacy is due to differing mechanism (190) T_C1 CD8+ cells may be the optimal CTL to use in adoptive therapy. Our *in vitro* generated CD8+ T cells produced large amounts of IFNγ indicating they were of a T_C1 phenotype. CD8-π-2 only secreted IFNγ in response to πBCL_1 when stimulated by a panel of different tumours indicating its specificity. Surprisingly, IL-2 was not detectable in the supernatants of any CTL cultures (data not shown, cultures stimulated with no exogenous IL-2). The lack of IL-2 could indicate that no IL-2 was being produced, that it remained below the level of detection, or that all IL-2 produced was consumed by the T cells as CD25 expression increased upon activation. After two weeks of culture the TNFα production by CD8-π-1 cells decreased in the antigen stimulated group. This decrease could be due to the lymphoma binding the cytokine. Therefore, a true T_C1 phenotype was not confirmed but is suggested, as no production of the T_C2 cytokines (IL-4, IL-5 and IL-10) was observed by either line.

The TCR skewing shown by RT-PCR, coupled with the *in vivo skewing* detailed in chapter 3 suggests that the response to BCL_1 *in vivo* during anti-CD40 therapy is polyclonal. The *in vitro* skewing shown to Vβ10 and Vβ8 shows that *in vitro,* πBCL_1-specific clones or more than one clone with the same β chain of the TCR can outgrow other T cells in the culture to progressively skew the lines.

There could be many different epitopes recognised by different TCR or indeed different TCR which recognise the same epitope. The polyclonal nature of the *in vivo* response is supported by the fact than even when both anti-Vβ10 and anti-Vβ6 antibodies are used to block these particular T cells *in vivo*; the tumour is still cleared (personal communication Dr. R. French). Furthermore, more than one epitope could be recognised by the same β and α type chain with a differeing hypervariable region.

Thus, the skewing to Vβ10 by CD8-π-3 CTL does not conclusively show that all the T cells in culture recognise the same epitope and are therefore clonal. The CD8+Vβ10+ cells (whether they were clonal or not) were activated in response to lymphoma, as shown by an increase in forward scatter. The fact CD8-π-3 were not cytotoxic against A20 (data not shown) but that A20/BCL1 CD8+ T cells are cross protective *in vivo*, is also indicative that the CTL grown out *in vitro* are not truly polyclonal for all of the tumour antigens recognised *in vivo*.

Intracellular IFNγ staining was used to assess production of IFNγ in a non-skewed CTL line (CD8-π–1) to determine whether the CTL specifically activated by πBCL_1 possess a particular TCR. Although activation of the CTL was seen to be πBCL_1 dependent, no skewing of the TCR repertoire was observed.

In summary, the data in this chapter shows that CTL can be grown from CD8+ T cells from cured mice and that memory CD8+ T cells incubated with antigen are capable of killing target lymphoma. Memory CD8+ T cells incubated for the same time without πBCL_1 survive but cannot kill. Lines CD8-π-1 and CD8-π-2 have shown specific cytotoxic activity for πBCL_1 and not for other tumours against which they were tested. Line CD8-π-2 proliferated and produced T_C1 cytokines only in response to πBCL_1 out of a panel of tumours tested. However, other CTL generated showed cytotoxic activity against other tumours, P815, WEHI 231 and A20 suggesting the existence of shared tumour antigens. Preferential usage of Vβ10, a Vβ8 and a Vβ6 TCR was observed *in vitro* and this has also been observed *in vivo*, suggesting several tumour antigens being recognised by a polyclonal T cell response. However, *in vitro* it is evident that lines generated may not be polyclonal as in the CTL line CD8-π-3 over 60% of T cells possessed a Vβ10 TCR and in another line generated by G. Crowther over 90% had skewed to a Vβ8 phenotype.

Chapter 5 Generation of tumour-specific CD4+ T cell lines *in vitro*.

5.1 Introduction.

Previous studies detailed in chapter 1, in both human and mouse models have shown that CD4+ T cells can be effective in adoptive therapy regimes either to directly target the tumour and cause direct cytotoxicity through cytokine production or more importantly, to provide specific help for CTL in the eradication of tumour.

In the BCL_1 model, CD4+ T cells respond to lymphoma in the presence and absence of anti-CD40 administration by proliferation and activation. We wished to address whether CD4+ T cell lines could be generated in a similar manner to the CTL lines detailed in chapter 4 for possible use in adoptive therapy.

5.2 Materials and Methods.

5.2.1 CD4+ T cell culture

CD4+ T cells from immune mice were isolated using the MACS method detailed in materials and methods and were cultured as CD8+ T cells detailed in chapter 4 (section 4.2.1). CD4+ T cell lines characterised in this chapter are typical of several others generated. CD4+ T cells cultured in the absence of πBCL_1 were typically co-cultured with normal B cells purified from splenocytes from BALB/c mice on using MACS as detailed in materials and methods and in Chapter 4 (section 4.2.1)

5.2.2 IFNγ, IL-5,TNFα and IL-10 ELISA

Standard ELISAs were carried out as stated in materials and methods Chapter 2 and as detailed in chapter 4 section 4.2.2.

5.3 RESULTS

5.3.1 Proliferation of CD4+ T cells cultured *in vitro* with or without πBCL_1

To investigate whether CD4+ T cells could respond to tumour *in vitro*, CD4+ T cells were positively purified using MACS from a cured mouse and were cultured under the conditions stated in materials and methods. After three weeks of weekly re-stimulation under these conditions the proliferative response by the CD4+ T cells was assessed by ^{3}H-thymidine uptake. Figure 5.3.1 shows the ^{3}H-thymidine uptake by *in vitro* stimulated CD4+ T cells either co-cultured with irradiated πBCL_1 and cytokines or cultured with cytokines alone 48 hours after re-stimulation. CD4+ T cells cultured with cytokines alone proliferated much less (<500CPM) than those co-cultured with cytokines and tumour (>6000CPM). The irradiated πBCL_1 did not contribute to the proliferation as shown by the πBCL_1 control in the far left column of this figure. Importantly, flow cytometry showed that both CD4+ T cell groups had equivalent viability and CD4+ T cell numbers at the time of re-stimulation (data not shown).

Having shown that CD4+ T cells responded to πBCL_1 *in vitr*, using CD4+ T cells purified from spleen D20-D65 of anti-CD40 therapy, co-cultured with πBCL_1, we attempted to establish long term tumour-specific CD4+ T cell lines. Figures 5.3.2-5.3.8 show the characterisation of two such established, *in vitro* generated CD4+ T cell lines (CD4-π-1 and CD4-π-2).

5.3.2 Proliferation of an established CD4+ T cell line in response to antigen.

The proliferative responses of two established CD4+ T cell lines (CD4-π-1 and CD4-π-2) cultured *in vitro* for 6 weeks previously, were investigated to determine whether they would proliferate in response to antigen. CD4+ T cell lines were CFSE labelled and cultured for 6 days in the presence or absence of πBCL_1 with 20 units/ml IL-2 and the proliferative response investigated by observation of progressive dilution of CFSE intensity in FL-1 by flow cytometry. Naïve B cells were again used as a control. CD4+ T cells were labelled with anti-CD4-PE conjugate (YTA3.1.2-PE) and only CD4+ T cells are shown. Figure 5.3.2 shows the proliferative response of one line, CD4-π-2. Over the 6 day period CD4-π-2

Figure 5.3.1) Proliferation of CD4+ T cells cultured *in vitro* with or without πBCL_1

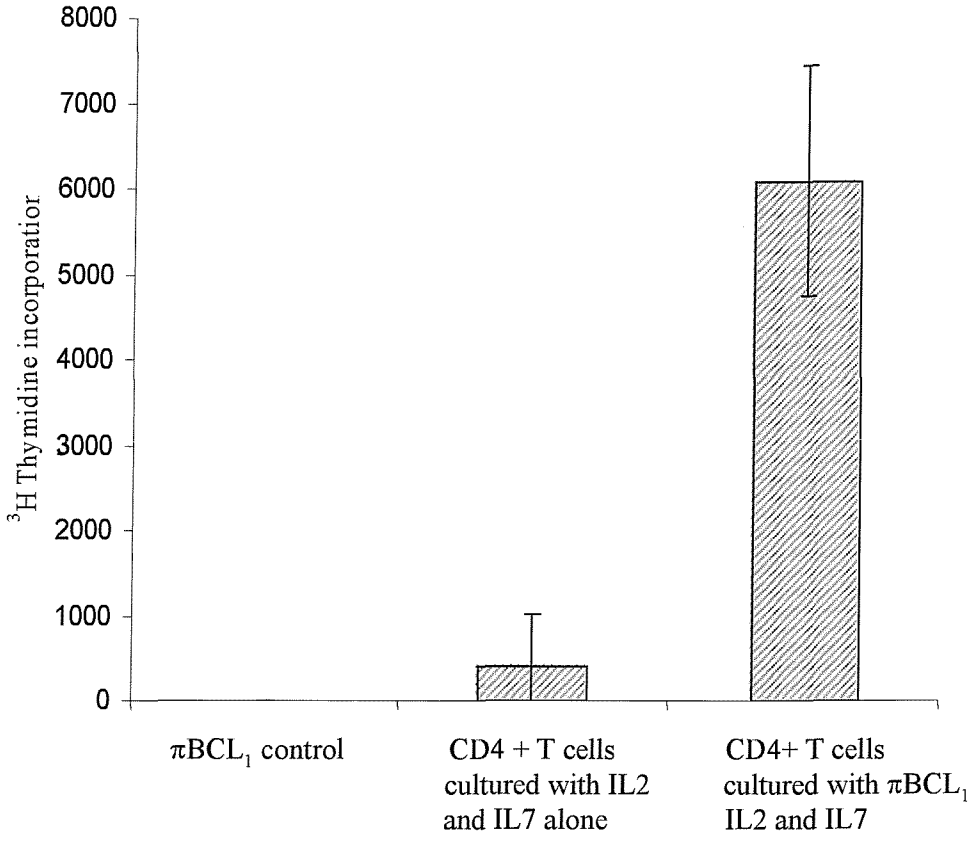


Figure 5.3.1) Proliferation of *in vitro* CD4+ T cells cultured with or without πBCL_1. CD4+ T cells cultured previously for 3 weeks under standard conditions were cultured with or without πBCL_1 in the presence cytokines IL7 and IL2 48 hours before 16 hour ^{3}H Thymidine pulse.

Figure 5.3.2) Proliferation of an established CD4+ T cell line(anti-π-2) cultured *in vitro* with πBCL_1 or B cells.

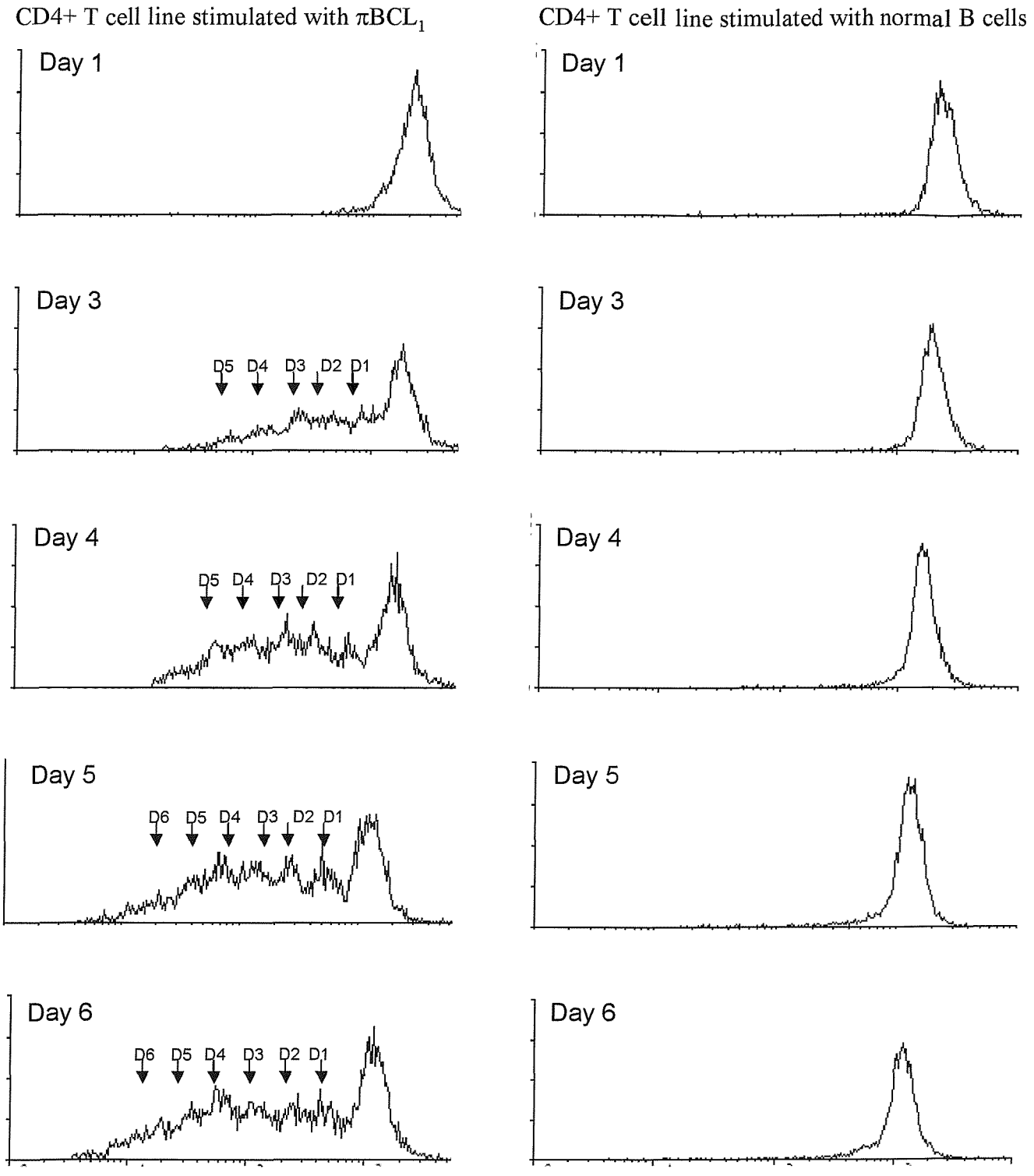


Figure 5.3.2) Proliferation of an established *in vitro* generated CD4+ T cells cultured with πBCL_1 or B cells was assessed using CFSE labelling and incubation over 6 days with irradiated πBCL_1 or B cell control.CD4+ T cells cultured previously for 6 weeks under standard conditions were CFSE labelled and cultured with πBCL_1 or B cells in the presence of IL2 (20 units/ml) in 96 well fromat at a density of 1x 10^5 cells/well (πBCL_1 was used at 5 x 10^4 /well). CD4+ T cells were labelled with anti-CD4-PE conjugate and were analysed by flow cytometry day 1-6 to investigate the division of the cells ,seen by progressive dilution of the CFSE staining in FL-1 (D1-D6).

incubated without πBCL_1 did not proliferate as shown by the lack of CFSE dilution. CD4-π-2 incubated over 6 days in the presence of πBCL_1 proliferated strongly over the 6 day period as indicated by dilution of the CFSE. CD4-π-1 showed the same response to tumour (data not shown).

5.3.3 Activation of an established CD4+ T cell line in response to antigen.

CD4 T cell lines were re-stimulated weekly as described in materials and methods. To investigate whether expression of activation and adhesion molecules on these CD4+ T cells changed between the day of re-stimulation (day 0) and the 7 days thereafter, expression of OX40, CD25, 41BB, CD49d, CD62L, ICAM-1 and LFA-1 were observed before re-stimulation when they were rested (day 0), and on days 1-7 post re-stimulation with irradiated πBCL_1 and IL-2. CD4 line CD4-π-2, which had been established *in vitro* for 6 weeks was used in this experiment. Figure 5.3.3 shows the observed change in expression between day 0 and day 4 post re-stimulation with tumour and IL-2. 4 days after re-stimulation with tumour and IL-2 the CD4+ T cells show a pronounced increase in expression from day 0 of OX40, CD25, CD49d and LFA-1 and a decrease in the level of CD62L. CD4-π-2 also showed a slight increase in both 41BB and ICAM-(data not shown) expression at this time point although both ICAM-1 and LFA-1 expression were high throughout the experiment. However, after 6 days the level of expression of all markers observed had decreased (or increased in the case of CD62L) to near resting levels as seen on day 0 prior to re-stimulation. Figure 5.3.3b shows the expression of OX40, CD25, CD49d, CD62L and LFA-1 between days 0 and 6. In summary CD4-π-2 CD4+ T cells became activated in response to πBCL_1 and IL-2 (but not to IL-2 alone, data not shown) and this level of activation peaked at day 4 post re-stimulation, expression levels of all activation and adhesion markers observed returned to basal levels after seven days.

Figure 5.3.3a) Activation of an established CD4+ T cell line cultured *in vitro* with πBCL_1. Comparison of day 0 and day 4 post re-stimulation with tumour.

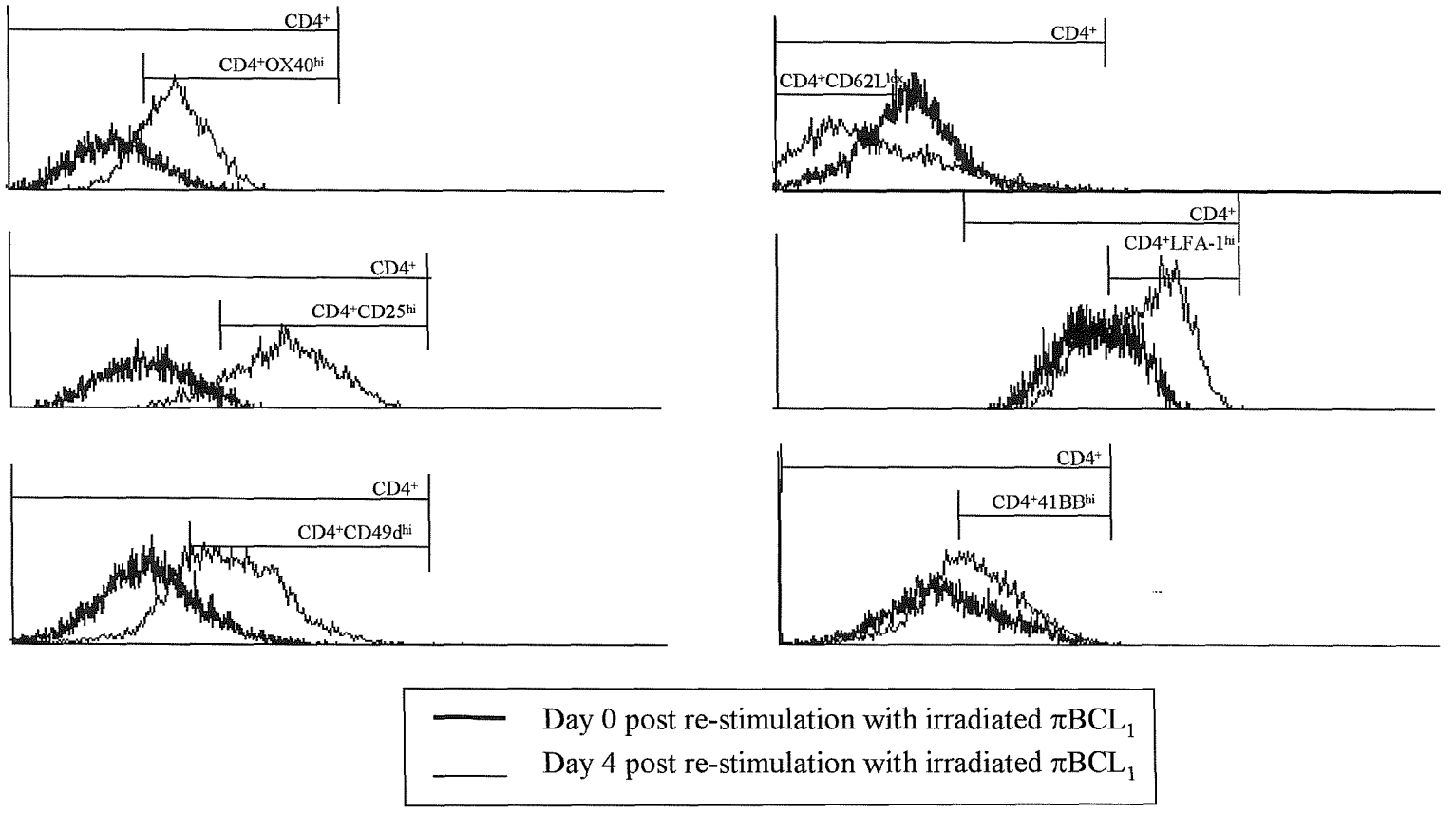


Figure 5.3.3a) Activation of an established *in vitro* generated CD4+ T cell line cultured with irradiated πBCL_1. CD4+ T cells cultured previously for 6 weeks under standard conditions were re-stimulated with πBCL_1 (day 0) in the presence of IL2 (20 units/ml) in 96 well format at a density of 1x 10^5 cells/well (irriadiated πBCL_1 was used at 5 x 10^4 /well). Flow cytometry was used to establish the expression of activation and adhesion molecules OX40, CD25, CD49d, CD62L, LFA-1 and 41BB on CD4+ T cells in culture on day 0 and day 4 post re-stimulation.

Figure 5.3.3b) Activation from day 0 to day 6 of an established CD4+ T cell line cultured *in vitro* with πBCL_1.

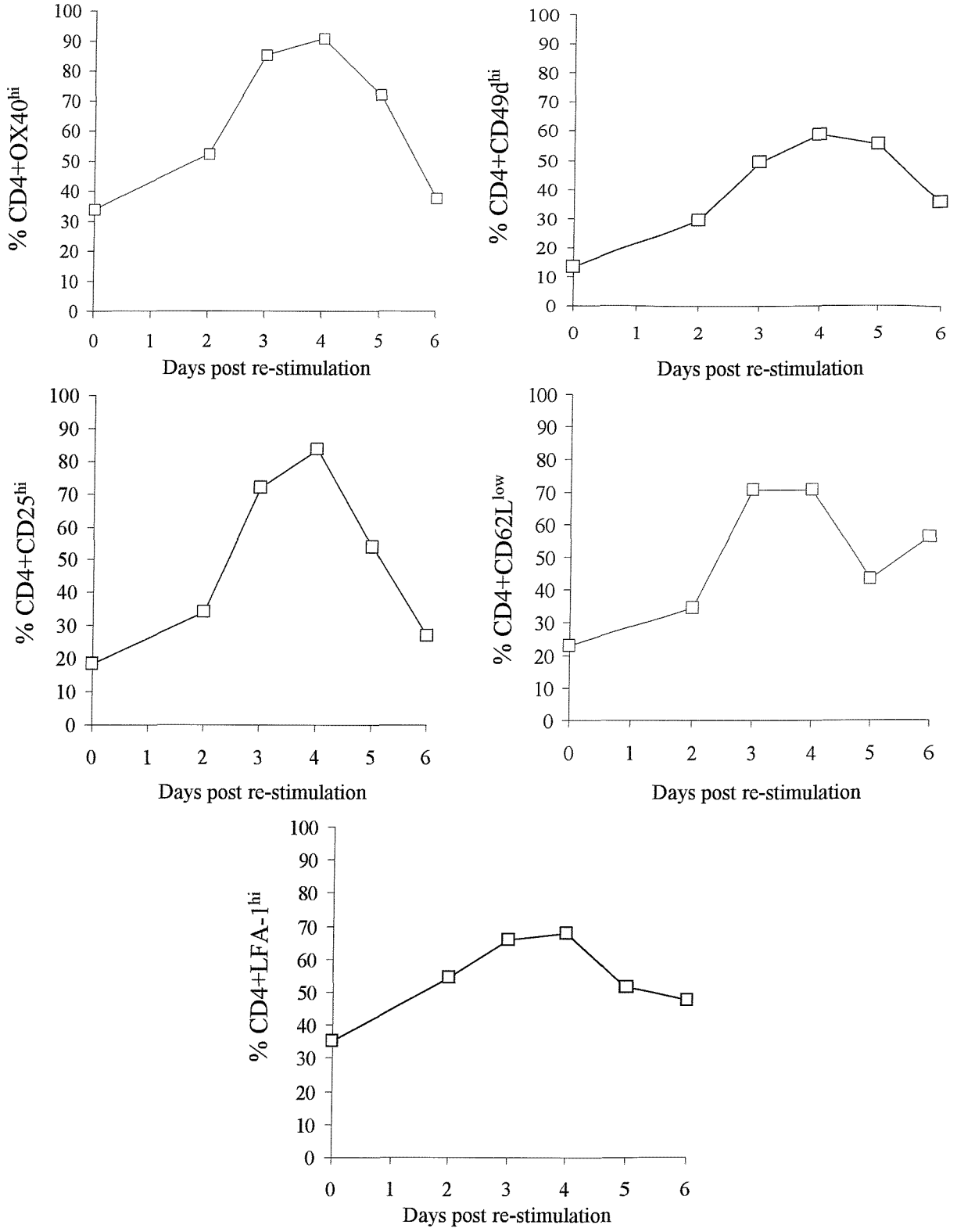


Figure 5.3.3b) Activation of an established *in vitro* generated CD4+ T cell line cultured with irradiated πBCL_1. CD4+ T cells cultured previously for 6 weeks under standard conditions were re-stimulated with πBCL_1 (day 0) in the presence of IL2 (20 units/ml) in 96 well format at a density of 1x 10^5 cells/well (irriadiated πBCL_1 was used at 5 x 10^4 /well). Flow cytometry was used to establish the expression of activation and adhesion molecules OX40, CD25, CD49d, CD62L, and LFA-1 on CD4+ T cells in culture on days 0-6 post re-stimulation.

5.3.4 Cytotoxic activity of *in vitro* generated CD4+ T cell lines.

Cytotoxic CD4+ T cell lines have been observed in both mouse and human studies(259) although their cytotoxic nature may be an artefact of *in vitro* generation (260) (261). We wished to asses whether *in vitro* generated CD4+ T cell lines had the capacity to kill target lymphoma.

Figure 5.3.4a shows cytotoxic activity by both CD4+ T cell lines CD4-π-1 and CD4-π-2. Data shows that over five weeks in culture CD4-π-2 showed increasing cytotoxic activity against πBCL_1. Taken directly from the spleen of cured mice, CD4+ T cells were not able to lyse πBCL_1 in a standard ^{51}Cr release assay in agreement with data from chapter 3 (CD8 T cells were depleted from spleen, and NK cells have previously been shown not to kill lymphoma at this time-point (258)). .After two weeks, over 30% lysis of target πBCL_1 was seen by CD4-π-2, increasing to over 60% lysis after five weeks. NK dependent killing was determined in this experiment by YAC cell lysis. Over the five weeks NK dependent lysis did increase but could not account for the large increase in killing by CD4+ T cells by CD4-π-2. Importantly, the same CD4+ T cells cultured without irradiated πBCL_1 were not cytotoxic after two weeks (data not shown). CD4-π-1 CD4+ T cell cytotoxic activity was not detected to the same extent. After five weeks *in vitro* these CD4+ T cells could only lyse <12% of target πBCL_1 and lysed over 6% of YAC cells thus were said to be non-cytotoxic.

Upon further investigation into the cytotoxic nature of CD4-π-2, dose-dependent lysis was shown, Figure 5.3.4b. Blocking of this lysis was achieved by anti-MHC class II mAb but not anti-CD8 mAb (Figure 5.3.4c) showing that the killing was CD4 dependent.

5.3.5 Tumour specificity of line CD4-π-2 after two and five weeks in culture.

Having previously determined that CD4+ T cell line (CD4-π-2) became cytotoxic after co-culture in the presence of irradiated πBCL_1, we wished to assess the specificity of this line. Thus, CD4-π-2 was tested for cytotoxic activity against other mouse tumours which also grow in BALB/c mice and which have the same class of MHC class II molecules. WEHI 231 ,A20 and P815 were assessed.

Figure 5.3.4) Cytotoxic activity of *in vitro* generated CD4+ T cell lines.

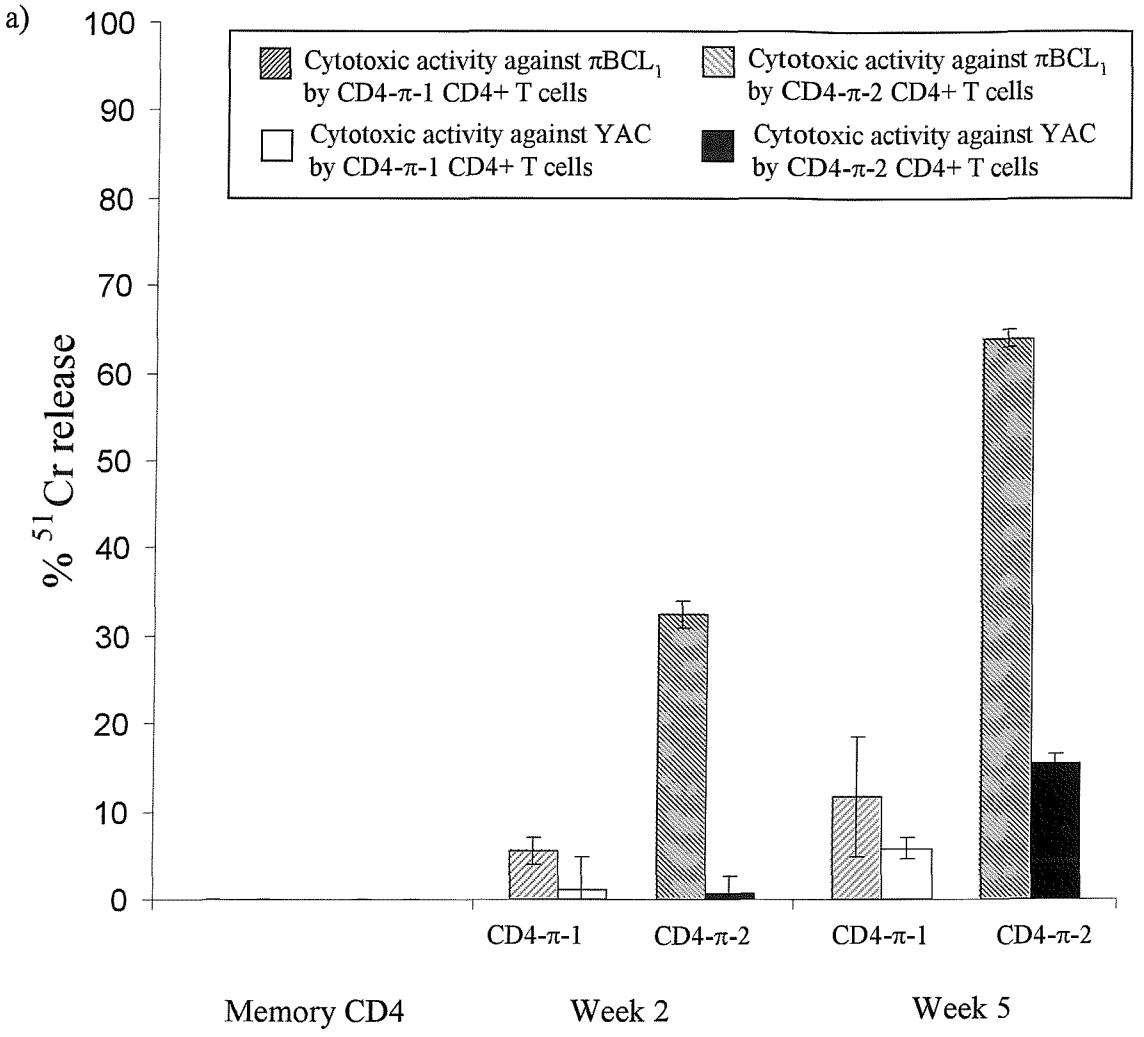


Figure 5.3.4) Increasing cyototoxic activity of CD4+T cells cultured *in vitro* over time in the presence of πBCL_1. Standard ^{51}Cr release assay was used to assess cytotoxicity against $\pi BCL_1$and YAC cells at an effector to target ratio of 20:1after 2 and 5 weeks culture in the presence of IL-7 week 1 and IL-2 thereafter.CD4+ T cells were previously assessed using flow cytometry to ensure all live cells in the culture were of a CD4 phenotype.

Figure 5.3.4) Cytotoxic activity of *in vitro* generated CD4+ T cell lines.

b)

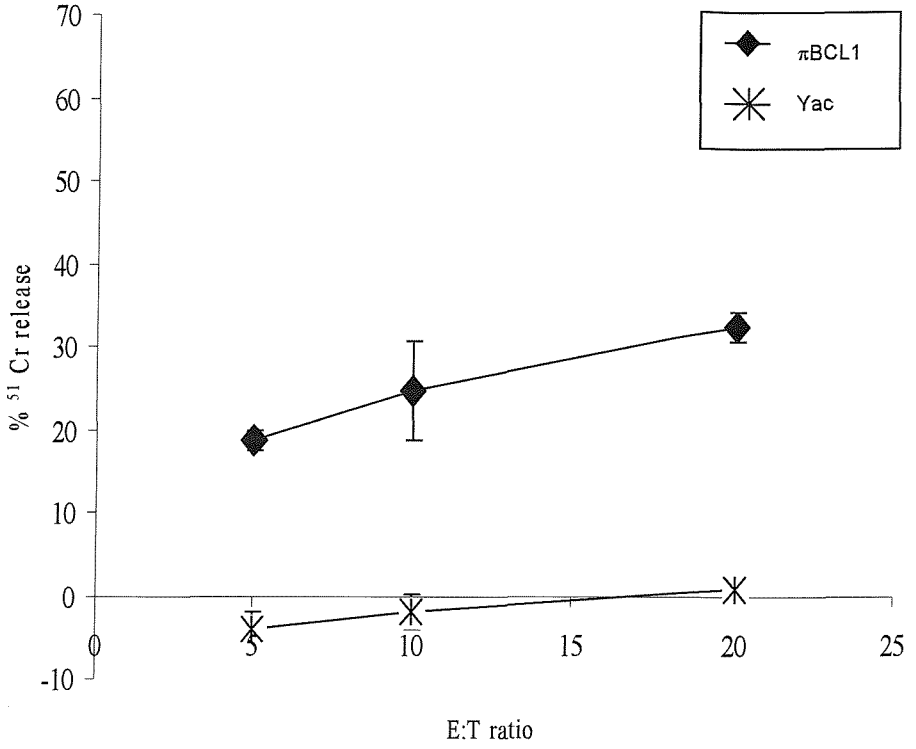


Figure 5.3.4b) Dose-dependent cytototoxic activity of CD4+T cells (anti-π-2) cultured *in vitro* over time in the presence of πBCL_1. Standard ^{51}Cr release assay was used to assess cytotoxicity against $\pi BCL_1$and YAC cells at effector to target ratio of 20:1, 10:1 and 5:1 after 2 weeks culture in the presence of IL-7 week 1 and IL-2 thereafter.

c)

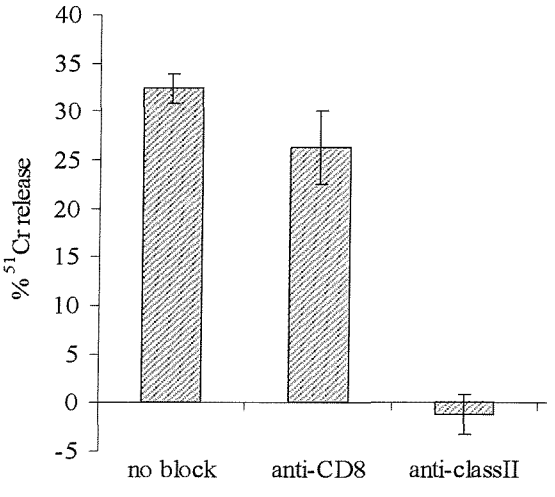


Figure 5.3.4c) Cytotoxic activity of CD4+T cells (anti-π-2) cultured *in vitro* over time in the presence of πBCL_1. Standard ^{51}Cr release assay was used to assess cytotoxicity against πBCL_1at effector to target ratio of 20:1 after 2 weeks culture in the presence of IL-7 week 1 and IL-2 thereafter. Anti-CD8 and anti-MHC class II mAb were used in assays at 50μg/ml.

Figure 5.3.5a shows that CD4-π-2 were able to effectively kill πBCL_1 as previously shown but these CD4+ T cells were not able to effectively kill any of the other lymphomas tested.

After five weeks in culture with πBCl_1 the CD4+ T cell line CD4-π-2 was able to more effectively lyse target πBCL_1 (Figure 5.3.4). We wished to investigate whether the *in vitro* generated CD4+ T cell line was still specific for πBCL_1 even with increased cytotoxic activity. Thus, we repeated the above experiment on the same T cells after five weeks. Figure 5.3.5b shows that CD4-π-2 was able to lyse target πBCL1 in a dose dependent manner killing over 60% of πBCL_1 at a 20:1 ratio and over 50% even at a 5:1 ratio of effector to target cells. CD4-π-2 lysed YAC cells in this assay at 15% at an E:T ratio of 20:1 (thus the majority of killing was through CD4+ T cells as no CD8 + T cells were present in the culture). The *in vitro* generated CD4+ T cell line was still unable to lyse WEHI 231 cells after 5 weeks. However, CD4-π-2 was able to lyse both A20 cells (over 30% lysis at a 20:1 E:T ratio) and P815 cells (over 45% lysis at a 20:1 E:T ratio).

Having established that both cytotoxic and non-cytotoxic CD4+ T cell lines could be generated *in vitro* we wished to address the mechanisms of their cytotoxicity. Thus in the next section the cytokine profiles of both CD4-π-1 and CD4-π-2 were examined.

5.3.6 Cytokine production by cytotoxic and non-cytotoxic CD4+ T cell lines in response to πBCL_1.

Cytokine production by the non-cytotoxic CD4+ T cell line CD4-π-1 was examined over a two week period after one week of previous *in vitro* culture.

The levels of T_H1 cytokines IFNγ and TNFα as well as the T_H2 cytokine IL-5 were assessed by standard ELISA from supernatants taken at day 7 and day 14 of the assay. Figure 5.3.6a shows that after 1 week IFNγ levels detected in supernatants of CD4+ T cells stimulated with and without πBCL_1 were equivalent. However, after 2 weeks IFNγ was detected at a higher level in supernatants of CD4+ T cells cultured with πBCL_1 and IL-2 than in supernatants of the CD4+ T cells cultured without tumour. TNFα production was seen to decrease in all supernatants from week one to week two. However, there was more detectable secreted TNFα in

Figure 5.3.5) Cytotoxic activity of an *in vitro* generated CD4+ T cell line.

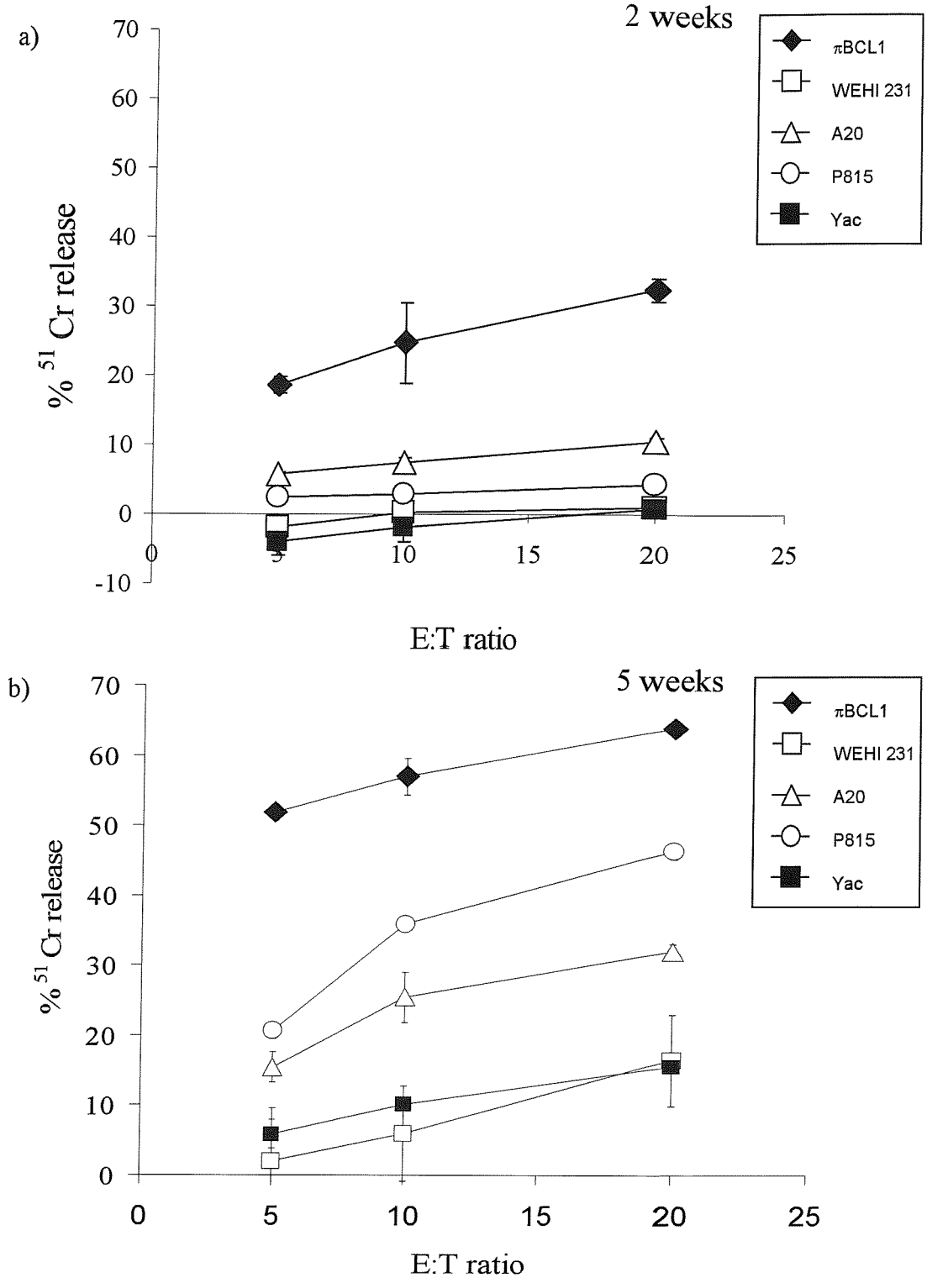


Figure 5.3.5) CD4+ T cells were grown in the presence of cytokines as previously described with πBCL_1 over 5 weeks and the cytotoxic activity against πBCL_1, A20, P815 and WEHI 231 was assessed after 2 weeks and 5 weeks. YAC killing control was performed to assess NK dependent killing.

Figure 5.3.6a) Cytokine secretion by *in vitro* cultured CD4+ T cells, grown in with or without πBCL_1.

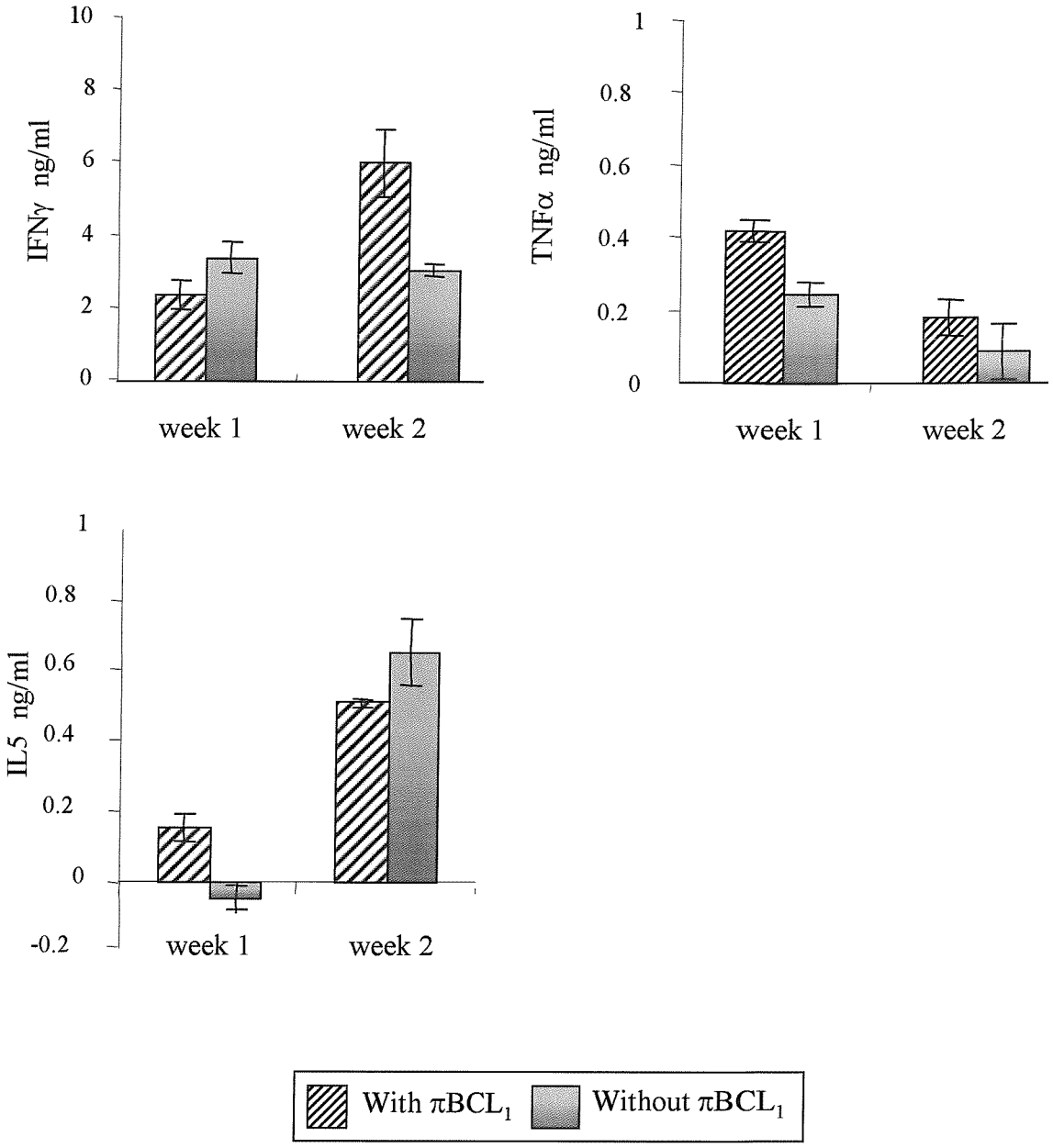


Figure 5.3.6a) Anti-π-1CD4+ T cells cultured previously for 3 weeks under standard conditions were cultured for 2 weeks with either irradiated πBCL_1, or B cells in the presence of IL2 (20 units/ml) in 96 well format at a density of 1x 10^5 T cells/well and 5 x 10^4 tumour or B cells/well. Supernatants were taken from triplicate wells on day 7 where CD4+ T cells were re-stimulated and day 14 and IFNγ, TNFα and IL5 levels were established by standard ELISA.

Figure 5.3.6b) IFNγ production by an established CD4+ T cell line generated *in vitro* with πBCL_1, in response to culture over 1 week with πBCL_1, A20, P815, WEHI 231 and B cells.

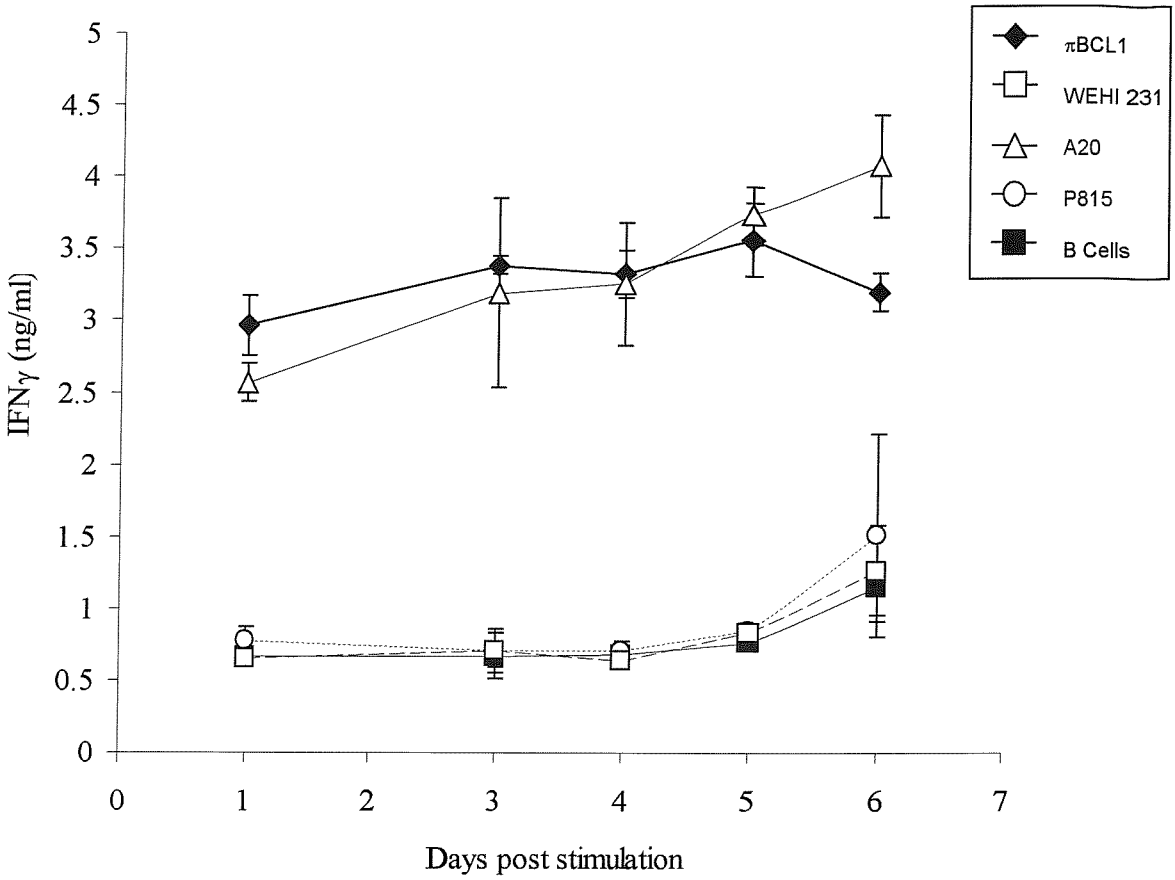


Figure 5.3.6b) Anti-π-2 CD4+ T cells cultured previously for 6 weeks under standard conditions were cultured for 1 week with either irradiated πBCL1,A20, P815, WEHI 231, or B cells in the presence of IL2 (20 units/ml) in 96 well format at a density of 1x 10^5 T cells/well and 5 x 10^4 tumour or B cells/well. Supernatants were taken from triplicate wells on day 1-6 post re-stimulation and IFNγ levels were established by standard ELISA.

supernatants of CD4+ T cells cultured in the presence of πBCL_1 than in the supernatants of CD4+ T cells cultured without πBCL_1. After two weeks the IL-5 secretion from CD4-π-1 was increased from levels detected after one week. However the increase was independent of the presence of tumour. Thus over the two week assessment of CD4-π-1, a defined skewing towards a T_H1 or T_H2 phenotype was not confirmed.

The cytokine response to πBCL_1, A20, WEHI 231 and P815 by the cytotoxic line CD4-π-2 was assessed to see if the response, unlike anti-π-1, was skewed to either T_H1 or T_H2. At the time of assay CD4-π-2 had been in *in vitro* culture for 6 weeks. Figure 5.3.6b shows IFNγ secretion by CD4-π-2 CD4+ T cells over a 6 day period in the presence of πBCL_1, A20, WEHI 231, P815 and normal murine B cells. The *in vitro* generated CD4-π-2 CD4+ T cell line produced the T_H1 cytokine IFNγ when stimulated with πBCL_1. The level of IFNγ remained between 2.5 - 3.5 ng/ml between days 1 and 6 post stimulation with irradiated πBCL_1. CD4-π-2 did not secrete IFNγ in response to the control irradiated B cells,WEHI or P815 but was able to secrete IFNγ in response to irradiated A20.

5.3.7 TCR Skewing of an *in vitro* generated CD4+ T cell line.

We wished to address whether *in vitro* generated CD4+ T cell lines showed preferential TCR usage in the presence of antigen. After 6 weeks of *in vitro* culture the Vβ usage of CD4-π-2 was investigated by flow cytometry and compared to naïve CD4+ T cells from BALB/c mice. CD4-π-2 cells were labelled with either anti-Vβ 3,6,8,9,10,13 or 14-FITC and anti-CD4-PE and the percentage of the different Vβ chain usage was calculated within the CD4 populations tested. FACS analysis of CD4-π-2 cells showing the percentage of Vβ 6, 8 and 10 positive CD4+ T cells is shown in Figure 5.3.7a and a comparison of TCR usage between CD4-π-2 cells and naïve CD4+ T cell is shown graphically in Figure 5.2.7b. Analysis of the TCR usage showed that no more than 10 % of naïve CD4+ T cells expressed any of the Vβ chains tested. However, TCR usage by CD4-π-2 cells was skewed, with more than 30% of CD4-π-2 cells expressing a Vβ8 TCR chain and more than 10% expressing Vβ6.

Figure 5.3.7) TCR skewing by CD4-π-2 CD4+ T cells

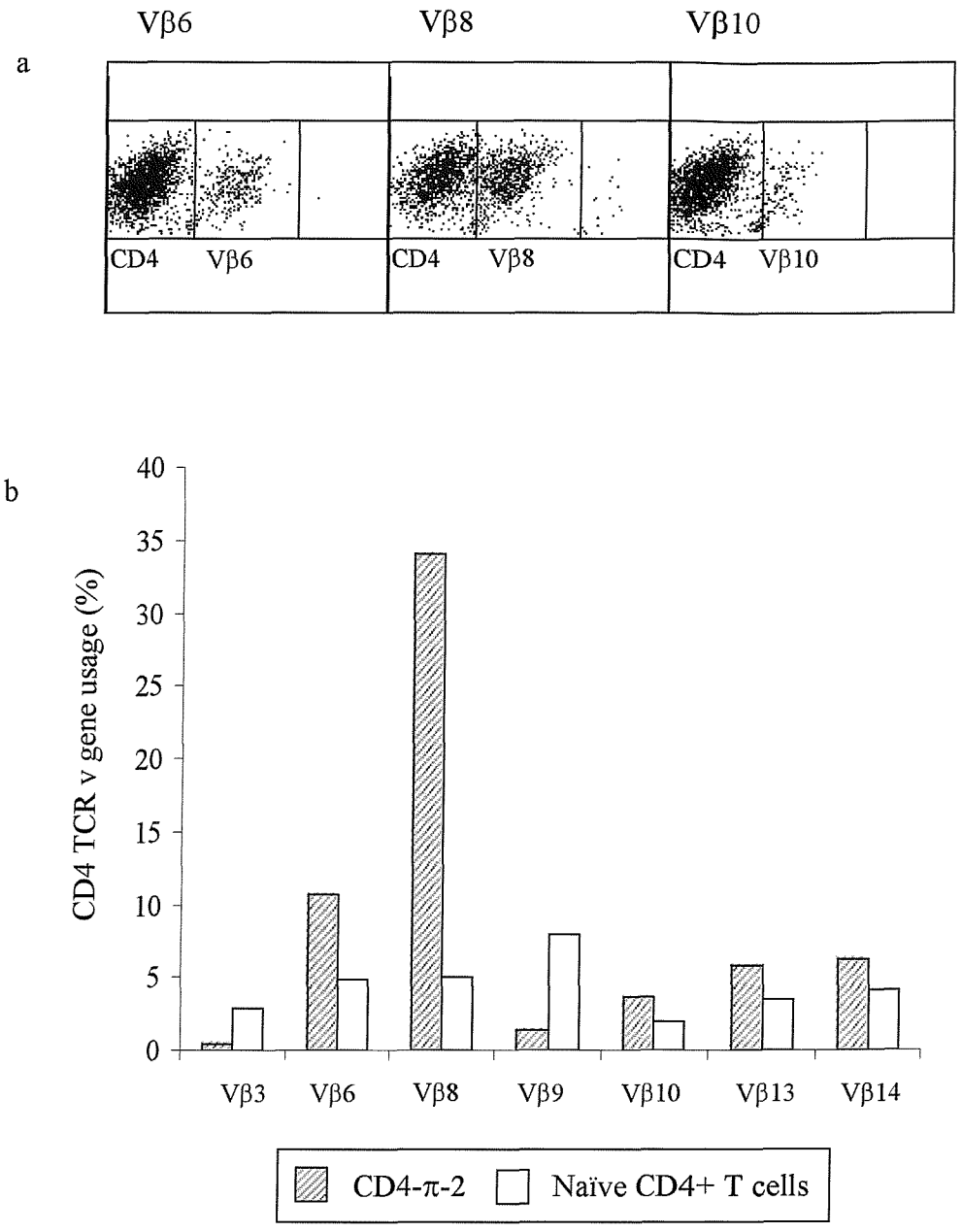


Figure 5.3.7) TCR Vβ skewing of CD4-π-2 CD4+ T cells. Skewing was assessed by immunoflurescence. Live CD4-π-2 (assessed by FSC bs SSC) cells were labelled with either anti-Vβ 3,6,8,9,10,13 or 14-FITC conjugate and anti-CD4-PE conjugate. Vβ skewed cells were worked out as a percentage of total live CD4+ T cells.

5.3.8 The proliferative response by CD4-π-2 cultured *in vitro* with πBCL$_1$, WEHI 231, P815, A20 or B cells.

The proliferative response of the CD4+ T cell line CD4-π-2 cultured *in vitro* for 7 weeks previously was investigated to determine whether CD4-π-2 would proliferate not only in response to πBCL$_1$ (as shown previously in Figure 5.2.2) but also in response to WEHI 231, P815 and A20 .

Figure 5.2.8 shows proliferation of CD4-π-2 in response to πBCL$_1$, WEHI 231, P815, and A20 all with the addition of IL-2 after five days. Over the six day period CD4-π-2 incubated on WEHI 231, A20, P815 and on B cells (data not shown) did not proliferate. The lack of CFSE dilution by CD4-π-2 cells on these tumours is evident after five days compared to the strong proliferation of CD4-π-2 incubated in the presence of πBCL$_1$, indicated by dilution of the CFSE.

Figure 5.3.8) Proliferation of an established CD4+ T cell line cultured *in vitro* with πBCL_1, WEHI 231, P815, A20 or B cells.

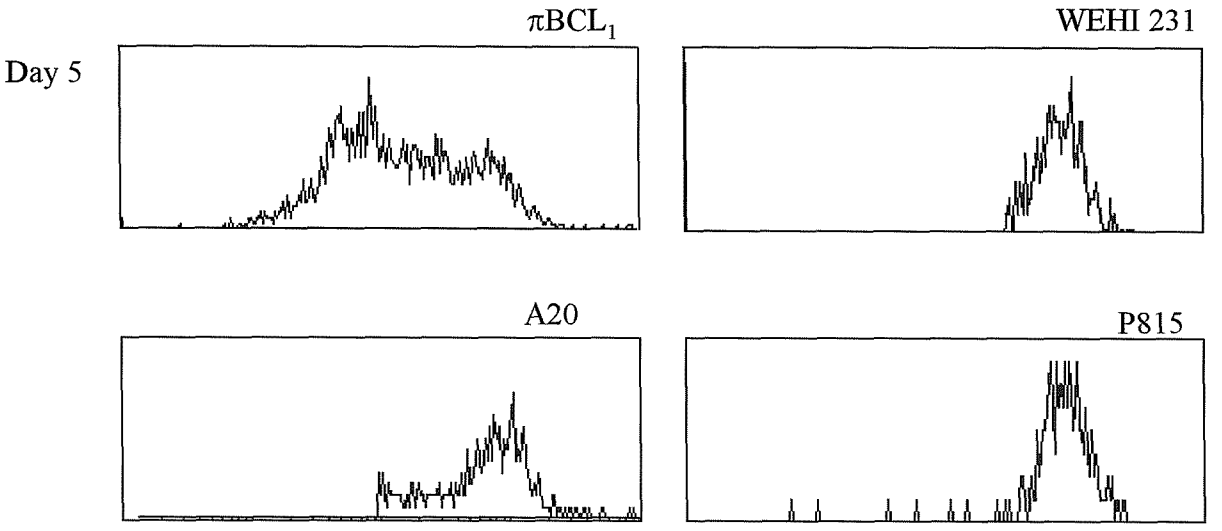


Figure 5.3.8) Proliferation of an established *in vitro* generated CD4+ T cells cultured with πBCL_1, WEHI, P815, A20 or B cells was assessed.CD4+ T cells cultured previously for 6 weeks under standard conditions were CFSE labelled and cultured with πBCL_1 WEHI, P815, A20 or B cell control.in the presence of IL-2 (20 units/ml) in 96 well format at a density of 1x 10^5 CD4+ T cells/well and 5 x 10^4 tumour or B cells/well. Cells were analysed by flow cytometry day 1-6 to investigate the division of the cells (seen by progressive dilution of the CFSE staining D1-D6). Days 5 only is shown.

5.3 Discussion

In this chapter we have shown that CD4+ T cells taken from cured mice were able to be grown in the presence of πBCL_1 (the *in vitro* line of the BCL_1 tumour). Importantly, an established line of 6 weeks proliferated on πBCL_1 and not on a B cell control in the presence of IL-2. This suggests that the *in vitro* CD4+ T cell lines generated proliferated in response to tumour antigen(s) presented by the B cell lymphoma in the context of MHC class II since the control B cells would also have been presenting antigens in the context of MHC class II but these were not tumour antigens and failed to stimulate the CD4+ T cell lines. Furthermore, in response to πBCL_1 (Figure 5.2.3) and not normal B cells (data not shown) the CD4+ T cell line (CD4-π-2) became activated and this activation peaked at day 4 post stimulation with irradiated πBCL_1, returning to basal levels (the level seen at day 0) by day 7 post stimulation. The increased expression of CD25 (the IL-2Rα chain) after culture with irradiated πBCL_1 could also explain why the proliferation of these T cell lines was so pronounced as IL-2 (20units/ml) was readily available throughout the culture period and would have acted upon the IL-2R.

Thus, the CD4+ T cells in culture which were tumour-specific would respond to weekly re-stimulations with the irradiated πBCL_1 by becoming activated and proliferating. Presumably only those CD4+ T cells which were tumour-specific would be able to survive prolonged periods *in vitro*. After two weeks CD4-π-2 CD4+ T cells were able to kill 30% tumour cells in standard ^{51}Cr release assays and after five weeks this cytotoxicity had doubled. This indicates that either the CD4+ T cells had become more effective killers or that more of the CD4+ T cells in the culture were tumour-specific. Importantly, cytotoxic activity was established to be both NK independent (^{51}Cr release assay Figure 5.3.4b) and CD8+ T cell independent (no CD8+ T cells were present in *in vitro* culture as established by flow cytometry, data not shown).

When cytotoxicity was tested against other tumours with the same MHC background it appeared that after two weeks the CD4-π-2 CD4+ T cells were πBCL_1 specific. However, the percentage lysis was low after two weeks and cytotoxicity against other tumour cells, A20 and P815 was seen albeit at below 10%. After five weeks in culture CD4-π-2 effectively lysed πBCL_1, A20 and P815 in standard ^{51}Cr release assays. This observation indicates that the CD4+ T cell line may have been recognising antigen(s)

which are not unique to (π)BCL_1 but are shared with other tumours. CD4+ dependent lysis of both P815 and A20 is an interesting observation as the presence of shared antigens between (π)BCl_1, A20 and P815 is suggested in re-challenge experiments where BCL_1 cured mice are resistant to re-challenge with A20 (258) .

Shared tumour antigens are also implicated with the generation of CD8+ CTL lines on irradiated πBCL_1 which show cytotoxic activity against both A20 and P815 aswell as to πBCL_1. Thus both MHC class I and MHC class II restricted and shared tumour antigens may exist in this model.

Cytokine profiles were examined for both cytotoxic and non-cytotoxic CD4+ T cell lines. Established CD4+ T cell lines showed either a mixed T_H1/T_H2 phenotype or a T_H1 phenotype (as shown in Figure 5.3.6). The established cytotoxic CD4-π-2 showed a T_H1 phenotype as indicated by IFNγ and not IL-5 production when stimulated with irradiated πBCL_1 and A20. Disappointingly, IFNγ was not detected when CD4-π-2 was stimulated with irradiated P815 as CD4-π-2 was able to kill this tumour at this time, however other T_H1 cytokines were not tested for at this time and could have been produced in response to P815.

Although CD4-π-2 was able to produce T_H1 cytokines in response to A20 and could kill both A20 and P815 in standard ^{51}Cr release assays, both of these tumours failed to induce proliferation in CFSE labelling experiments with CD4-π-2. Only πBCL_1 tumour could induce proliferation and cytokine, the *in vitro* line of the tumour with which the mice were originally immunised.

This may indicate that although some tumour antigens are shared, some (which the CD4-π-2 CD4+ T cells also recognised) may be unique to πBCL_1 / BCL_1. The proliferative response or lack thereof may be an consequence of the level of expression of CD25 on the CD4+ T cells in culture. Expression high on CD4-π-2 cells upon stimulation with irradiated πBCL_1 but was not assessed upon stimulation with other tumours. Other factors such as the level of co-stimulatory molecules on the different tumours may have also affected the level of proliferation of the *in vitro* CD4+ T cell lines.

Chapter 6 Antigen independent stimulation of CD8+ and CD4+ T cells

6.1 Introduction

In order for adoptive transfer to be effective against tumour *in vivo*, large numbers of tumour-specific CD8+ and CD4+ T cells are required. We have established in Chapters 3 and 4 that CD8+ tumour-specific T cells can be generated both *in vivo*, at the peak of response to anti-CD40, and also *in vitro* from spleens of mice cured of BCL_1. The latter requires the co-culture of CD8+ T cells with irradiated lymphoma. Similarly, Chapter 5 shows that CD4+ T cell lines can also be generated *in vitro* in this manner.

In this chapter we wished to investigate whether *ex vivo* expansion of T cells was possible in the absence of tumour as the availability of tumour may be limiting in the clinical setting.

We therefore tested various different methods of tumour independent stimulation of T cells using mAb against both CD3 and co-stimulatory molecules to see whether these methods could yield high numbers of viable and specific T cells.

6.2 Materials and methods

6.2.1 Stimulation of lymphocytes with anti-CD3, anti-CD28 and anti-4-1BB

Soluble mAb (anti-CD3; KT3 and 1452C11, anti-CD28; 37.51 and anti-4-1BB; LOB 12.3) were diluted in 10% RPMI, sterile filtered and used at the final concentrations stated. For experiments where plate bound anti-CD3 was required, anti-CD3 mAbs (KT3 and 1452C11) were diluted in PBS to a working concentration of 4 x final concentration required and sterile filtered. Plates were coated using 50µl anti-CD3 in PBS for 2 hours at 37°C and washed twice with sterile PBS before adding cells. Stimulation of cells on beads was carried out using M-450 Tosylactivated Dynabeads® which had been coated o/n with either KT3, 37.51 or both in accordance with manufacturers instructions (Dynal (U.K) Ltd).

Experiments were carried out in 96 or 24 well format. Cells were routinely used at a density of 1 x 10^6/ml in 10% RPMI and volumes used were either 100µl/well or 500µl/well for 96 and 24 well assays respectively. Dynabeads were used at a 1:2 ratio beads: cells. Total well volume was rouinely 200µl.

Feeder cells such as those expressing CD32 were routinely irradiated (50Gy), seeded at a density of 10^4/well and allowed to adhere for at least 2 hours at 37°C before supernatant was removed to carry out the co-culture.

6.2.2 Assessment of Apoptosis

Apoptisis was assessed using Annexin V and PI staining and cells dead cells or those undergoing apoptosis were visualised using flow cytometry as described in materials and methods, Section 2.6.

6.3 Results

6.3.1 Stimulation of *in vivo* generated CTL on anti-CD3 and anti-CD28 mAb.

In initial experiments we decided to investigate the response of CD8+ T cells to stimulation with anti-CD3 and anti-CD28 mAb with the addition of IL-2 in the culture system. *In vivo* generated CTL were investigated first, as these tumour-specific cells were readily available from the spleen of mice at the peak of response to anti-CD40 (D9). Effector splenocytes which had been enriched by MACS depletion for *in vivo* CTL (as detailed in materials and methods, Section 2.7.1) were stimulated with anti-CD3 and anti-CD28 mAb using three different methods of stimulation: with plate bound anti-CD3 and anti-CD28 mAb, with soluble anti-CD3 and anti-CD28 mAb and with anti-CD3 and anti-CD28 conjugated to dynabeads.

Figure 6.3.1 shows the proliferative response by the splenocytes enriched for effector CTL (50% CD8+ T cells) when cultured under these three conditions for 72 hours. Firstly, cells were stimulated in the presence of either plate bound or soluble anti-CD3 (using either KT3 or 1452C11 anti-CD3 mAb) with or without the addition of soluble anti-CD28 and the proliferative response measured by standard ^{3}H thymidine assay. Effector cells proliferated strongly in response to high concentrations (10μg/ml) of plate bound anti-CD3 mAb (both KT3 and 1452C11) in this experiment, and this proliferative response was augmented by the addition of 10μg/ml of soluble anti-CD28. At lower concentrations of plate bound anti-CD3 (1μg/ml) proliferation was not observed with or without anti-CD28 addition. Soluble anti-CD3 mAb were also able to stimulate effector cells but the proliferative response was always less than that of plate bound anti-CD3. The proliferative response was the same with and without the addition of 20units/ml of IL-2 with both soluble and plate bound mAb stimulation.

The level of proliferation when effector cells were stimulated with mAb coated dynabeads was consistently lower than on plate bound mAb, as also shown in Figure 6.3.1. Anti-CD28 coated dynabeads were unable to stimulate effector cells into proliferating whereas anti-CD3 coated dynabeads could, and this proliferation was augmented by the addition of anti-CD28 on the same bead only (cis-stimulation) and could not be mimicked by the addition of anti-CD28 on separate beads (trans-

stimulation). Again there was no additional proliferative response upon the addition of 20 units/ml of IL-2 to the cultures.
The greatest proliferation of effector cells was consistently observed using plate bound anti-CD3 and soluble anti-CD28.

During these experiments the viability of the effecotr cells was also assessed. The most viable cultures after four days stimulation were those stimulated on 10μg/ml plate bound anti-CD3 (KT3) in the presence of soluble anti-CD28 (>40% viability). The addition of anti-CD28 was important for the survival of these cells as viability without anti-CD28 was 10% after four days. Viability of effecotor cells after four days in culture with soluble mAb and stimulated with mAb coated dynabeads was <10%.
Importantly, in these experiments despite the effector cells being enriched for CTL, CD4+ T cells were also present and these could also proliferate on anti-CD3 and anti-CD28. Typically these cells made up less than 10 % of the cultures when peak cells were stimulated on plate bound or soluble mAb (data not shown). However, stimulation upon mAb coated dynabeads led to cultures in which CD4+ T cells outgrew the CD8+ T cells.

We therefore decided to investigate the proliferative response of *in vivo* generated CTL further by using plate bound anti-CD3 with the addition of anti-CD28, as this had yielded the most viable cells. We also positively purified CD8+ T cells in order to eliminate non-CD8+ cells from cultures. Furthermore, we also investigated the role of an additional co-stimulatory molecule 4-1BB in this system using a mAb against 4-1BB (LOB12.3) to see whether the effect of anti-CD28 on proliferation and viability could be bettered. Studies have demonstrated an important role for 4-1BB/4-1BBL in T cell activation including the production of IL-2 as well as T cell proliferation for CD8+T cells (67).

Figure 6.3.1) Proliferation of *in vivo* generated CTL *ex vivo* on anti-CD3, and anti-CD28 mAb

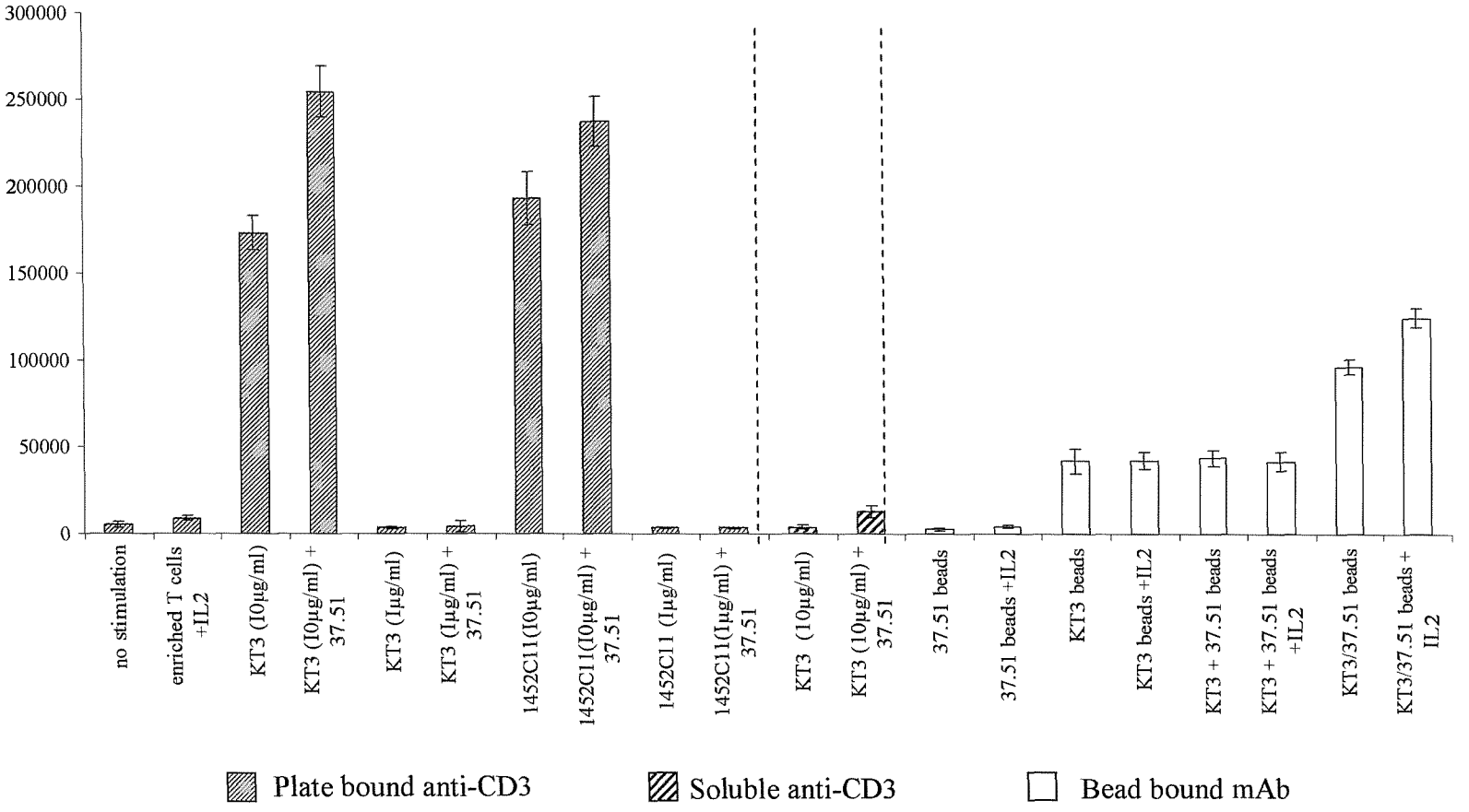


Figure 6.3.1) Proliferation of *in vivo* generated CTL on anti-CD3(KT3 or 1452C11) and anti-CD28 (37.51) mAb.The anti-CD3 mAb KT3 and 1452C11 were either coated to 96 well culture plates (plate bound) (1 hour at 37°C in PBS) , were soluble or were coated to beads. Cells were plated at 1 x 10^5 cells /well and beads were used a 1:2 ratio beads: cells, having previously performed a titration. Proliferation was assessed by ^{3}H-thymidine incorporation, 16 hour pulse.

6.3.2 Investigation of the effect of co-stimulation on proliferation of *in vivo* generated CTL on plate bound anti-CD3

In the previous experiment the proliferative effect of plate bound anti-CD3 (KT3) was investigated on relatively impure populations of CD8+CTL taken from mice D9 of anti-CD40 treatmentt (effector splenoctyes). We wished to purify the CD8+ T cells further using MACS. The addition of anti-CD28 and anti-4-1BB to cultures was assessed with respect to augmentation of the proliferative response. We also investigated the proliferative response of both naïve CD8+ T cells and CD8+ T cells taken from cured mice D20-65 of treatment. The three pools of CD8+ T cells; naïve, effector and those from cured mice were stimulated over five days on plate bound anti-CD3 (KT3), at a range of concentrations (0-10μg/ml) with and without the presence of either soluble anti-CD28 or soluble anti-4-1BB at a final concentration of 10μg/ml. The proliferative response was measured after 48 hours, 72 hours (data not shown) and five days by standard ^{3}H-thymidine incorporation assay (16 hour pulse). All CD8+ T cells were >97% pure prior to stimulation.

After 48 hours of stimulation (Figure 6.3.2a) all three pools of CD8+ T cells had proliferated in a dose dependent manner which reached a plateau after 1μg/ml. Effector CD8+ T cells (CTL) proliferated less well than either naïve CD8+ T cells or those taken from cured mice in this direct comparison. Neither anti-CD28 nor anti-41BB was seen to augment the proliferation of effector cells to the same extent as for both other types of CD8+ T cells (seen at 1μg/ml anti-CD3 concentration). After five days in culture (Figure 6.3.2b) the proliferation by effector CTL was poor and less than both other CD8+ T cell types.

6.3.3 Viability of CD8+ T cells after stimulation on anti-CD3 with and without co-stimulation

We wished to assess the viability of the three types of CD8+ T cell upon stimulation with mAb to investigate whether this method would yield enough viable CTL for use in adoptive transfer. Therefore, we assessed the percentage of live cells contained within cultures stimulated over five days on plate bound anti-CD3 at a concentration of 1μg/ml as this had led to maximal proliferation of effector cells after 48 hours, with and without the presence of either soluble anti-CD28 or anti-4-1BB. Viability was measured by annexin V and PI staining. Figure 6.3.3 shows viability of the three

Figure 6.3.2a) Proliferation of CD8+ T cells on anti-CD3 with and without co-stimulation, 48 hours after stimulation.

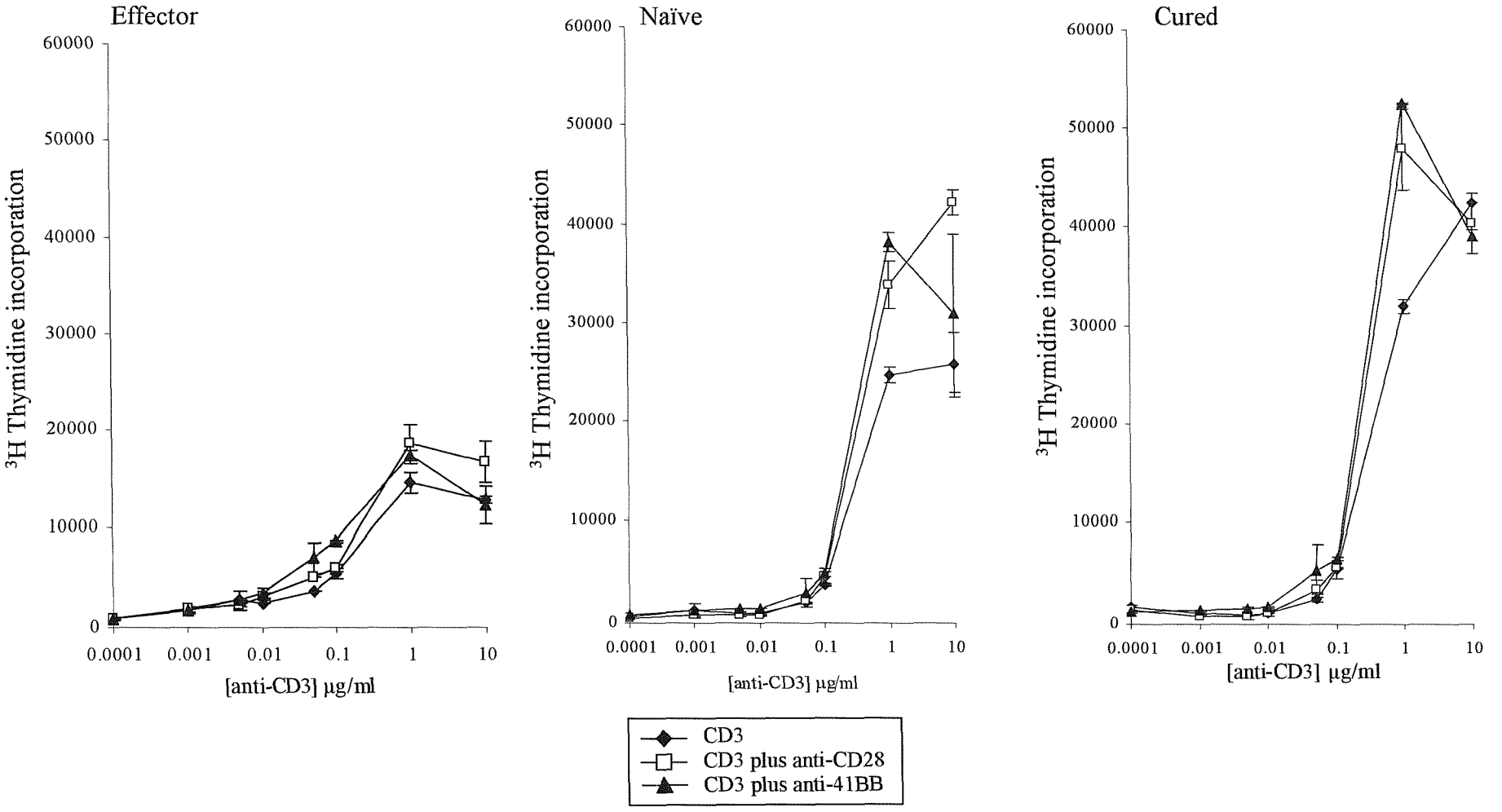


Figure 6.3.2a) Proliferation of CD8+ T cells taken from the peak of response to BCL_1 naïve CD8+ T cells, and CD8+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD8+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3)(concentration ranging from 0.0001-10µg/ml) at a density of 5 x 10^4 cells/well. Anti-CD28 and anti-41BB were added to wells at a final concentration of 10µg/ml. Proliferation was measured by adding 25µCi/well ^{3}H-thymidine (16 hour pulse) and harvesting samples after 48 hours.

Figure 6.3.2b) Proliferation of CD8+ T cells on anti-CD3 with and without co-stimulation, five days after stimulation.

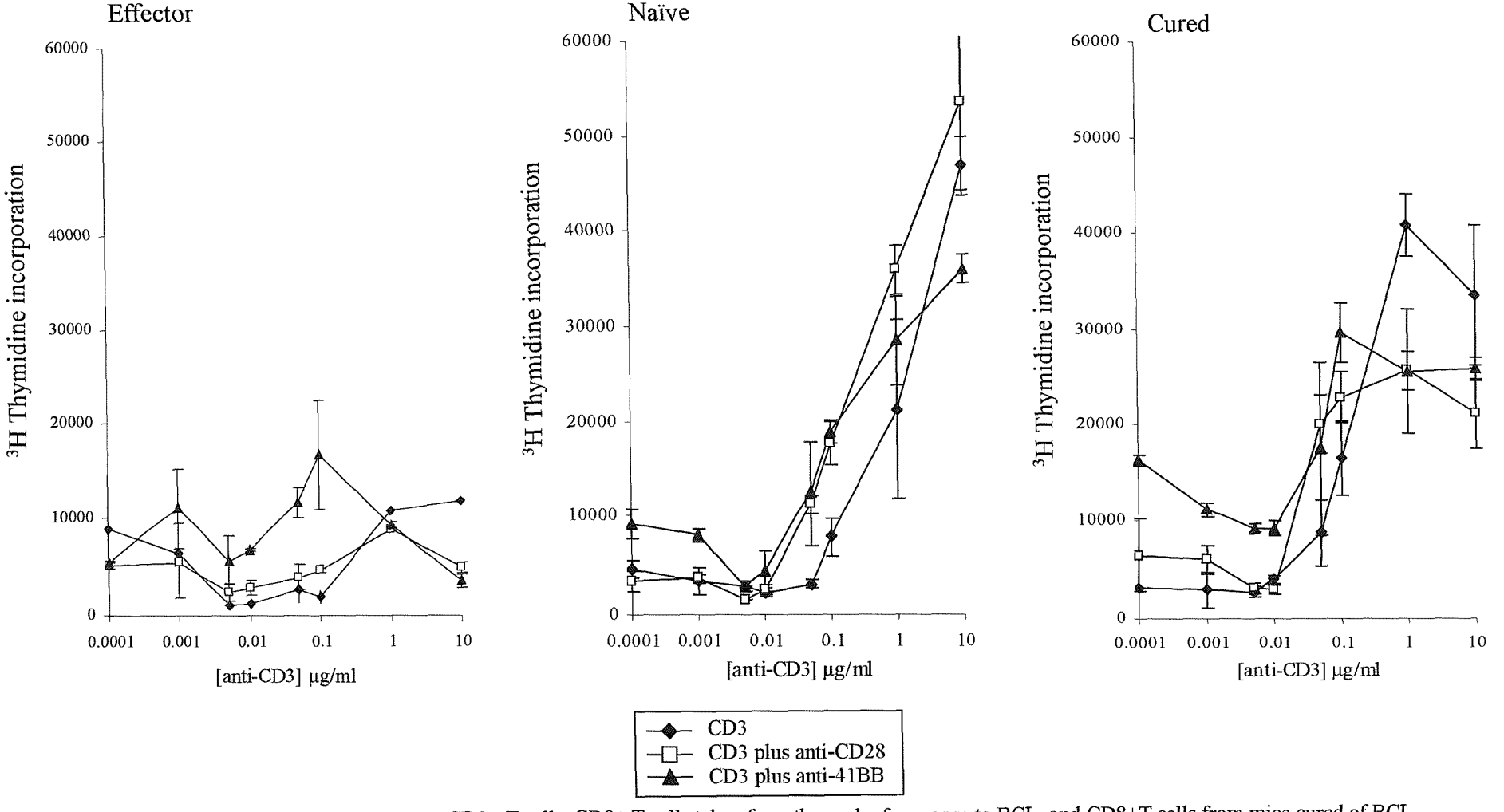


Figure 6.3.2b) Proliferation of naïve CD8+ T cells, CD8+ T cells taken from the peak of response to BCL_1 and CD8+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD8+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3)(concentration ranging from 0.0001-10µg/ml) at a density of 5 x 10^4 cells/well. Anti-CD28 and anti-41BB were added to wells at a final concentration of 10µg/ml. Proliferation was measured by adding 25µCi/well ^{3}H-thymidine (16 hour pulse) and harvesting samples after 5 days.

types of CD8+ T cells. Despite the viability of effector, naïve and CD8+ T cells from cured mice being nearly 100% at the start of the culture period, after only one day of mAb stimulation, effector CTL viability was less than 25% and this decreased over the five day period to less than 5 %. Coupled with the poor proliferation (Figure 6.3.2b), this decrease in viability left very few effector cells in culture after five days. Anti-CD28 and anti-41BB made no difference to effector cell viability.

Naïve CD8+ T cell viability decreased steadily over the five day period of mAb stimulation from approximately 50% after one day of stimulation to approximately 10% after five days. Again neither anti-CD28 nor anti-4-1BB increased the viability of these cells. By contrast CD8+ T cells taken from cured mice survived the best, remaining 30% viable after five days when stimulated in the presence of anti-CD3 and anti-4-1BB; indeed the CD8+ T cells from cured mice were the only pool of CD8+ T cells for which viability was affected by the addition co-stimulatory mAbs (10% viability without 4-1BB after five days compared with 10% viability without).

6.3.4 Viability of peak CTL upon stimulation with low concentrations of anti-CD3 with and without co-stimulation.

The disappointing survival of the effector CTL at 1μg/ml anti-CD3 led to further investigation of the viability of effector cells stimulated at lower anti-CD3 concentrations. It was hoped that stimulation on lower concentrations of anti-CD3, despite sub-maximal proliferation, would yield higher net CTL for further use due to increased peak cell viability. Viability studies were carried out as previously stated on peak cells stimulated with 0.1, 0.01 and 0.001 μg/ml plate bound anti-CD3 with and without the addition of anti-CD28 and anti-41BB.

Figure 6.3.4 shows that the viability of effector cells when simulated on plate bound anti-CD3 was poor even when low concentrations of anti-CD3 were used. Viability was consistently below 30% and numbers of effector CTL were never sufficient to carry out further work.

After five days in culture the only CD8+ T cells which had proliferated and survived well enough for further use were naïve and those from cured mice stimulated on low concentrations of anti-CD3 (data not shown). These cells were tested in standard ^{51}Cr release assays and were found to be non-cytotoxic (data not shown) and were not used in further work.

Figure 6.3.3) CD8+ T cell viability over five days on anti-CD3 with and without co-stimulation.

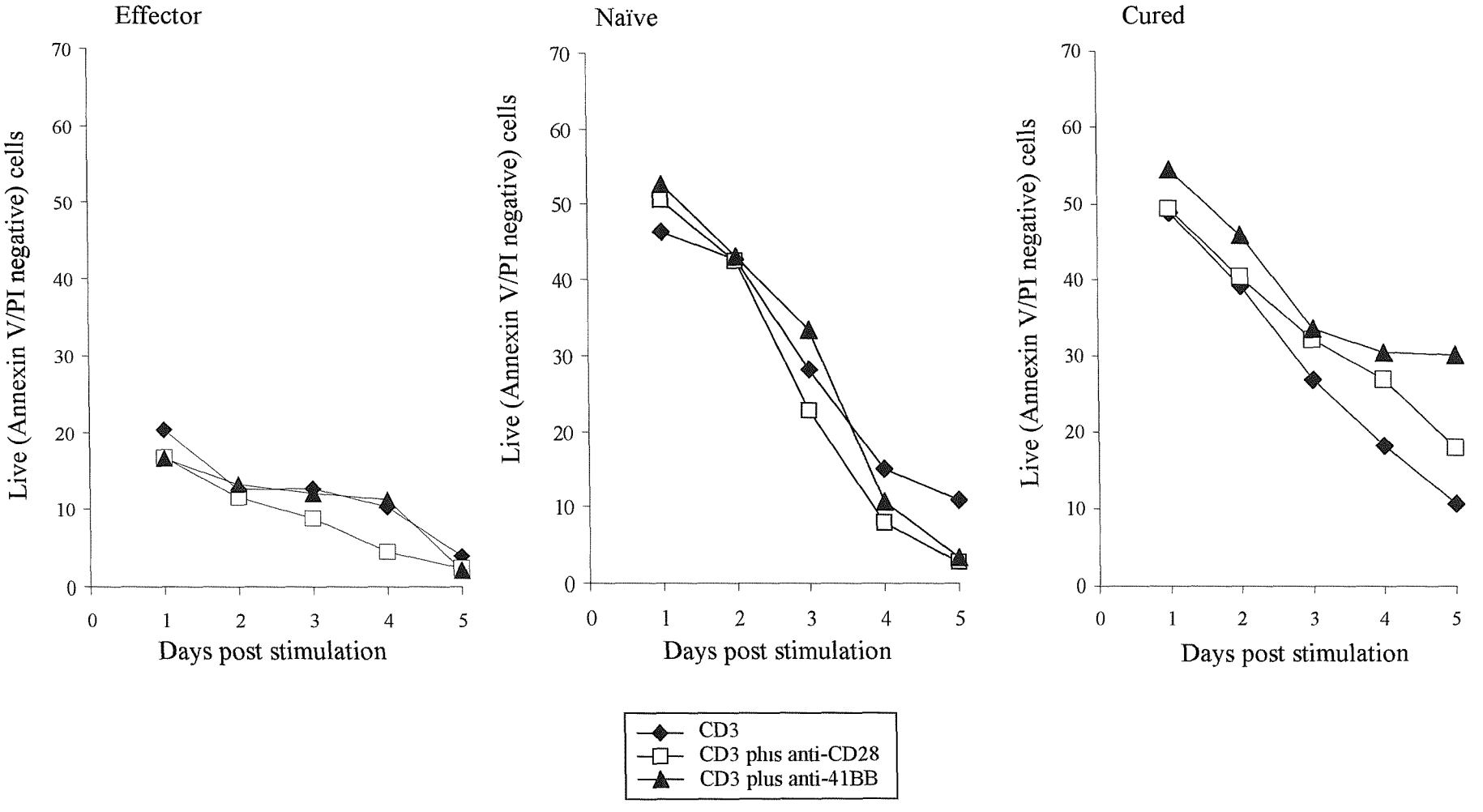


Figure 6.3.3) Viability of naïve CD8+ T cells, CD8+ T cells taken from the peak of response to BCL_1 and CD8+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD8+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3) (concentration 1μg/ml) at a density of 5 x 10^5 cells/well (24 well plate). Anti-CD28 and anti-41BB were added to wells at a final concentration of 10μg/ml. Cells were taken at day 1-5 and were analysed by flow cytometry using standard annexin V/ propidium iodide assay.

Figure 6.3.4) Effector CD8+ T cell viability over five days on low concentrations of plate bound anti-CD3 with and co-stimulation.

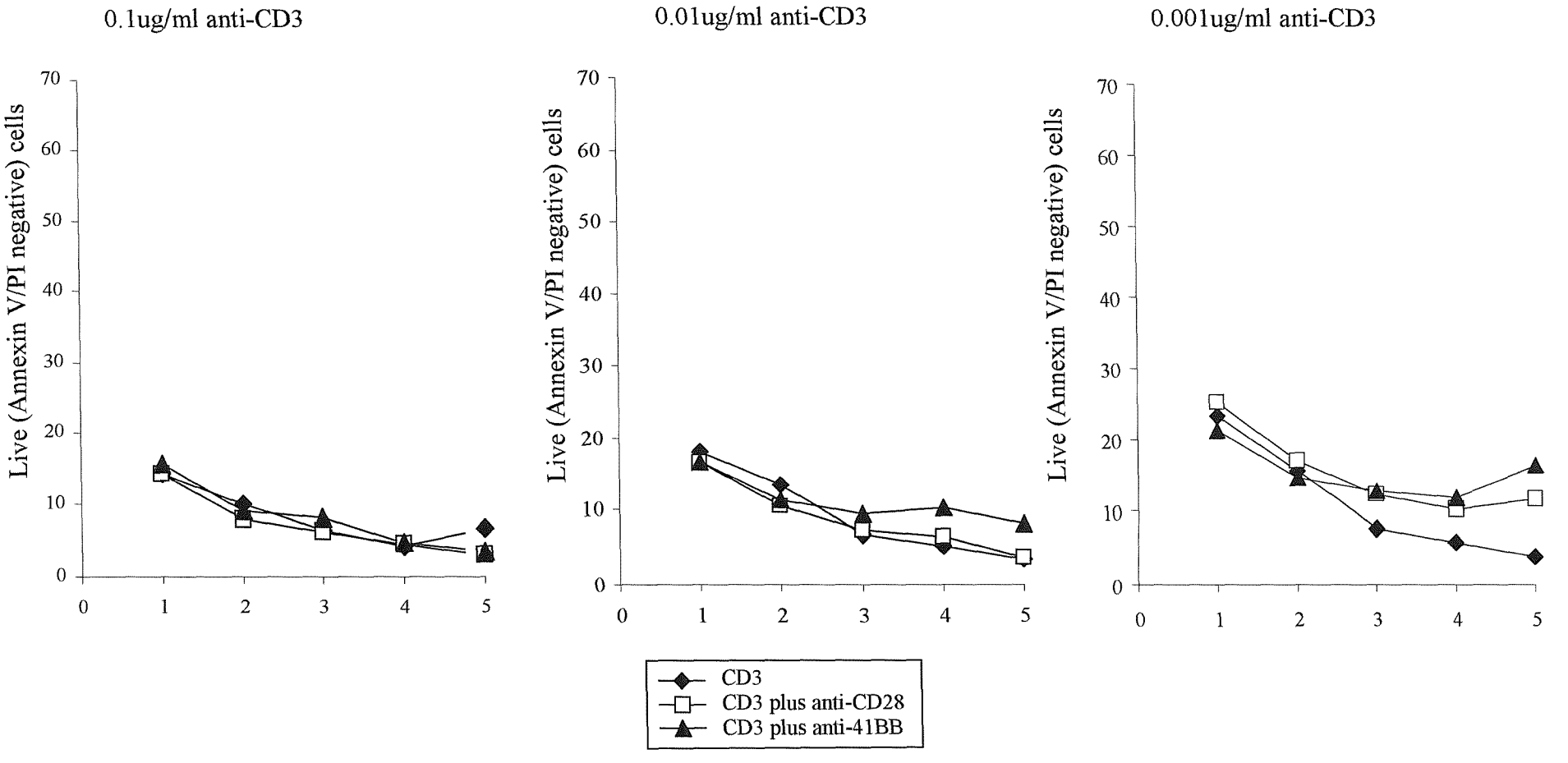


Figure 6.3.4) Viability of CD8+ T cells taken from the peak of response to BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD8+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3) (concentrations of 0.1, 0.01 and 0.001μg/ml) at a density of 5 x 10^5 cells/well (24 well plate). Anti-CD28 and anti-41BB were added to wells at a final concentration of 10μg/ml. Cells were taken at day 1,2,3,4 and 5 and were analysed by flow cytometry using standard annexin V/ propidium iodide stain.

6.3.5 IFNγ production by the three types of CD8+T cells on anti-CD3 with and without co-stimulation.

To investigate whether the poor viability of effector CTL was due to the over activation of these cells, the secretion of the T_C1 cytokine IFNγ by the peak, naïve and CD8+ T cells from cured mice was examined. The three different pools of CD8+ T cells were stimulated over five days on plate bound anti-CD3 at a range of concentrations (0-10μg/ml) with and without soluble anti-CD28 or anti-4-1BB at a final concentration of 10μg/ml and the level of IFNγ in the supernatants measured by standard ELISA. Levels of IFNγ after 48 hours, 72 hours (data not shown) and five days were determined.

Figure 6.3.5a shows IFNγ production after 48 hours. Effector CTL were producing high concentrations of IFNγ at this early time point in response to anti-CD3 in a dose-dependent manner and were even responding well to low concentrations of anti-CD3. The response was augmented by addition of either anti-CD28 or anti-4-1BB, especially anti-4-1BB. Naive CD8+ T cells produced lower levels of IFNγ at this time and, albeit in a dose dependent manner, these cells required much more stimulus through CD3 to produce the same amount of IFNγ as the peak cells. For example, to produce 4ng/ml IFNγ naïve CD8+ T cells needed to be stimulated with 1000x the amount of anti-CD3. Production of IFNγ for naïve CD8+T cells was augmented by anti-CD28 and anti-41BB to a greater extent than with the effector cells. CD8+ T cells from cured mice responded to lower levels of anti-CD3 than naïve CD8+ T cells but did not produce as much IFNγ as effector cells in response to lower concentrations of anti-CD3.

After five days stimulation (Figure 6.3.5b) all the pools of CD8+ T cells were producing large amounts of IFNγ, more than they had been at 48 hours, with all cells producing 6-7ng/ml with maximal stimulation. Compared to the dose response for the other two CD8+ T cell types, the curve for the effector cells was shifted to the left, showing that the effector cells were responding to lower levels of anti-CD3. This would indicate that the effector cells were the most activated throughout the *ex vivo* treatment.

Having established that *in vivo* generated CTL could not be expanded directly *ex vivo* using anti-CD3 and anti-CD28 or anti-4-1BB we wanted to assess whether *in vitro*

Figure 6.3.5a) IFNγ production by CD8+ T cells stimulated on anti-CD3 with and without co-stimulation after 48 hours stimulation.

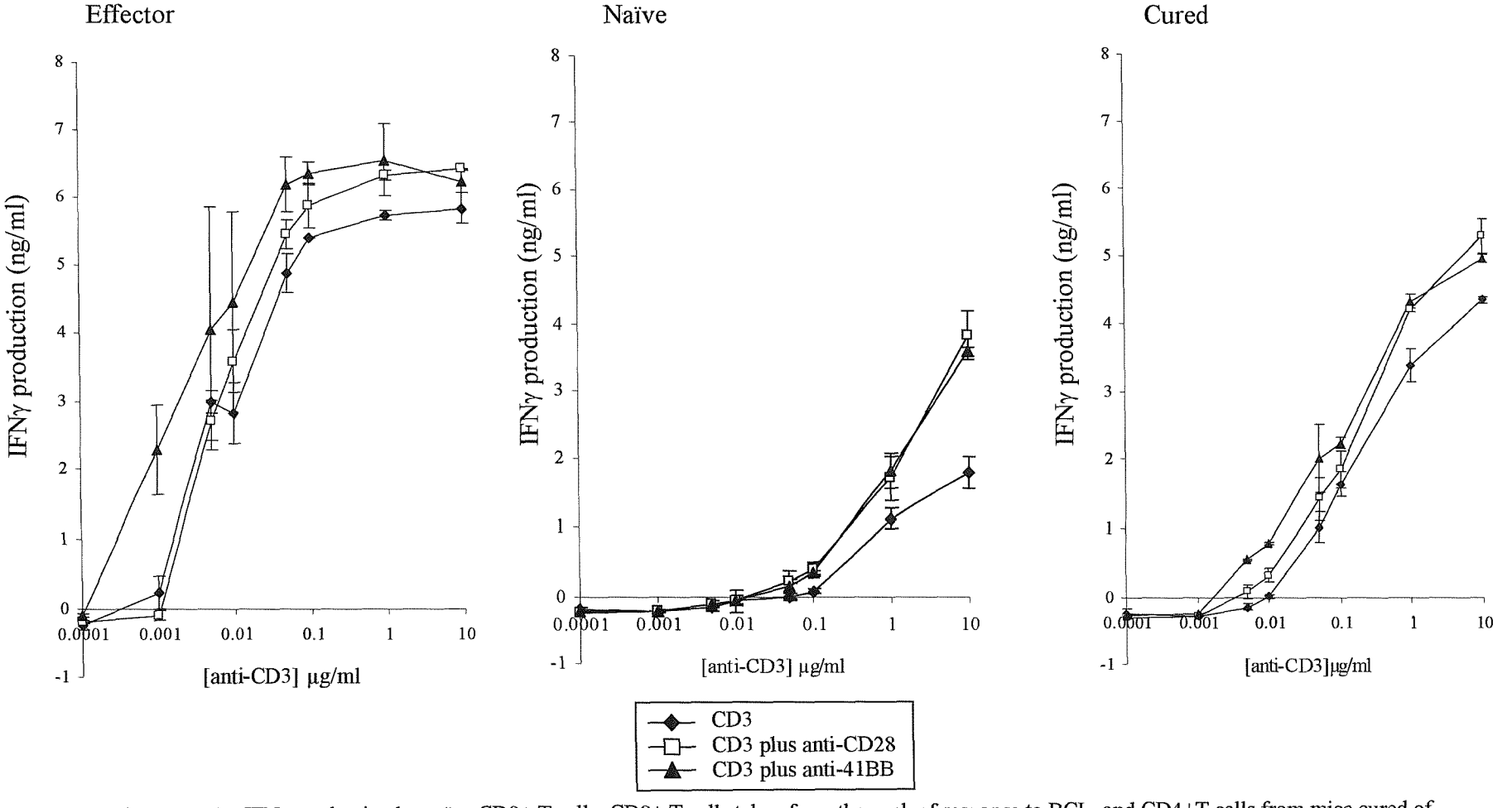


Figure 6.3.5a) IFNγ production by naïve CD8+ T cells, CD8+ T cells taken from the peak of response to BCL_1 and CD4+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD8+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3)(concentration ranging from 0.0001-10µg/ml) at a density of 5 x 10^4 cells/well. Anti-CD28 and anti-41BB were added to wells at a final concentration of 10µg/ml. Supernatants were taken at the 48 hour time point and IFNγ was measured by standard ELISA

Figure 6.3.5b) IFNγ production by CD8+ T cells on anti-CD3 with and without co-stimulation after five days stimulation.

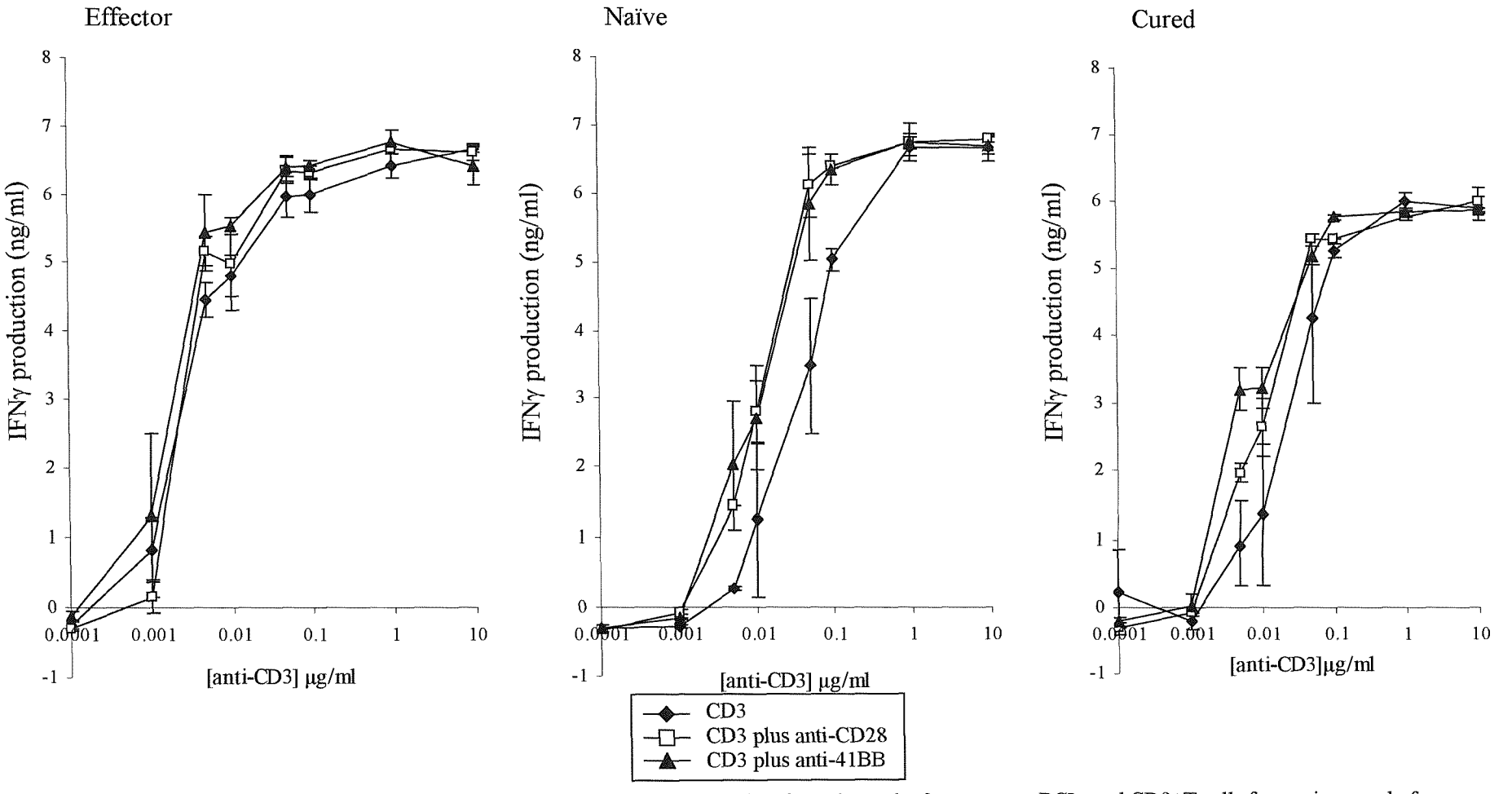


Figure 6.3.5b) IFNγ production by naïve CD8+ T cells, CD8+ T cells taken from the peak of response to BCL_1 and CD8+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD8+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3)(concentration ranging from 0.0001-10µg/ml) at a density of 5 x 10^4 cells/well. Anti-CD28 and anti-41BB were added to wells at a final concentration of 10µg/ml. Supernatants were taken at the 5 day time point and IFNγ was measured by standard ELISA

CTL lines, initially stimulated on irradiated tumour (detailed in chapter 4), would be more amenable.

6.3.6 Proliferation of *in vitro* generated CTL using antibodies.

Proliferation of *in vitro* CTL lines was carried out via stimulation with plate bound anti-CD3 at three concentrations 0.01, 0.1 and 1μg/ml. Plate bound anti-CD3 had yielded the highest proliferation in studies on *in vivo* generated CTL, and 1μg/ml was the concentration at which CD8+ T cells proliferated maximally. Again anti-CD28 and anti-4-1BB stimulation were included to investigate whether co-stimulation increased proliferation. Cultures were also incubated with and without the presence of anti-TNFα and a Fas-fc fusion protein mix to investigate whether blocking of these death pathways could improve the survival and thus increase the numbers of the CTL upon stimulation with anti-CD3 and anti-CD28/anti-4-1BB.

In vitro generated CTL (line CD8-π-4 shown here) were incubated on plate-bound anti-CD3 and co-stimulatory mAbs after 2-6 weeks *in vitro* culture with πBCL_1. Cells were rested for seven days after stimulation with irradiated tumour prior to incubation on mAb. Proliferation of the CD8-π-4 cells after 72 hours with mAb only is shown in Figure 6.3.6. Without blocking of TNFα and Fas-L, stimulation with anti-CD3 alone caused a low level of proliferation with a maximum of 1000 CPM observed. This proliferation was not augmented by the addition of anti-4-1BB. At low concentrations of plate bound anti-CD3, addition of anti-CD28 to cultures was able to increase proliferation two fold to 2000 CPM. With the addition of anti-TNFα and Fas-fc fusion protein no significant difference was seen except in cultures where anti-CD3 and anti-CD28 had been used to stimulate CTL. Here anti-TNFα and Fas-fc increased proliferation only slightly (Figure 6.3.6).

However, viability studies showed that as with the *in vivo* generated CTL, after four days in culture less than 5% of the *in vitro* generated CTL had survived, irrespective of the stimulation they had received (data not shown).

6.3.7 Proliferation of *in vivo* generated CD4+ T cells on anti-CD3 with and without co-stimulation.

Despite disappointing expansion of CD8+CTL, it was of interest to investigate whether CD4+ T cells taken from the peak of their response to tumour *in vivo* could

Figure 6.3.6) Proliferation of an *in vitro* generated CTL line (CD8-π-4) on plate bound anti-CD3 with and without co-stimulation, in the presence or absence of anti-TNFα and Fas-Fc fusion protein.

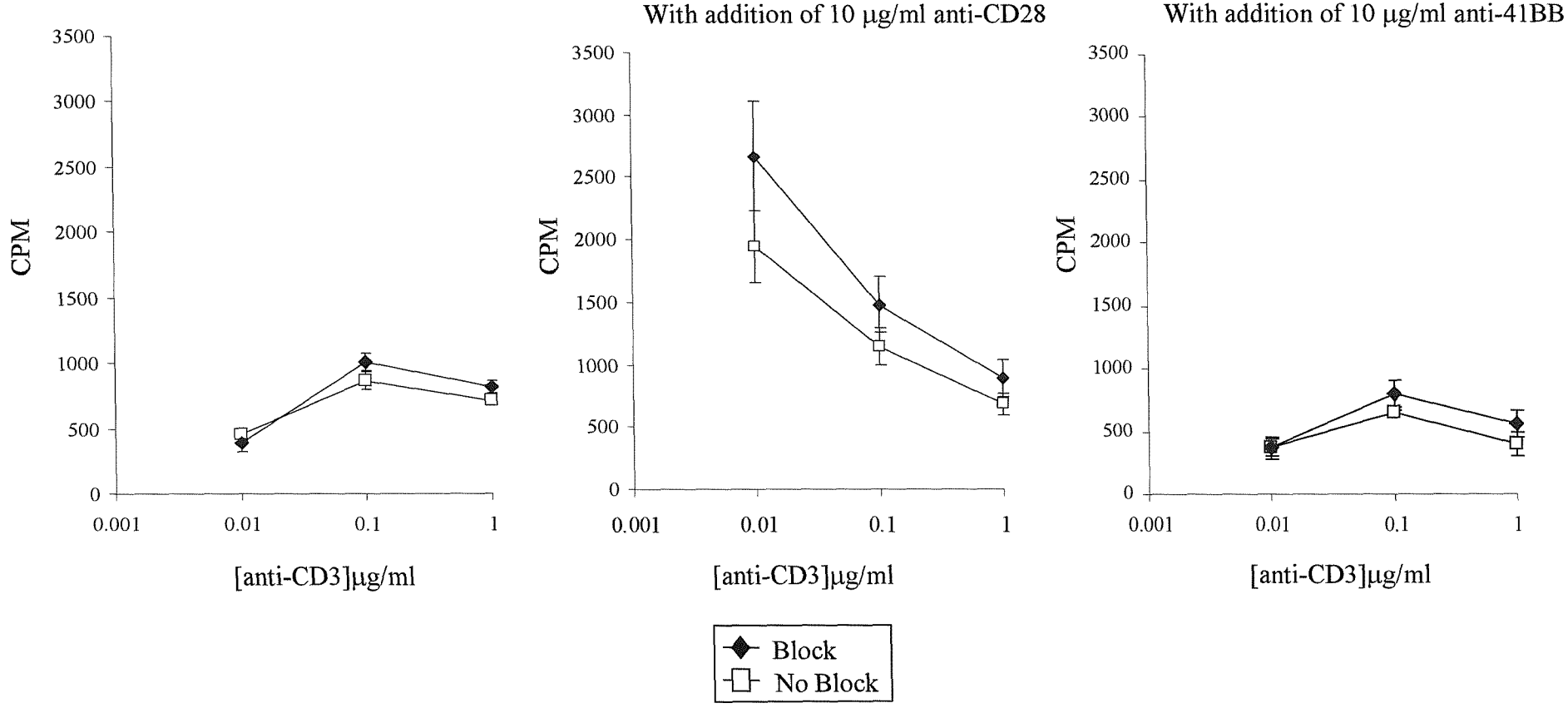


Figure 6.3.6) Proliferation of CD8-π-4 T cell line when stimulated in the presence of plate bound anti-CD3 (KT3 at a concentration of 0.01, 0.1 and 1μg/ml) and in the presence and absence of either soluble anti-CD28 (37.51 at a concentration of 10μg/ml) or anti-41BB(LOB 12.3 at a concentration of 10μg/ml).Cultures were also incubated in the presence (block) and absence (no block) of anti-TNFα mAb (at 50μg/ml) and a Fas-fc fusion protein (at a final concentration of 100μg/ml).Proliferation was determined by standard ^{3}H-thymidine incorporation after 72hours (16 hour pulse).

be expanded on anti-CD3 with anti-CD28/anti-4-1BB to generate enough numbers for use in adoptive therapy, as CD4+ T cells may be more compliant with mAb stimulation than CD8+ T cells.

As previously described for CD8+ T cells the three pools of CD4+ T cells; naïve, peak (D7 of therapy) CD4+ T cells from cured mice (D20-65 of therapy) over five days on plate bound anti-CD3 (KT3) at a range of concentrations (0-10μg/ml), with and without either soluble anti-CD28 or soluble anti-4-1BB. The proliferative response was again measured after 48 hours, 72 hours (data not shown) and five days. Figures 6.3.7a and b show the ^{3}H-thymidine incorporation after 48, and five days respectively. After 48 hours (Figure 6.3.7a) proliferation of all CD4+ T cell groups had occurred at anti-CD3 concentrations of 0.1μg/ml and above and proliferation of all CD4+ T cells, unlike their CD8+ counterparts was similar, with peak cells showing the greatest proliferation. The naïve CD4+ T cell proliferation was augmented by the addition of both anti-CD28 and anti-4-1BB, particularly evident at the 1μg/ml concentration of anti-CD3. However upon addition of anti-41BB at an anti-CD3 concentration of 10μg/ml proliferation decreased. This was also seen in the CD4+ T cells taken from cured mice where cells stimulated with 10μg/ml concentrations of anti-CD3 took up less ^{3}H-thymidine after 48 hours than those stimulated with 1μg/ml anti-CD3. Anti-CD28 augmented the response to anti-CD3 at concentrations of anti-CD3 lower than 0.1μg/ml in peak cells and CD4+ T cells from cured mice.

After five days (Figure 6.2.8b) there was a more marked difference between the proliferative response of the three CD4+T cell groups although not as marked as with the CD8+ T cells at this time point. Naïve CD4+ T cell proliferation was higher than it had been at the 48 hour and 72 hour (data not shown) time points and higher than both the peak and T cells from cured mice at this time (a maximum of 250,000 CPM seen at 1μg/ml anti-CD3 plus anti-4-1BB). Anti-CD28 was seen to decrease the proliferation of these cells at higher anti-CD3 concentrations. Peak CD4+ T cells proliferated marginally less well after five days than at any other time point tested and proliferated less with anti-CD3 alone than on anti CD3 with either anti-41BB or anti-CD28. The addition of anti-CD28 more than doubled the ^{3}H-thymidine uptake of these cells at a concentration of 1μg/ml anti-CD3. CD4+ T cells from cured mice showed a similar ^{3}H-thymidine uptake on day five to the 72 hour time point, that is

Figure 6.3.7a) Proliferation of CD4+ T cells on anti-CD3 with and without co-stimulation ,48 hours after stimulation.

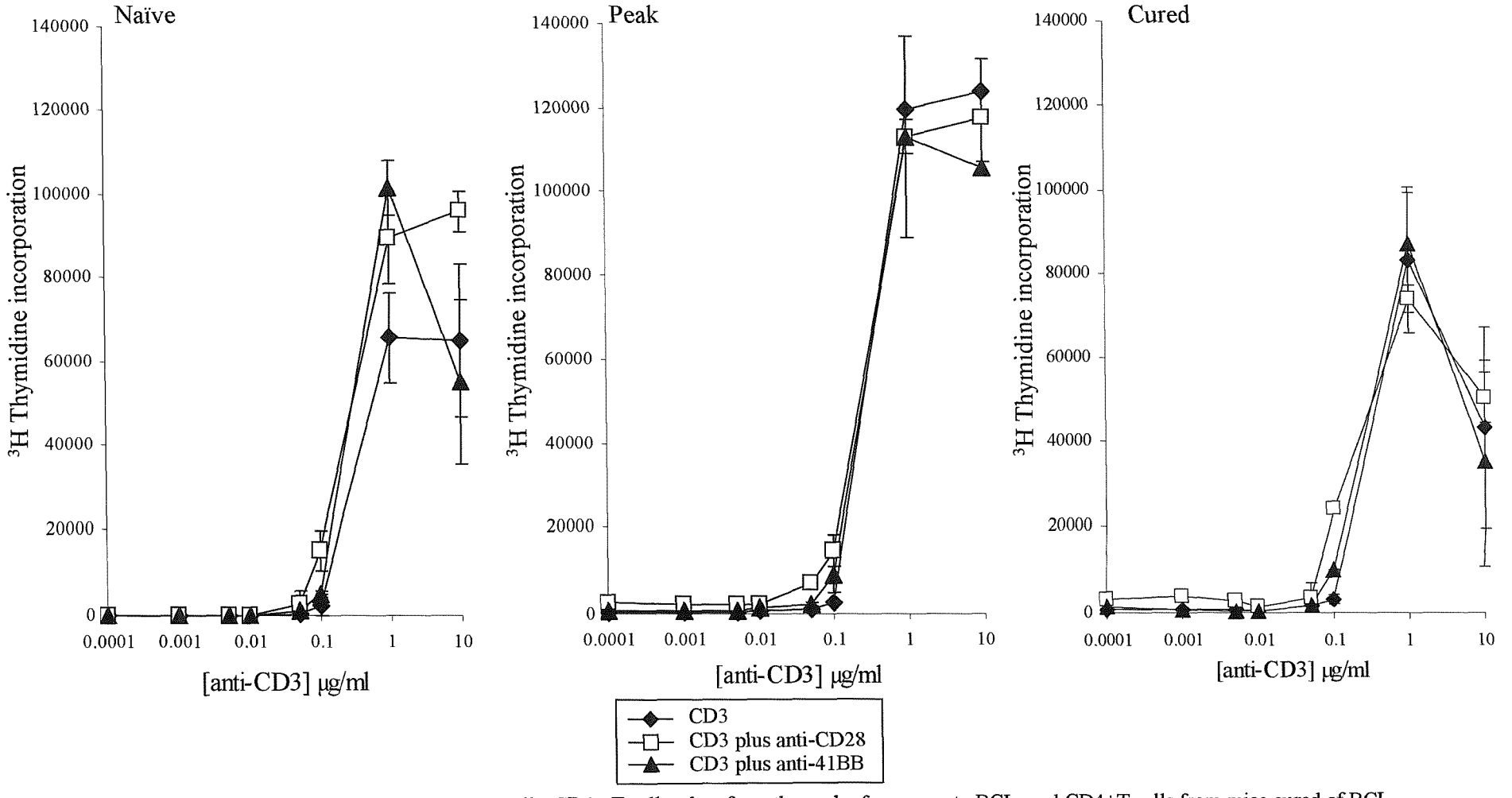


Figure 6.3.7a) Proliferation of naïve CD4+ T cells, CD4+ T cells taken from the peak of response to BCL_1 and CD4+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD4+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3)(concentration ranging from 0.0001-10µg/ml) at a density of 5 x 10^4 cells/well. Anti-CD28 and anti-41BB were added to wells at a final concentration of 10µg/ml. Proliferation was measured by adding 25µCi/well ^{3}H-thymidine (16 hour pulse) and harvesting samples after 48 hours.

Figure 6.3.7b) Proliferation of CD4+ T cells on anti-CD3 with and without co-stimulation, five days after stimulation.

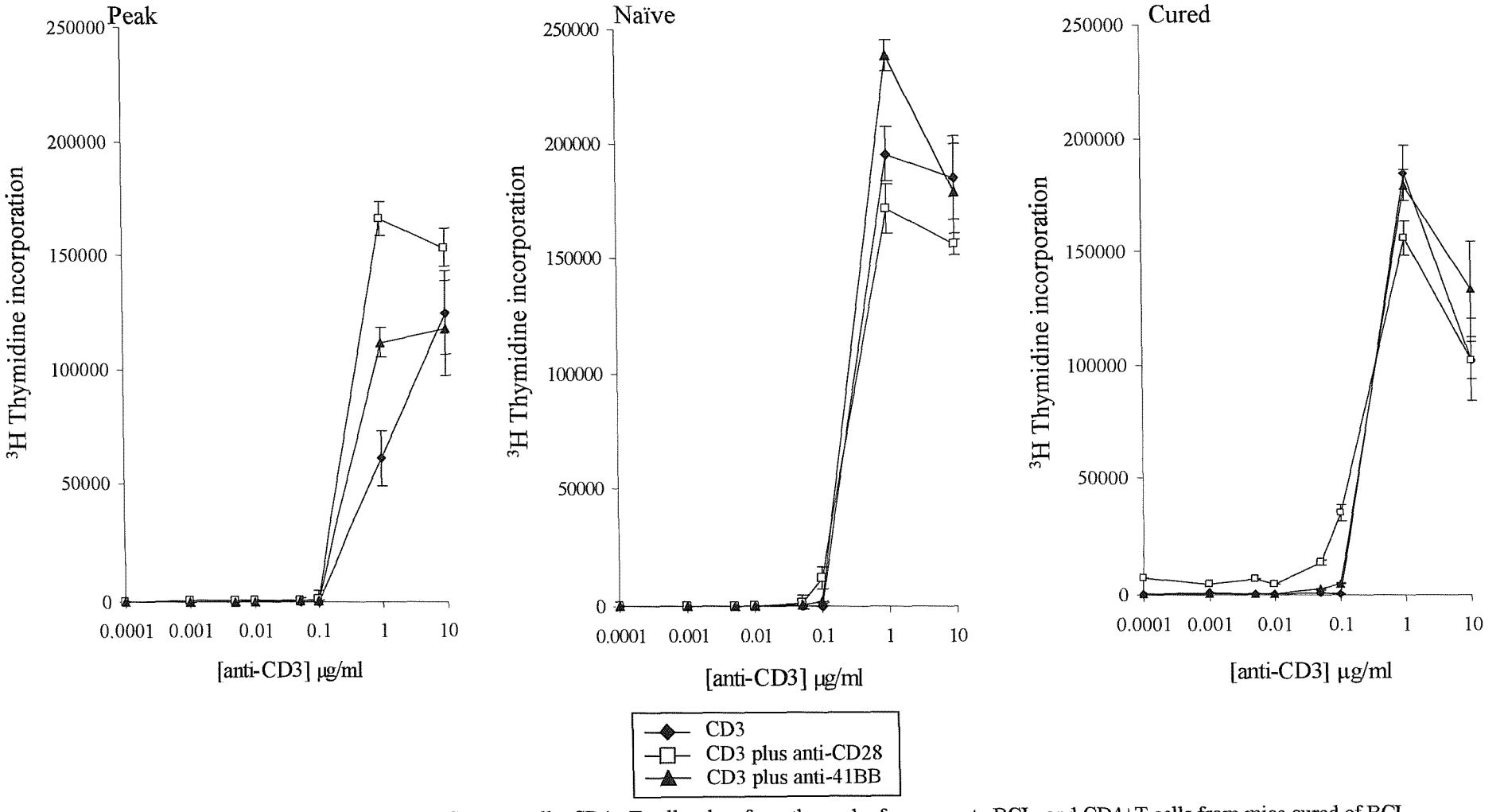


Figure 6.3.7b) Proliferation of naïve CD4+ T cells, CD4+ T cells taken from the peak of response to BCL_1 and CD4+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD4+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3)(concentration ranging from 0.0001-10µg/ml) at a density of 5 x 10^4 cells/well. Anti-CD28 and anti-41BB were added to wells at a final concentration of 10µg/ml. Proliferation was measured by adding 25µCi/well ^{3}H-thymidine (16 hour pulse) and harvesting samples after 5 days.

they proliferated less well upon the addition of anti-CD28 at the two highest concentrations of anti-CD3 and anti-CD28 augmented proliferation at concentrations of anti-CD3 lower than 0.1μg/ml.

6.3.8 IFNγ production by CD4+ T cells on anti-CD3 with and without co-stimulation.

The three pools of CD4+ T cells were stimulated over five days on plate bound anti-CD3 at a range of concentrations (0-10μg/ml) with and without either soluble anti-CD28 or soluble anti-41BB at a final concentration of 10μg/ml; the level of the T_H1 cytokine IFNγ in the supernatants was then measured.

Figure 6.3.8 shows IFNγ production after 48 hours of stimulation, all three pools of CD4+ T cells produced increasing levels of IFNγ with increasing anti-CD3 concentration. In all cases and at all anti-CD3 concentrations, the IFNγ production was augmented by the addition of anti-CD28 or anti-4-1BB, with anti-CD28 together with anti-CD3 stimulation yielding the highest IFNγ concentrations. Both naïve and CD4+ T cells from cured mice showed similar profiles, with peak CD4+ T cells producing slightly more IFNγ under these conditions.

By 72 hours (data not shown) the level of IFNγ in the supernatants was higher for all CD4+ T cells tested than at 48 hours, with peak cells showing slightly higher production than the other two pools. Once again, the CD4+ T cells stimulated with anti-CD28 or anti-4-1BB along with anti-CD3 showed the highest IFNγ production.

After five days (data not shown) all CD4+ T cell tested were still producing high and equivalent levels of IFNγ and all groups

6.3.9 Viability of CD4+ T cells on anti-CD3 with and without co-stimulation

During the previous experiments where naïve, peak and CD4+ T cells from cured mice were allowed to proliferate on plate bound anti-CD3 with or without anti-CD28 or anti-4-1BB the viability of the CD4+ T cells was assessed. For this type of experiment purified CD4+ T cells were stimulated on 10, 1, 0.1 and 0 μg/ml plate bound anti-CD3 with or without soluble anti-CD28 or anti-4-1BB at 10μg/ml; the viability was assessed days 2-5 after stimulation using standard annexin V and PI staining.

Figure 6.3.8) IFNγ production by CD4+ T cells on anti-CD3 with and without co-stimulation after 48 hours stimulation.

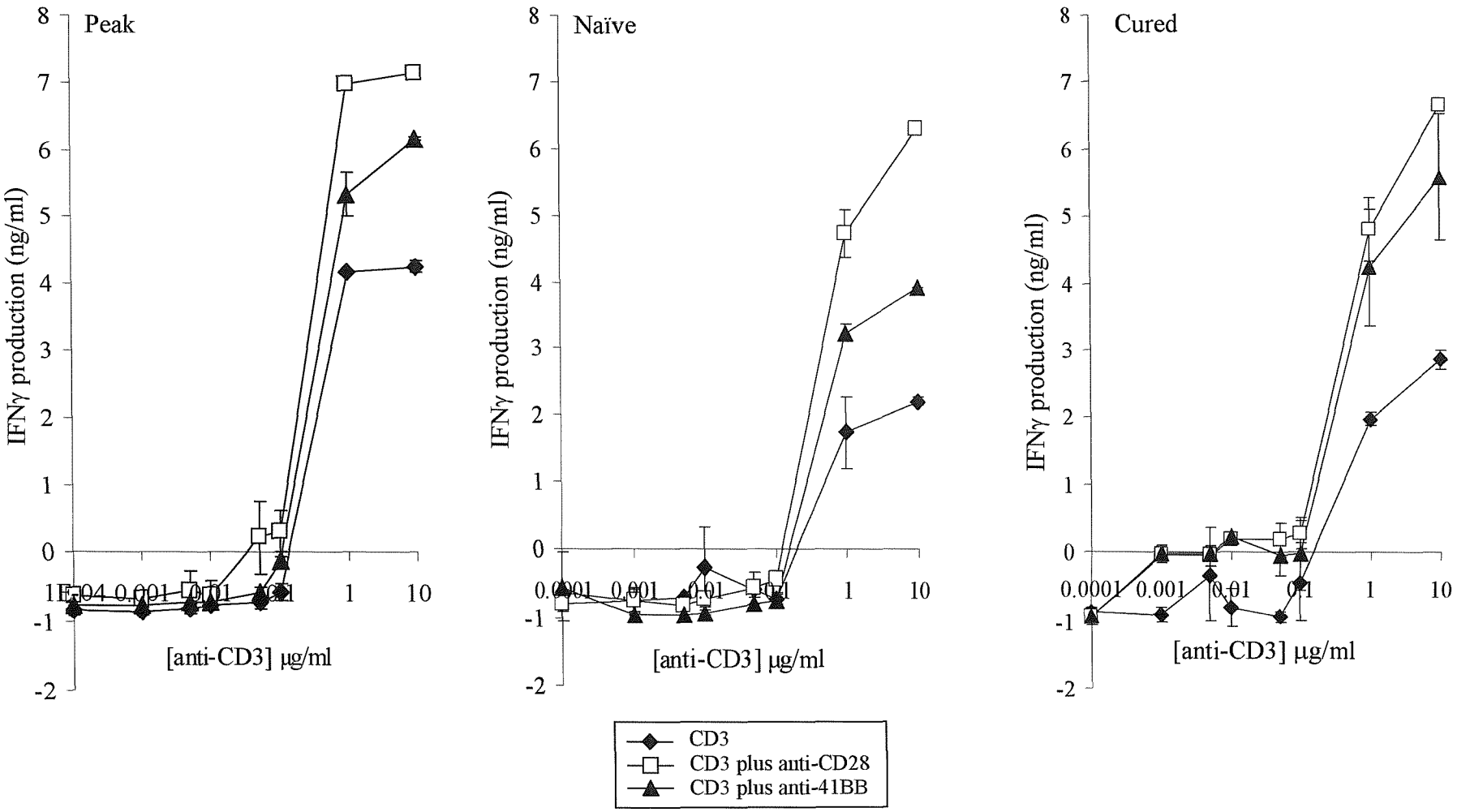


Figure 6.3.8) IFNγ production by naïve CD4+ T cells, CD4+ T cells taken from the peak of response to BCL_1 and CD4+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD4+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3)(concentration ranging from 0.0001-10µg/ml) at a density of 5 x 10^4 cells/well. Anti-CD28 and anti-41BB were added to wells at a final concentration of 10µg/ml. Supernatants were taken at the 48 hour time point and IFNγ was measured by standard ELISA

Figure 6.3.9) Viability over five days of naïve CD4+ T cells, CD4+ T cells taken from the peak of response to BCL_1 and CD4+T cells from mice cured of BCL_1 on anti-CD3 with or without anti-CD28 or anti-41BB.

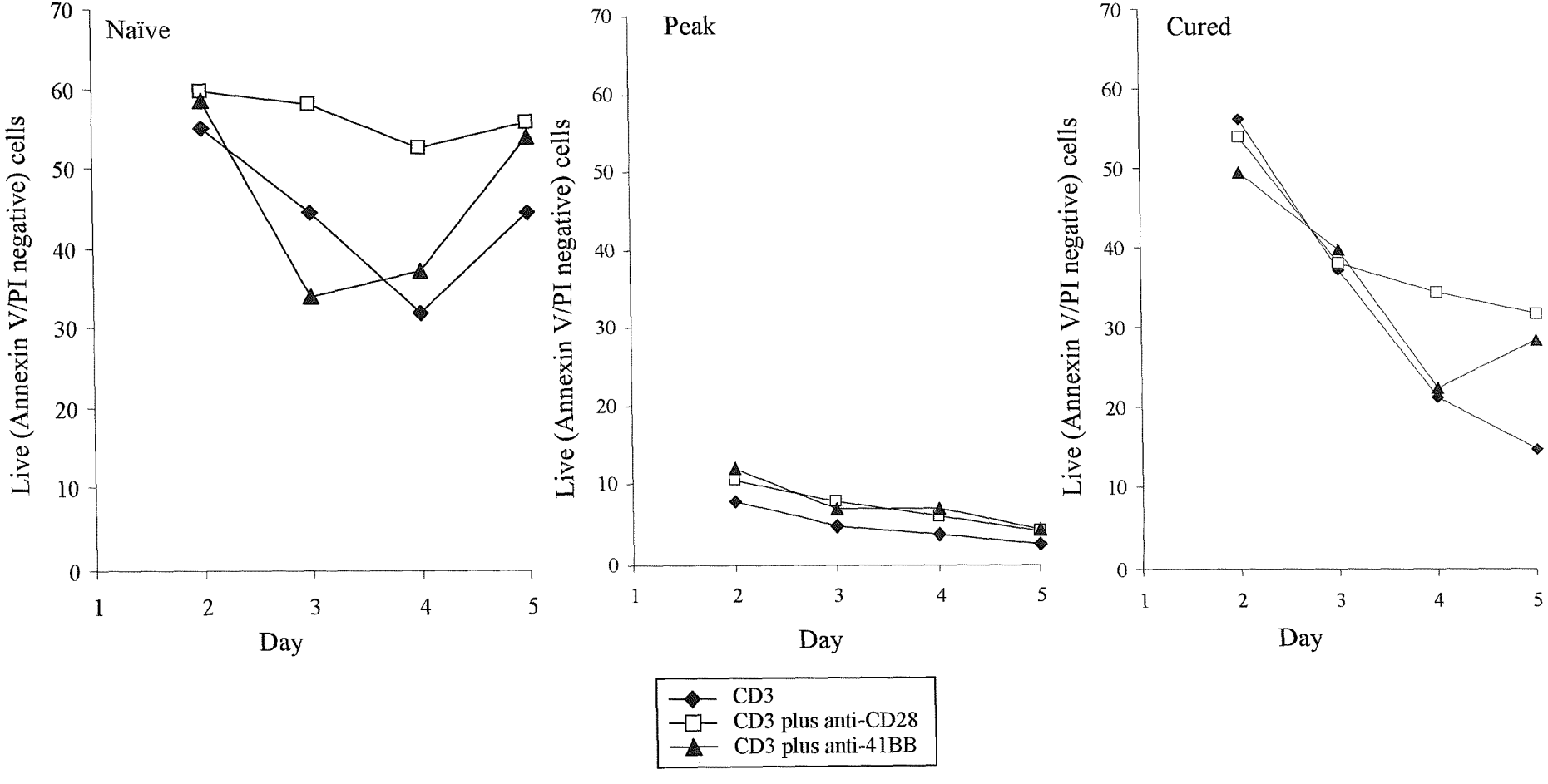


Figure 6.3.9) Viability of naïve CD4+ T cells, CD4+ T cells taken from the peak of response to BCL_1 and CD4+T cells from mice cured of BCL_1 on anti-CD3 with and without anti-CD28 and anti-41BB. CD4+ T cells were isolated using MACS and seeded onto plate- bound anti-CD3 (KT3) (concentration 1μg/ml) at a density of 5 x 10^5 cells/well (24 well plate). Anti-CD28 and anti-41BB were added to wells at a final concentration of 10μg/ml. Cells were taken at day 2,3,4 and 5 and were analysed by flow cytometry using standard annexin V/ propidium iodide stain.

Figure 6.3.9 shows the viability of all three pools of CD4+ T cells when stimulated on 1μg/ml anti-CD3 with and without the addition of either anti-CD28 or anti-41BB. For all other anti-CD3 concentrations used the viability observed was less than at 1μg/ml, thus figure 6.3.9 shows the highest viability observed for all pools of CD4+ T cells in this experiment.

60% Naïve CD4+ T cells in the cultures were viable at day two post stimulation and in cultures stimulated with anti-CD3 and anti-4-1BB or with anti-CD3 alone, viable cell numbers decreased to 30% on days three and four respectively increasing to between 45 and 60 % on day five. Cultures stimulated with both anti-CD3 and anti-CD28 remained approximately 60% viable throughout the experiment.

Peak cells, however, were less viable upon mAb stimulation with all combinations of mAb yielding less than 15% viable cells after two days decreasing to less than 5% after five days. The addition of anti-CD28 and anti-4-1BB was seen to increase viability only slightly.

The viability of CD4+ T cells from cured mice also decreased steadily over the five day period. Cells cultured on anti-CD3 alone were least viable by day five at 15% viability compared to approximately 30% viability when either anti-CD28 or anti-4-1BB had been included in the cultures.

6.4 Discussion

Adoptive transfer of T cells has been shown to be effective in the regression of some tumours in experimental models (118,262). From these previous studies it is evident that for adoptive immunotherapy to be effective against tumour a large quantity of anti-tumour T cells have to be transferred. Thus, any means of expanding reactive cells must generate sufficient numbers of cells whilst maintaining or augmenting their tumour-reactivity.

Anti-CD3 antibodies were shown to be mitogenic for both human and mouse T lymphocytes almost two decades ago (263,264). Many methods have since been utilised to attempt to expand cells in an antigen independent manner in both animal and human studies for adoptive therapy regimes. Anti-CD3, anti-CD28 and IL-2 have been shown to expand T cells as detailed in Chapter 1 Section 1.6.2.2.2.

Artificial antigen presenting cells (also detailed in Section 1.6.2.2.2) have also been utilised in attempts to proliferate both CD4+ and CD8+ T cells. These have included mAb coated beads and cell lines stably transfected with CD32 the low affinity Fcγ receptor and loaded with anti-CD3 and anti-CD28 and more recently also transfected with 4-1BB ligand (4-1BBL).

In our first experiments, cultures were made up of mixed splenocyte populations, including some CD4+ T cells which would also be stimulated by anti-CD3 mAb and anti-CD28. However, the use of peak effector splenocytes did allow the investigation into various methods of stimulation of CTL *ex vivo*. The bead-based system gave the lowest rates of proliferation in our hands using enriched peak splenocytes and in accordance with data from other laboratories using human cells, preferentially expanded CD4+ T cells (208). Differing bead:cell ratios were investigated and the best proliferation seen is shown in Figure 6.1.3. Not only was CD8+ T cell expansion poor on beads but the use of a bead based system also has other problems when being transferred to the clinic, such as the expense and removal of the beads before administration of the cells into patients. Therefore, for these reasons the bead based system was not examined further. After extensive investigation in to proliferation using both soluble and plate bound mAb using a wide range of mAb concentrations (data not shown), the greatest proliferation of *in vivo* generated CTL in the mixed

splenocyte system was achieved using high concentrations of plate bound anti-CD3. Therefore experiments on MACS purified CD8+ T cells were carried out using this system. Due to the observation that anti-CD28 in the culture system augmented the proliferation of the CTL enriched effector splenocytes, the effects of co-stimulation upon pure CD8+ T cells was also investigated in latter experiments.

Of the three pools of CD8+ T cells on which proliferation was attempted using plate bound anti-CD3 it soon became apparent that effector CTL were likely to be terminal effector cells, as they consistently proliferated less well than both naïve CD8+ T cells and those taken from cured mice, taking up less ^{3}H-thymidine. This was presumably due to their poor viability. As soon as one day after antibody stimulation, this was greatly diminished. The higher levels of IFNγ produced by these cells compared with both memory and naïve CD8+T cells and the fact that very low concentrations of anti-CD3 could induce high secretion of IFNγ also indicated that these cells were highly activated to would be likely AICD was occurring. Thus, effector CTL were not compliant with stimulation on plate bound mAb. Both naïve CD8+ T cells and those taken from cured mice proliferated well the latter pool showing comparable viability to those stimulated on antigen, but neither naïve nor CD8+ T cells from cured mice proliferated *ex vivo* on mAb could kill target tumours and thus were not further utilised in adoptive therapy.

CD4+ T cells from the peak of their response to BCL_1 proliferated better than peak CD8+ CTL, however viability of these cells was still low.

We were also unable to proliferate *in vitro* generated CTL lines sufficiently on plate bound anti-CD3 and anti-CD28 or anti-4-1BB mAb. The culture system on πBCL_1 could be used to generate specific, cytotoxic CTL, but expansion of CTL was always the limiting factor for adoptive therapy regimes as had been experienced by other groups (211). Even when a cell based artificial APC system (L-cells expressing CD32 loaded with anti-CD3 and anti-CD28) (210) was used CTL could not be expanded enough to facilitate adoptive therapies (data not shown). Future work would aim to investigate the cell based artificial APC system as it has been reported that the transfection of 4-1BBL into these cells allows the stimulation of CD4+ T cells, and more recently CD8+ T cells (216). Thus the same system could potentially be used for both sets of T cell lines.

Chapter 7 Adoptive therapy of BCl_1 by transfer of CD8+ CTL.

7.1 Introduction.

In order to elicit effective immunotherapy of tumours, large numbers of anti-tumour lymphocytes need to be generated. There are a number of ways in which to generate anti-tumour lymphocytes, specifically CTL. The identification of certain tumour antigens has allowed immunisation regimes to try and create these *in vivo* Clinical trials, however, have previously been disappointing (reviewed in (123)). Adoptive transfer regimes provide a method by which CTL can be generated *ex vivo* and placed into recipient hosts for eradication of tumour. An advantage of using adoptive transfer over, for example, systemic administration of anti-CD40 is that tumour might be specifically targeted. Furthermore in a clinical setting, the patient's own cells could potentially be expanded *ex vivo* and put back in to the same individual thus eliminating the need for MHC matching. However, prior clinical trials have shown that even when highly active anti-tumour T cell clones have been adoptively transferred back into patients, engraftment, meaning the persistence and survival of these tumour-reactive cells failed to occur (215). Recently, work has shown that engraftment of melanoma specific T cell clones can be achieved if patients were previously lymphodepleted. However in these trials tumour antigen(s) have been isolated (225). In the BCL_1 model of lymphoma, antigens are yet to be identified. In previous chapters we have shown that both *in vivo* and *in vitro* CTL can be generated and we wished to assess their efficacy in adoptive therapy regimes. We also wanted to investigate the efficacy of *in vitro* generated CD4+ T cell lines with respect to the provision of help for CTL. As described in Chapter 1, studies in mice have shown that adoptive therapy including CD4+ T cells can be effective . However in transgenic systems, CD4 help is not always needed due to the large numbers of tumour specific CTL. We wished to assess whether in our system where numbers of CTL were limited and where antigen was not known, CD4 T cell lines have a role.

7.2 Materials and methods

7.2.1 Adoptive transfer of *in vivo* and *in vitro* generated CTL

To investigate the efficacy of both *in vivo* and *in vitro* generated CTL against BCL_1, adoptive therapies were carried out in the following ways.

7.2.1.1 Adoptive therapies using *in vivo* generated CTL

Donor mice were immunised with 5 x 10^7 BCL_1 (D0) and treated with 1mg anti-CD40 (3/23) (D4) as previously detailed in materials and methods and chapter 3 figure 3.2. Spleen were harvested from donor mice typically on D9 and total splenocyte numbers were counted and flow cytometry was used to calculate the numbers of CD8+ T cells, CD4+ T cells and BCL_1 cells present. MACS was used to deplete BCL_1 cells and CD4+ T cells if depletion was required in adoptive therapy studies. CD8+CTL were transferred in to recipient mice i.p. at the same time as administration of BCL_1 at the numbers stated in the results section. Numbers stated refer to the final numbers of CD8+ T cells transferred in total splenocytes.

7.2.1.1 Adoptive therapies using *in vitro* generated CTL and CD4+ T cell lines

CTL and CD4+ T cell generated using methods detailed in Chapter 4 and Chapter 5 were administered to recipient mice at the same time as BCL_1 as previously described. Prior to adoptive transfer cell numbers in culture were counted and both CD8+ T cell and CD4+ T cell percentages in culture were calculated by flow cytometry. Numbers stated in the results section refer to final CD8+CTL and final CD4+ T cell numbers.

7.2.2 Administration of IL-2

10,000 units murine IL-2 in PBS (the production of which is detailed in materials and methods) was administered i.p. over four days, 2,500 units on the day of adoptive transfer and 2,500 units for three days thereafter.

7.2.3 Administration of anti-CD4 (YTA3.1.2)

For CD4 depletion studies, anti-CD4 (YTA3.1.2) was administered at a concentration of 300μg on days -1, 0, 1 and 2 of adoptive therapy studies. Administration at day -1 was i.v, administration was i.p thereafter.

7.3 Results

7.3.1 Therapy of BCL_1 by adoptive transfer of CTL generated *in vivo.*

To ascertain whether CTL generated *in vivo* were effective in adoptive therapy regimes, CTL taken from the peak of response, D9 of therapy, were adoptively transferred in to recipient mice which were simultaneously inoculated with BCL_1. Figure 7.3.1 shows results obtained when adoptive transfer was carried out as stated in materials and methods using an E:T ratio of 100:1. In the adoptive transfer study, mice injected with BCL_1 i.p. succumbed to tumour 27 days after it was administered. Mice which also received adoptively transferred CTL were protected from tumour for a maximum of 11 days. This protection was significant ($p<0.02$) but all mice in this group succumbed to tumour.

Prior to transfer, CTL were tested in a standard ^{51}Cr release assay to ensure they were capable of killing πBCL_1 *in vitro*. The insert in Figure 7.3.2 shows the cytotoxic activity of the CTL upon removal of the CTL from donor spleens was 50% (at an E:T ratio of 10:1) and was blocked by both anti-CD8 and anti-MHC class I mAb.

7.3.2 The effect of CD4+ T cell depletion on therapy of BCL_1 by adoptive transfer of CTL generated *in vivo.*

In the above therapy *in vivo* generated CTL were transferred among total splenocytes. The total splenocyes included CD4+ T cell from the donor animal. We wished to assess whether these CD4+ T cells contributed to the therapy observed. In addition to CD4+ T cells transferred from donor mice, recipient mice would have an endogenous CD4+ T cell population which could provide help. In order to investigate whether transferred CD4+ T cells or the recipients' own CD4+ cells were important in adoptive therapy using *in vivo* generated CTL, an experiment was performed after these cells had been deleted. Firstly, CD4+ T cells were depleted from donor spleens using MACS (depletion reducing numbers from 12% to 0% CD4+ T cells, data not shown). The remaining splenocytes, of which 35% were CD8+ T cells, were adoptively transferred in to recipient mice (total number CTL 1×10^7) with 1×10^5 BCL_1. A second group of mice received total splenocytes, of which 25.5% were

Figure 7.3.1) Adoptive therapy of BCL_1using CD8+ CTL taken from the peak of the anti-CD40 response.

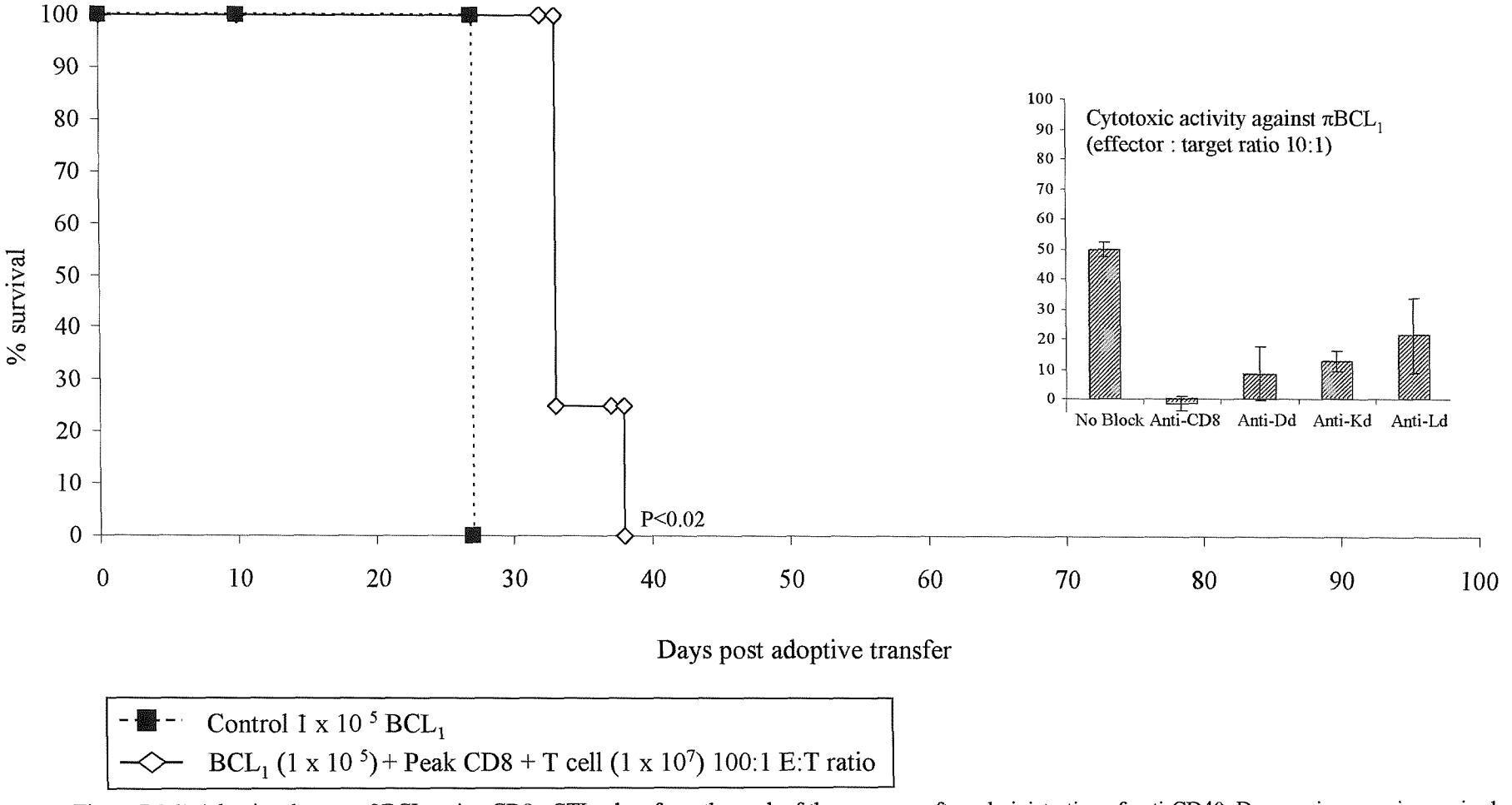


Figure 7.3.1) Adoptive therapy of BCL_1 using CD8+ CTL taken from the peak of the response after administration of anti-CD40. Donor mice were immunised with 5 x 10^7 BCL_1 D0 and treated with anti-CD40 on D4. D9 CD8+ CTL were harvested from the spleens of these mice and checked for cytotoxic activity in a standard ^{51}Cr release assay. 1 x 10^7 CD8+ CTL were transferred in total splenocytes into recipient mice i.p. with 1 x 10^5 BCL_1.Control mice received 1 x 10^5 BCL_1 (n=4 for both experimental groups). Insert shows cytotoxic activity of transferred CTL at an E:T ratio of 10:1 with and without the presence of blocking antibodies against CD8 and MHC class I (Kd, Dd and Ld).

Figure 7.3.2) Effect of CD4 depletion on adoptive therapy of BCL_1

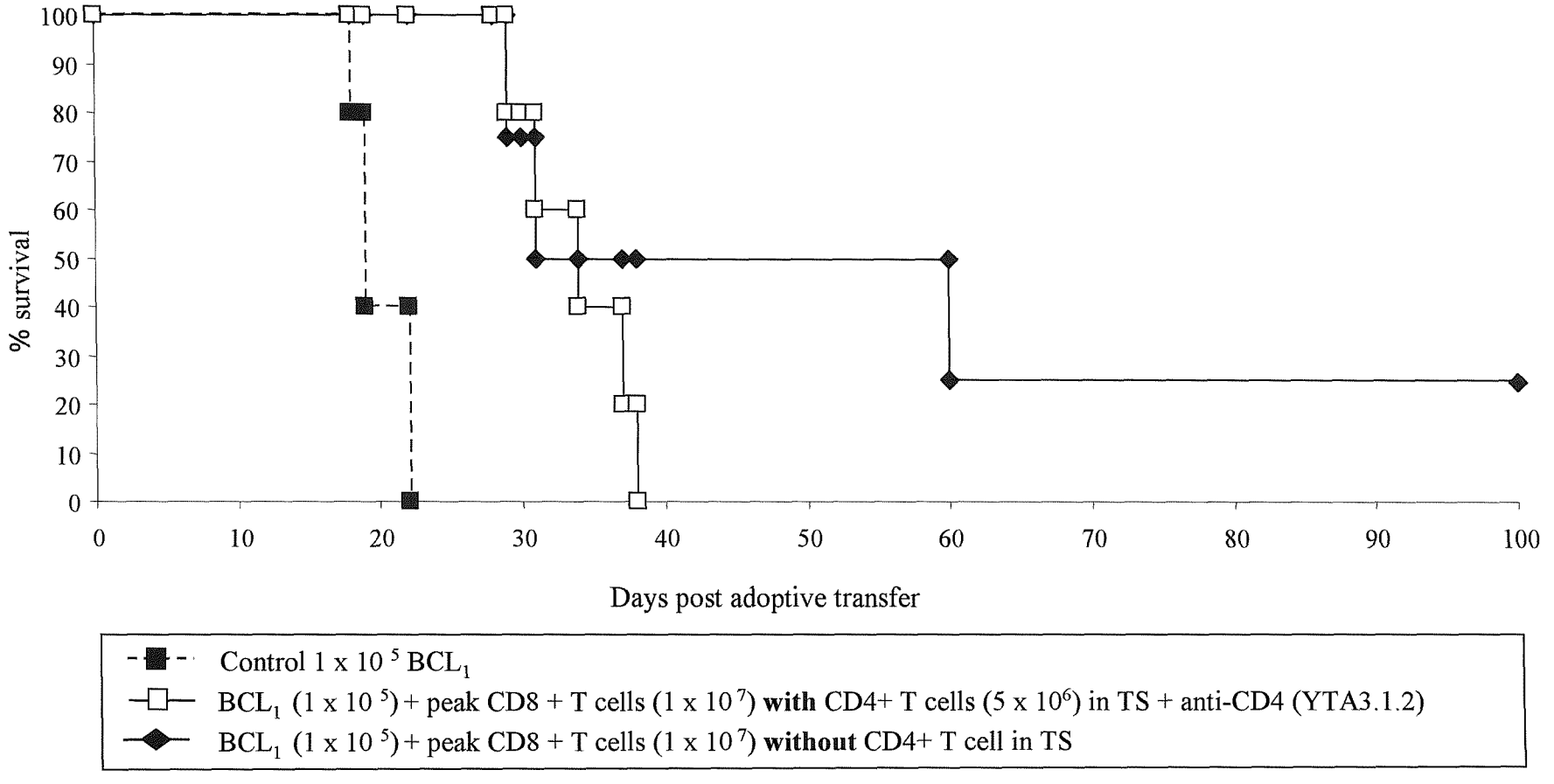


Figure 7.3.2) Adoptive therapy of BCL_1 using CD8+ CTL taken from the peak of the response to anti-CD40. Donor mice were immunised with 5 x 10^7 BCL_1 D0 and treated with anti-CD40 on D4. D9 total splenocytes containing CD8+ CTL were harvested from the spleen of donor mice. CD4+ T cells were removed from total splenocytes using MACS and remaining splenocytes including 1 x 10^7 CD8+CTL were transferred into recipient mice with 1 x 10^5 BCL_1. Total splenocytes containing 1 x 10^7 CD8+ CTL were injected into mice which had been administered with anti-CD4 (YTA 3.1.2) i.p. on days -1, 0,1,2 (300μg each day -1.2mg total administration over 4 days). Control mice were injected with 1 x 10^5 BCL_1 i.p.

CD8+T cells, including CD4+ T cell (total number CTL 1 x 10^7, total number CD4+ T cells 4.7 x 10^6) with 1 x 10^5 BCL_1, but were depleted of endogenous CD4+ T cells by administration of 4 i.p. injections of anti-CD4 from day -1 to day 3 of the adoptive transfer.

Figure 7.3.2 shows the results from these experiments. All control mice had died by day 22. In mice where *in vivo* generated CTL had been administered without CD4+ T cells from donor mice 50% of the mice survived to 60 days and there was one long term survivor. Mice in this group survived significantly longer than controls ($p<0.01$). When CTL were administered to mice in total splenocytes including CD4+ T cells but endogenous CD4+ T cells were depleted mice again survived significantly longer than controls but there were no long term survivors. There was, however no statistical difference between the effect of endogenous and transferred CD4+ T cells ($p>0.5$). Thus, these experiments showed there was no role for CD4+ T cells in adoptive transfer of *in vivo* generated CTL.

7.3.3 Effect of E:T ratio on adoptive therapies using *in vivo* generated CTL.

In this experiment we addressed the question as to whether survival was dependent upon the numbers of CTL originally transferred. Thus CTL were transferred with simultaneous BCL_1 injection at E:T ratios of 10:1, 20:1, 50:1 and 100:1. Figure 7.3.3 shows that despite over 70% killing shown by these cells in standard ^{51}Cr release assay at the time of transfer at a 50:1 E:T ratio (insert) oly one mouse (in the 20:1 E:T group) was fully cured. In all other groups progression of tumour was retarded by 5-15 days. There was no correlation between CTL numbers transferred and survival.

7.3.4 Effect of administration of IL-2 on adoptive therapies using *in vivo* generated CTL.

CD4+ T cells might provide help for CTL in adoptive transfer in the form of soluble factors such as IL-2. We aimed to establish whether IL-2 administered at the time of transfer and three days thereafter would increase the survival of the mice which had received CTL. IL-2 was, therefore administered to mice who received CTL:BCL_1 at a 10:1 ratio and compared to those mice who did

Figure 7.3.3) Effect of E:T ratio on adoptive therapy of BCL_1.

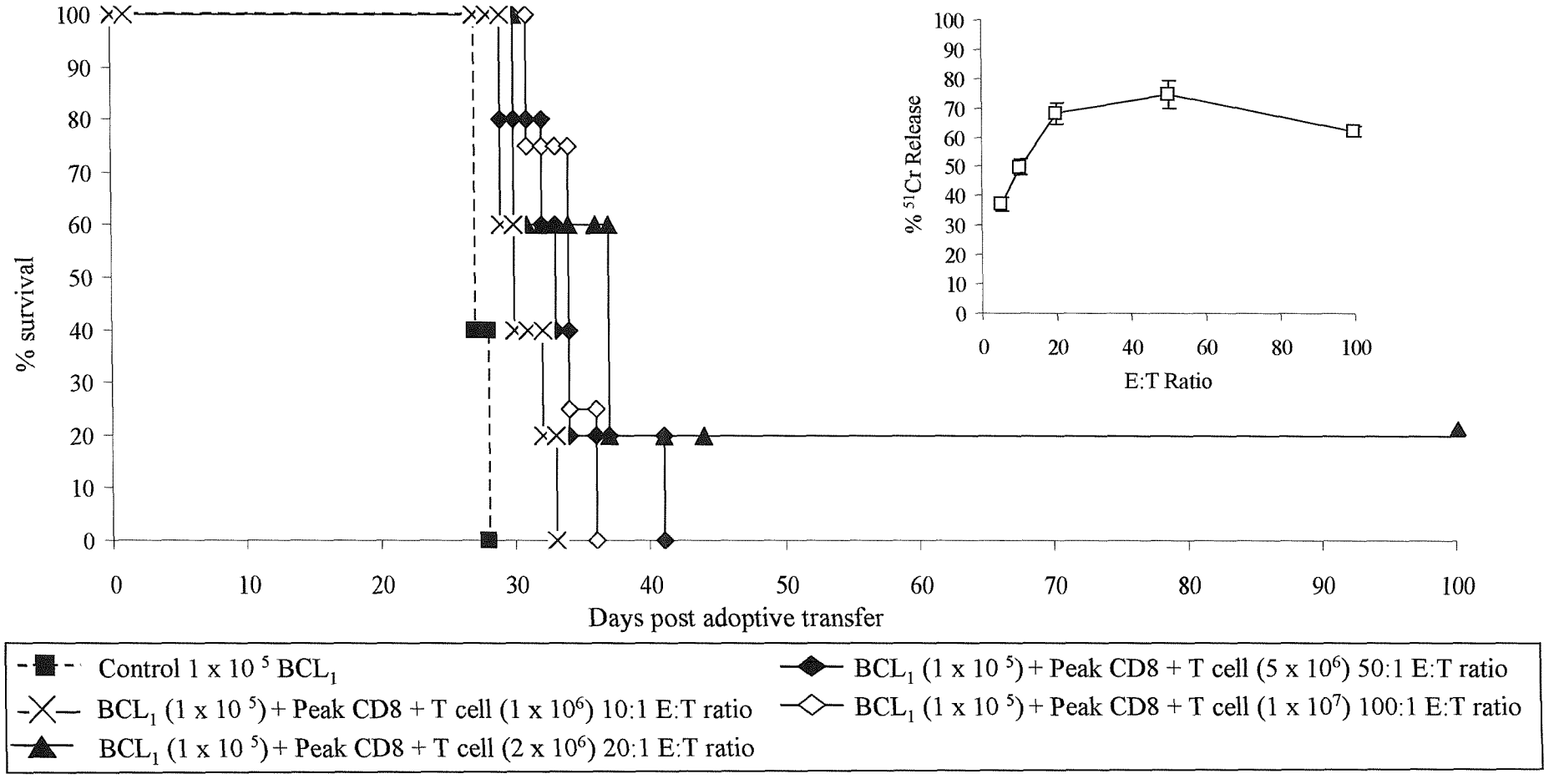


Figure 7.3.3) Adoptive therapy of BCL_1 using CD8+ CTL taken from the peak of the response to anti-CD40. Donor mice were immunised with 5 x 10^7 BCL_1 D0 and treated with anti-CD40 on D4. D9 CD8+ CTL were harvested from the spleens of donor mice and assayed for cytotoxic activity in a standard ^{51}Cr release assay. 1 x 10^7, 5 x 10^6, 2 x 10^6 or 1 x 10^6 CD8+ CTL were transferred in total splenocytes into recipient mice with 1 x 10^5 BCL_1.
(n=5 for all experimental groups).

Figure 7.3.4) Effect of IL-2 administration on adoptive therapy of BCL_1

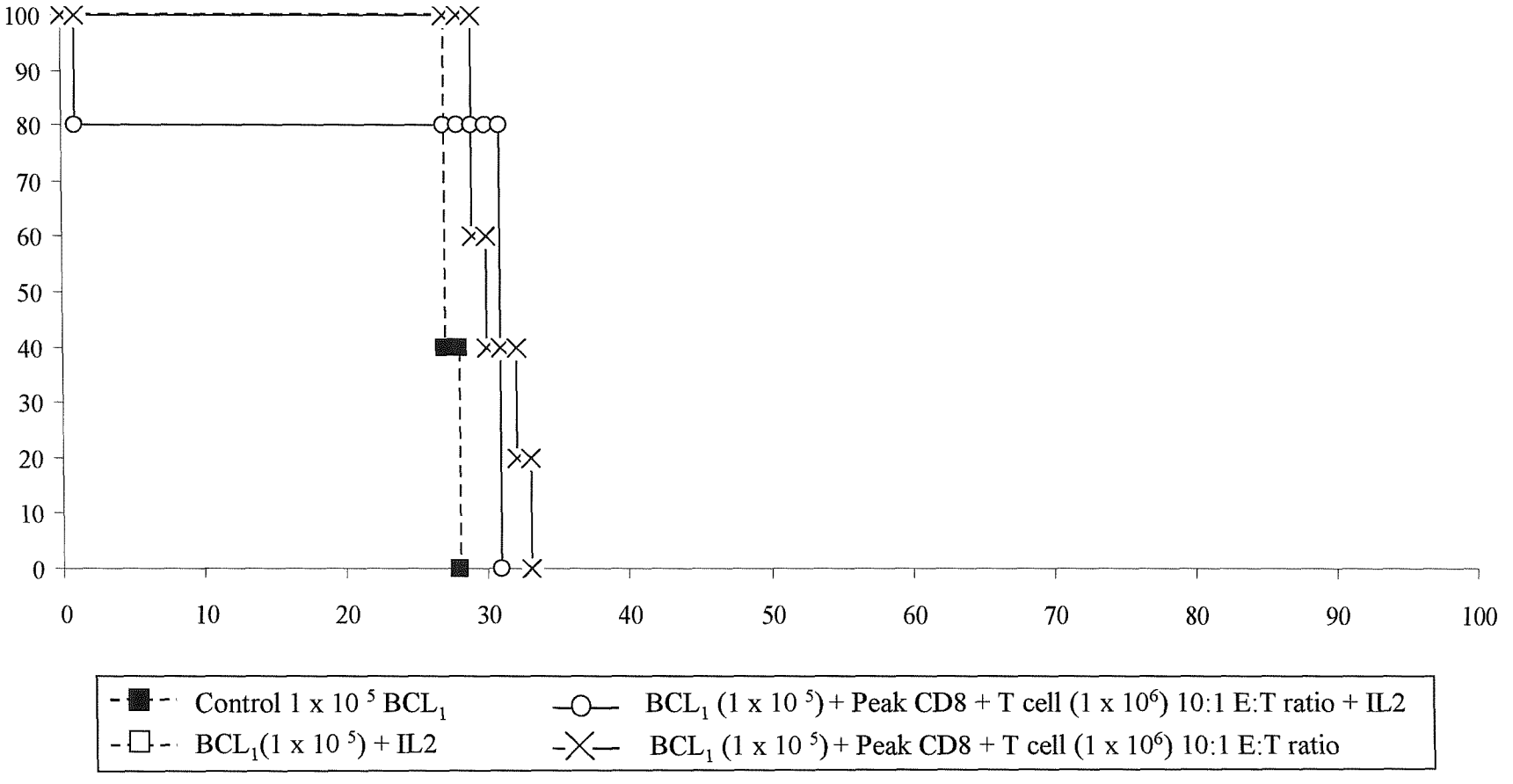


Figure 7.3.4) Adoptive therapy of BCL_1 using CD8+ CTL taken from the peak of the response to anti-CD40. Donor mice were immunised with 5 x 10^7 BCL_1 D0 and treated with anti-CD40 on D4. D9 CD8+ CTL were harvested from the spleens of donor mice and assayed for cytotoxic activity in astandard ^{51}Cr release assay. 1 x 10^6 CD8+ CTL were transferred in total splenocytes into recipient mice with 1 x 10^5 BCL_1. IL-2 was administered I.P. in to one 10:1 E:T group at 10,000 units over 4 days. Control mice received 1 x 10^5 BCL_1 (n=5 for all experimental groups).

not receive IL-2 but who received CTL at the same ratio. A control group was also added which received IL-2 and BCL_1 but no CTL, to assess whether IL-2 had any effect on its own. Figure 7.3.4 shows that when CTL were transferred at an E:T ratio of 10:1 IL-2 administration had no effect on survival. The mice which received IL-2 only succumbed to tumour at the same time as controls.

7.3.5 The effect of the activation status of CTL in adoptive transfer using *in vivo* generated CTL.

To assess whether the activation status of the *in vivo* generated CTL had any bearing on survival in adoptive therapy regimes, *in vivo* generated CTL were separated according to CD62L expression. The adhesion molecule was chosen firstly because it was shown to go down upon activation and because its expression levels yielded two distinct populations which could be separated using MACS. Thus activated $CD62L^{low}$ splenocytes were separated from a mixed splenocyte population of which 19% were CD8+ CTL cells (data not shown). The graph insert shows that in the mixed population at the peak of activation, 63% of CD8+ T cells were CD62low, after MACS separation this was increased to 96%. The $CD62L^{hi}$ CD8+ T cells were only modestly increased from 37% to 56%. In all therapy groups ($CD8+CD62L^{hi}$, $CD8+CD62L^{low}$ and mixed population) a final number of 1×10^7 CTL were used and administered at the same time as 1×10^5 BCL_1. Figure 7.2.4 shows that all control mice succumbed to tumour by day 27, the $CD8+CD62L^{hi}$ group provided only 3 days protection from tumour, the mixed CD8+ T cell population provided 7 days protection and the activated $CD8+CD62L^{low}$ cells provided the most protection with 1 mouse surviving to 47 days. Protection by adoptive transfer of CTL contained in splenocytes in all groups was significant compared to controls ($p<0.01$). Importantly, the additional protection conferred by the $CD62L^{low}$ group compared to both the mixed population and $CD62L^{hi}$ groups was shown to be highly significant ($P>0.01$).

Despite some protection against BCL_1 being achieved using *in vivo* generated CTL, adoptive transfer carried out with these cells failed to produce long term survivors in the majority of cases. Chapters 4 and 5 established that both CD8+

Figure 7.3.5) Effect of CD62L expression on CD8+ CTL in adoptive therapy of BCL_1.

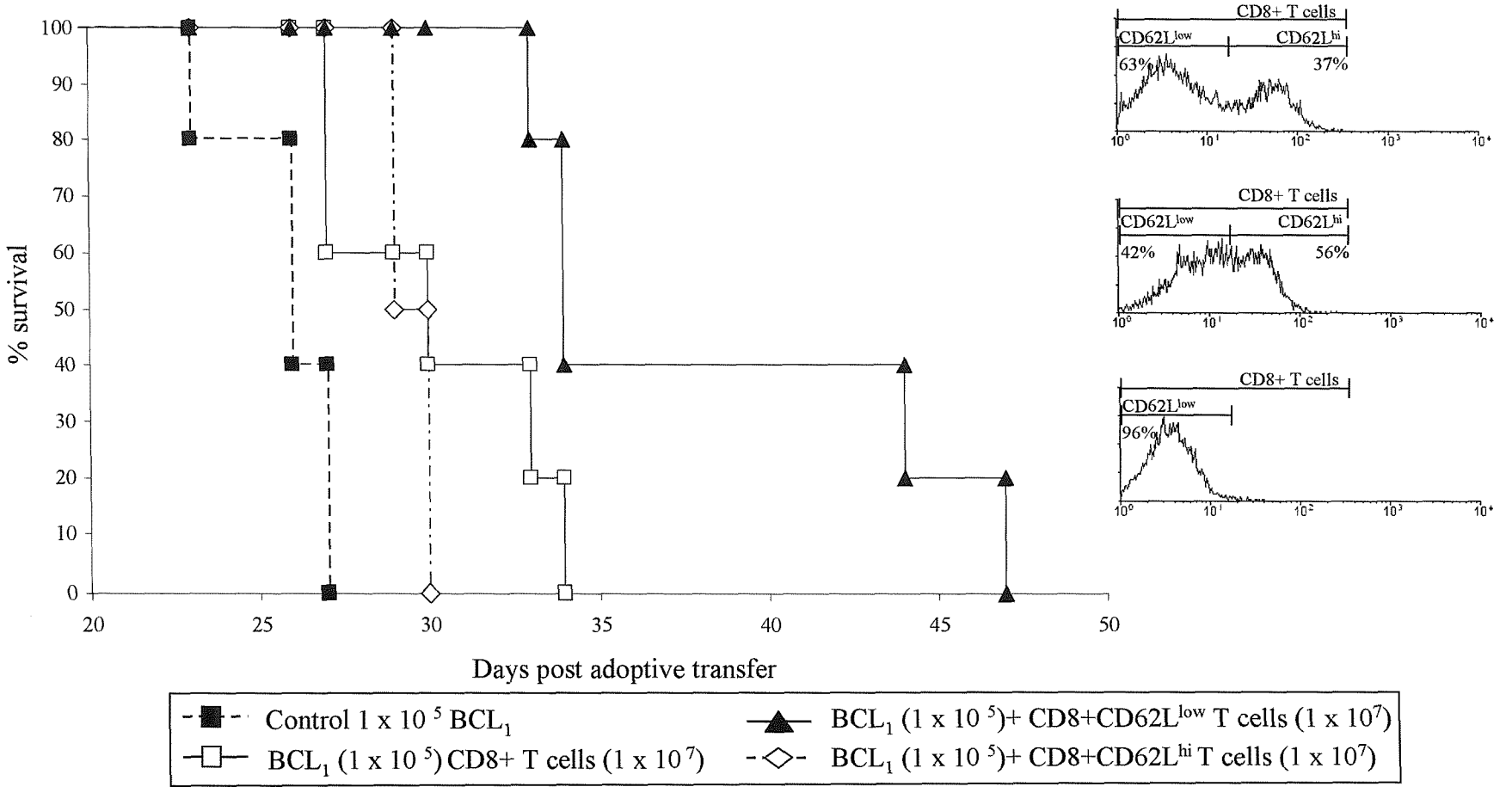


Figure 7.3.5) Comparison of adoptive therapy of BCL_1 using CD8+ CTL taken from the peak of the response to anti-CD40 and purified according to CD62L expression. Donor mice were immunised with 5 x 10^7 BCL_1 D0 and treated with anti-CD40 on D4. D9 CD8+ CTL were harvested from the spleens of donor mice and MACS was used to sort splenocytes (shown in insert).CD62L ^{low} (bottom histogram) and CD62L ^{hi} (middle histogram) cells were separated from the mixed popualtion (top histogram) by anti-CD62L-FITC conjugate on anti-FITC beads. All histograms were gated on CD8+ T cells only using anti-CD8-PE conjugate and visualised by flow cytometry . 1 x 10^7 CD8+ CTL were transferred in total splenocytes into recipient mice with 1 x 10^5 BCL_1.Control mice received 1 x 10^5 BCL_1 (n=5 for all experimental groups except CD62Lhi where n=4).

and CD4+ T cell lines could be grown *in vitro*, and these lines were tested in adoptive transfer regimes.

7.3.6 Adoptive therapy of πBCL_1 using *in vitro* generated CTL.

An *in vitro* generated CTL line (CD8-π-1) which had been cultured for 4 weeks and assessed in standard ^{51}Cr release assays (chapter 4 Figure 4.3.3) was used in an adoptive transfer study to elucidate its efficacy against πBCL_1 *in vivo*. Adoptive transfer was carried out as stated in materials and methods using an E:T ratio of 100:1. Figure 7.3.6 illustrates that mice injected with πBCL_1 i.p. with *in vitro* generated CTL (CD8-π-1) did not succumb to tumour, whereas the control group (πBCL_1 alone) did. Indeed therapy using an *in vitro* CTL line seemed more effective than using *in vivo* generated CTL.

In this experiment πBCL_1 was used as BCL_1 was not available from routine passage of tumour *in vivo* thus control mice succumbed to tumour later than would be expected if using BCL_1. BCL_1 was used in all subsequent adoptive therapy studies.

7.3.7 Comparison of adoptive therapy of BCL_1 using *in vivo* generated CTL and *in vitro* generated CTL.

Given the previous result where all CTL treated animals were cured we wished to address the efficacy of *in vivo* versus *in vitro* generated CTL directly. Adoptive transfer was carried out as previously stated using an E:T ratio of 10:1.

Prior to adoptive transfer being carried out ^{51}Cr release assay was used to determine cytotoxic activity of both sets of CTL. In addition to this CD8+ T cell % was assessed by flow cytometry. *In vivo* and *in vitro* CTL lysed target πBCL_1 cells at a level 50% and 61% respectively (ratio of 10:1) (*in vivo* CTL were assessed on the day of adoptive transfer, *in vitro* two days before) (data not shown). *In vivo* CTL made up 35% of total splenocytes in this experiment whereas *in vitro* CTL were 20% of the culture (day 7 of culture cycle) on the day of adoptive transfer. Final numbers of CTL used for both groups were equivalent. Figure 7.2.6 shows again that *in vivo* generated CTL gave a small amount of protection over control mice. *In vitro* generated CTL however were

Figure 7.3.6) Adoptive therapy of πBCL_1 using *in vitro* generated CD8+ CTL.

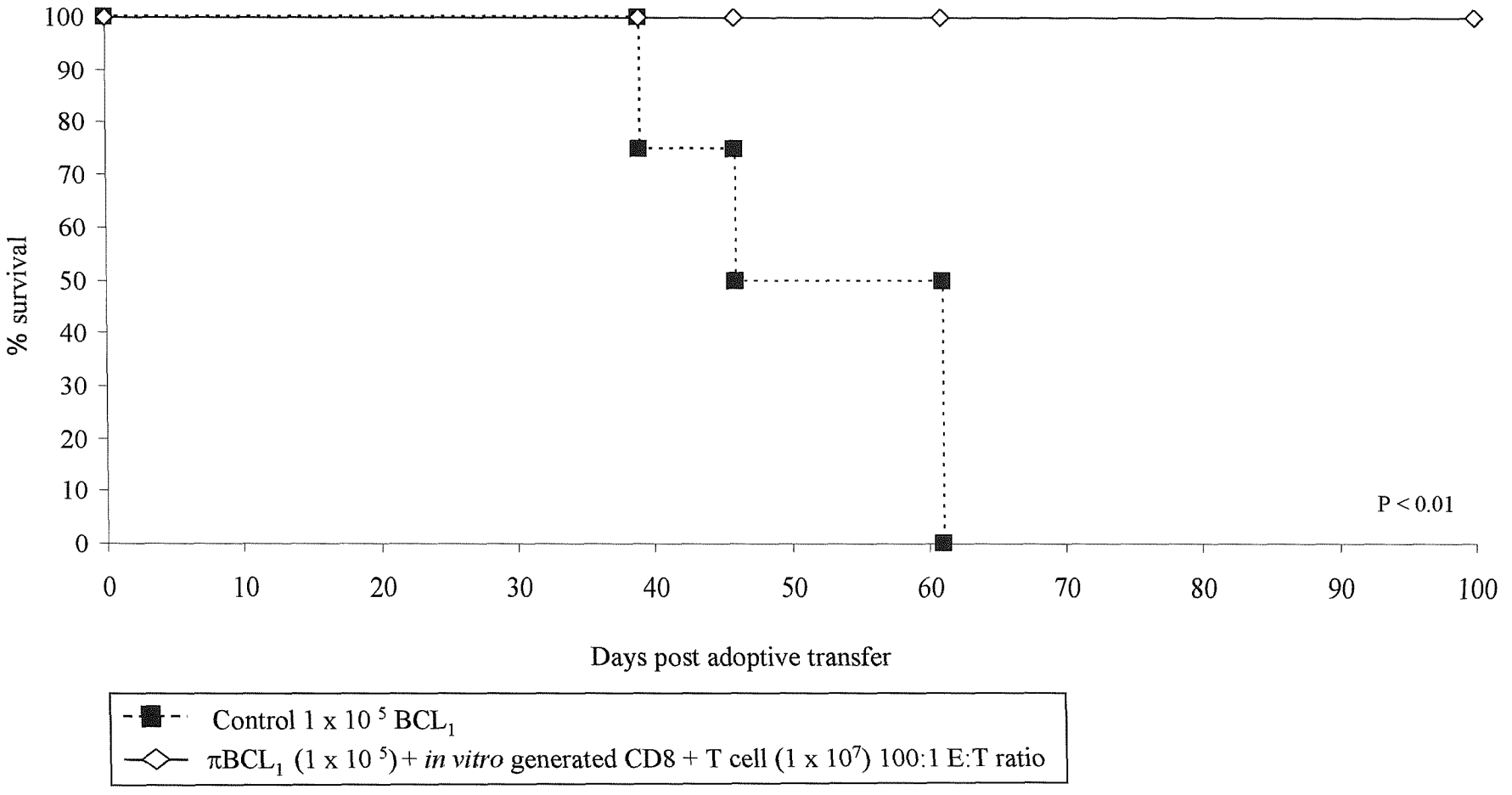


Figure 7.3.6 Adoptive therapy of πBCL_1 using in vitro generated CD8+ CTL. *In vitro* CD8+ CTL which had been co-cultured for 4 weeks with irradiated πBCL_1 were assessed for cytotoxic activity in standard ^{51}Cr release assay. 1 x 10^7 CD8+ CTL were transferred into recipient mice with 1 x 10^5 πBCL_1. Control mice received 1 x 10^5 BCL_1 (n=4 for both experimental groups).

significantly more effective than *in vivo* generated CTL ($p>0.01$) in this adoptive transfer experiment since even at a 10:1 E:T ratio, two mice out of five survived long term.

7.3.8 Effect of administration of a non-cytotoxic CD4+ T cell line (CD4-π-1) upon adoptive therapy using *in vitro* generated CTL (CD8-π-1).

To establish whether *in vitro* generated CD4+ T cells could provide help in adoptive therapy to *in vitro* generated CTL, the non-cytotoxic CD4+ T cell line CD4-π-1 was utilised in an adoptive transfer study. Adoptive transfer using CD8-π-1 cells was carried out as previously stated but this time with and without the addition of equivalent numbers of the CD4-π-1 CD4+ non-cytotoxic line. An E:T ratio of 10 :1 was used since at an E:T ratio of 100: 1 all mice had previously survived and thus help provided by the CD4+ line may have not be detected at this higher E:T ratio. CD4-π-1 were also assessed to see if they provided any therapy of BCL_1 on their own.

Figure 7.3.8 shows that despite CD4-π-1 CD4+ T cells possessing no intrinsic cytotoxicity and providing only 3 additional days of therapy compared to controls when administered alone, when administered with CD8-π-1 cells they appeared to increase the efficacy of the CTL line. Without addition of the CD4 line two out of five mice survived compared with four out of five mice upon administration of both CTL and CD4 lines ($p>0.3$).

7.3.9 Effect of IL-2 administration on adoptive therapy using *in vitro* generated CTL.

The effect of IL-2 administration was previously investigated for its effect on *in vivo* generated CTL (Figure 7.3.4). Here we assess the effect of IL-2 administration on *in vitro* generated CTL due to the observation that CD4-π-1 CD4+ T cells provided help to CD8-π-1 CTL. A low E:T ratio of 10:1 was again used to ensure any help provided was detected. IL-2 was administered over 4 days (days 0,1,2, and 3). Administration of IL-2 to *in vitro* generated CTL did not aid survival, interestingly the protection seen by *in vitro* generated CTL was absent when IL-2 was administered with mice in this group surviving

Figure 7.3.7) Direct comparison of adoptive therapy of BCL_1using *in vivo* generated and *in vitro* generated CD8+CTL

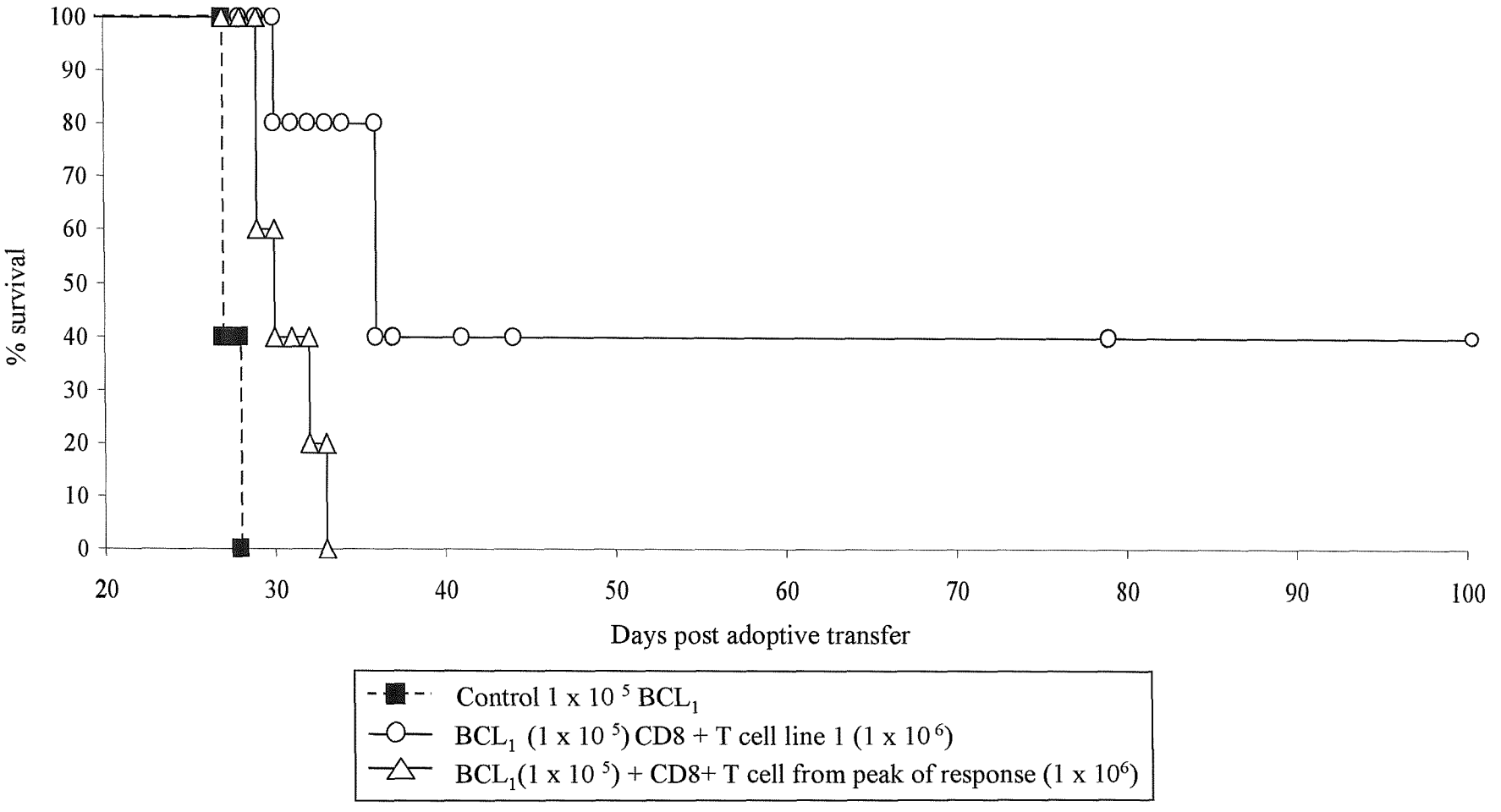


Figure 7.3.7) Comparison of adoptive therapy of BCL_1using CD8+ CTL taken from the peak of the response to anti-CD40 and *in vitro* generated CD8+CTL For peak adoptive therapies, donor mice were immunised with 5 x 10^7 BCL_1 D0 and treated with anti-CD40 on D4. D9 CD8+ CTL were harvested from the spleen of donor mice and 1 x 10^6 CD8+ CTL were transferred in total splenocytes into recipient mice with 1 x 10^5 BCL_1.Equivalent numbers of *in vitro* generated CD8+ CTL were transferred into recipient mice with 1 x 10^5 BCL_1.Control mice received 1 x 10^5 BCL_1
(n=5 for all experimental groups).

Figure 7.3.8) Effect of CD4+ T cell help on adoptive therapy of BCL_1using *in vitro* generated CD8+ CTL and a non-cytotoxic CD4+ T cell line (CD4-π-1)

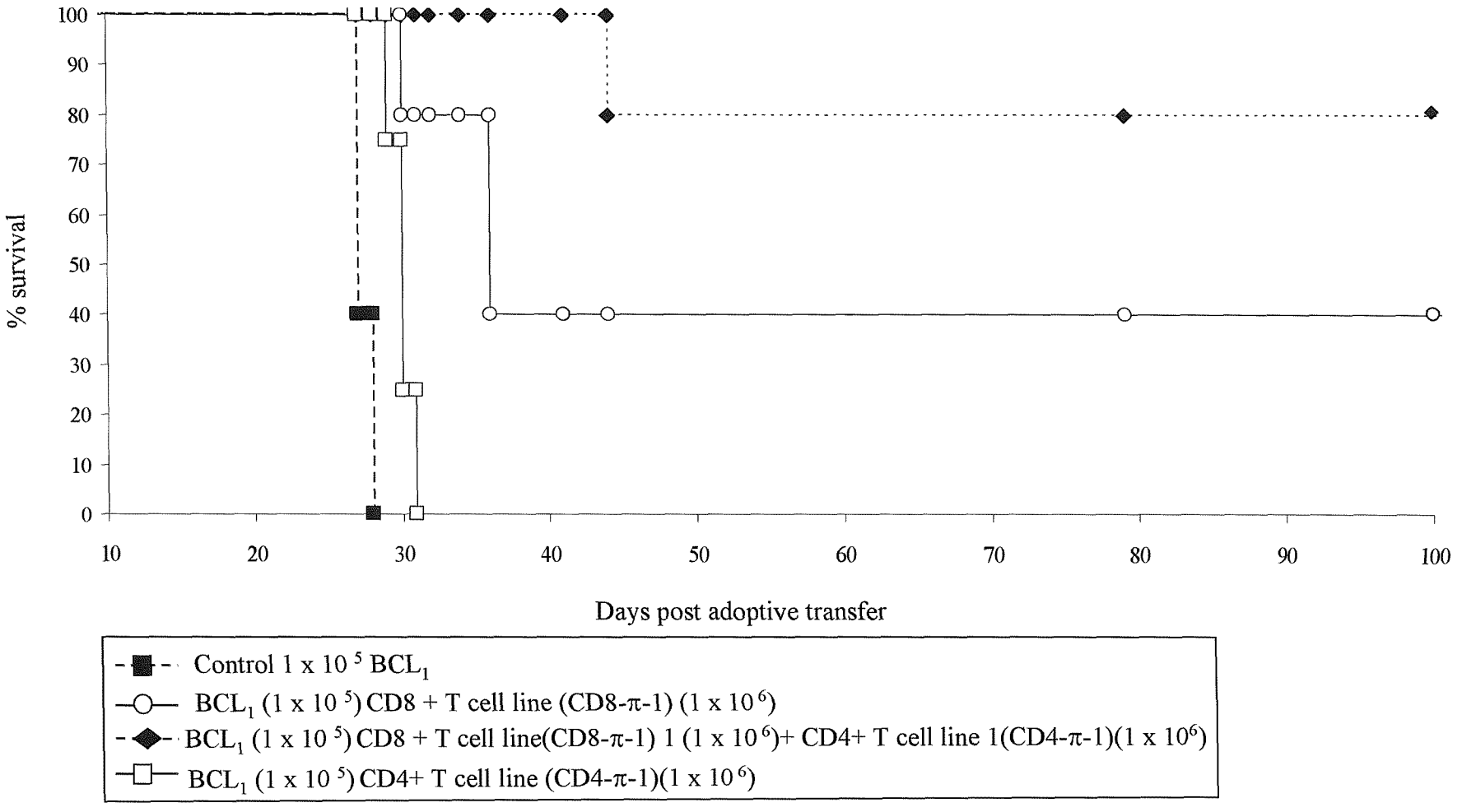


Figure 7.3.8) Comparison of efficacy of adoptive therapy of BCL_1using *in vitro* generated CD8+CTL at an E:T ratio of 10:1 and adoptive therapy where *in vitro* generated non- cytotoxic CD4+ T cells (CD4-π-1)were also administered. 1 x 10^6 *in vitro* generated CD8+ CTL (4 weeks in culture) were transferred into recipient mice with 1 x 10^5 BCL_1 with and without the addition of 1 x 10^6 CD4-π-1 which had also been grown *in vitro* for 4 weeks. Control mice received 1 x 10^5 BCL_1 (n=5 for all experimental groups).

Figure 7.3.9) Effect of IL-2 administration on adoptive therapy using *in vitro* generated CTL

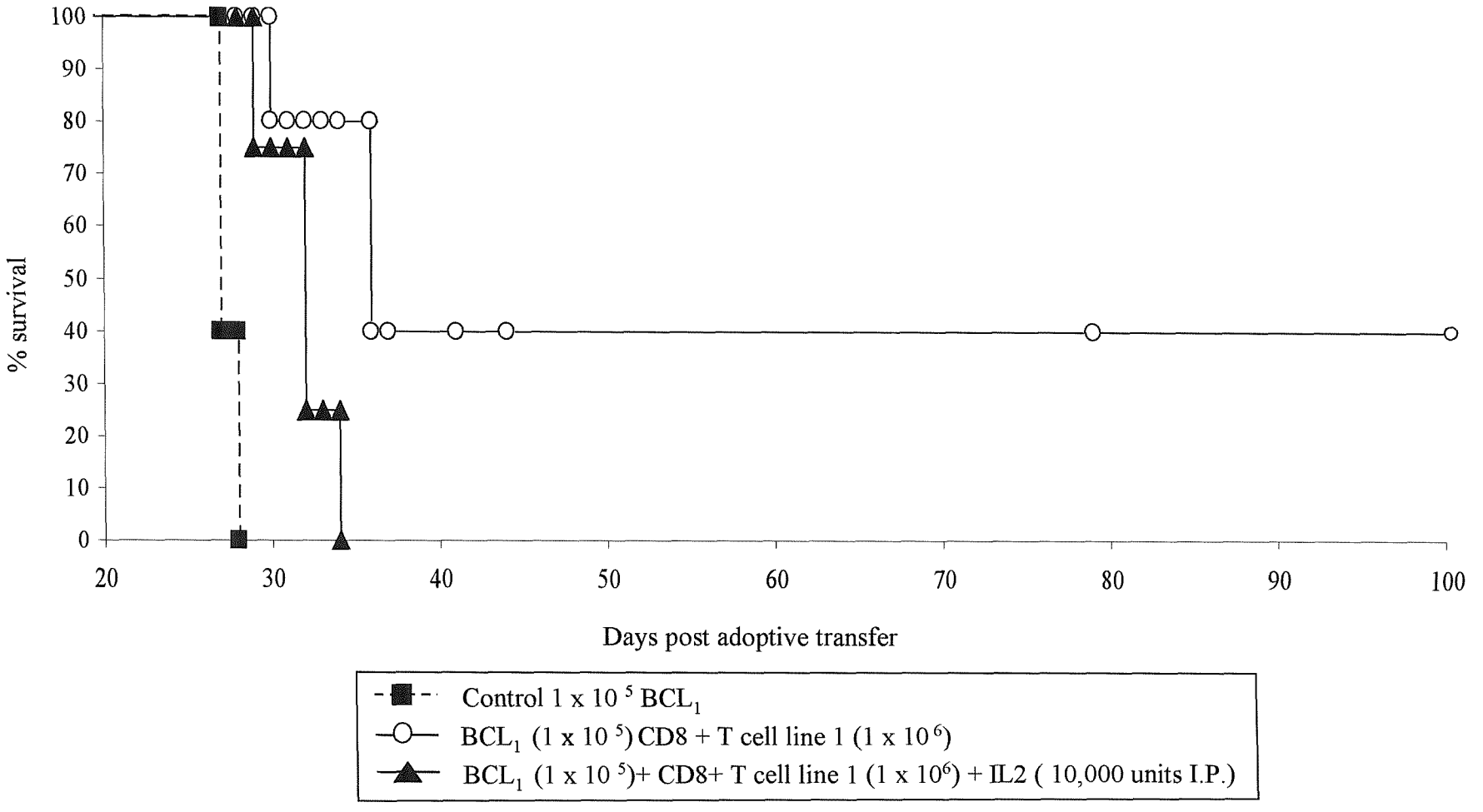


Figure 7.3.9) Effect of IL-2 administration on adoptive therapy using *in vitro* generated CTL. 1 x 10^6 *in vitro* generated CTL were transferred in to recipient mice with 1 x 10^5 BCL_1. IL-2 was administered i.p.at 10,000 units over 4 days. Control mice received 1 x 10^5 BCL_1 (n=5 for all experimental groups except where IL-2 was administered where n=4).

only as long as those administered with *in vivo* generated CTL seen in previous studies.

7.3.10 Effect of administration of a cytotoxic CD4+ T cell line (CD4-π-2) upon adoptive therapy using *in vitro* generated CTL (CD8-π-2).

The CD4+ T cell line CD4-π-2 was able to kill πBCL_1 in standard ^{51}Cr release assays (Chapter 5, Figure 5.3.4). Therefore, we wished to establish whether CD4-π-2, like CD4-π-1, was able to provide help to CTL in adoptive transfer studies as well as investigating whether it was effective itself in adoptive transfer regimes due to its cytotoxic behaviour.

Adoptive transfer was carried out as stated in materials and methods using an E:T ratio of 10:1. Figure 7.3.10 firstly supports data shown in Figure 7.3.8 in that the most effective therapy was seen upon administration of both CTL and CD4 lines (CD8-π-2 and CD4-π-2) where three out of four mice survived. This therapy is significantly more effective than the use of CD8-π-2 alone ($p<0.01$) where, even though mice survived longer than controls, four out of the five mice succumbed to tumour before day 30 with one mouse surviving to day 61. CD4-π-2 cells administered alone provided protection above control ($p<0.02$) with mice surviving 13 days longer than littermates who received tumour alone.

7.3.11 Effect of administration of naïve CD4+ T cells and endogenous CD4+ T cell depletion upon adoptive therapy using *in vitro* generated CTL (CD8-π-2).

The following experiment wished to investigate whether help provided by *in vitro* generated CD4+ T cell line (shown in Figures 7.3.8 and 7.3.10) could also be provided by naïve CD4+ T cells. Furthermore, the efficacy of CD8-π-2 was investigated when endogenous CD4+ T cells were depleted by anti-CD4 administration. 1 x 10^6 naïve CD4+ T cells were administered at the time of adoptive therapy of CD8-π-2 and anti-CD4 was administered day-1,0,1,and 2 of the therapy (300μg/day). Naïve CD4+ T cells were unable to recreate the effect of CD4-π-2 T cells when administered with CD8-π-2 in this therapy giving rise to one survivor out of a group of four mice. Naïve CD4+ T cells on their own provided no protection from tumour. Mice which were administered with anti-

Figure 7.3.10) Effect of CD4+ T cell help. Adoptive therapy of BCL_1 using *in vitro* generated CD8+ CTL with and without the administration of CD4-π-2, a cytotoxic CD4+ T cell line (after two weeks culture).

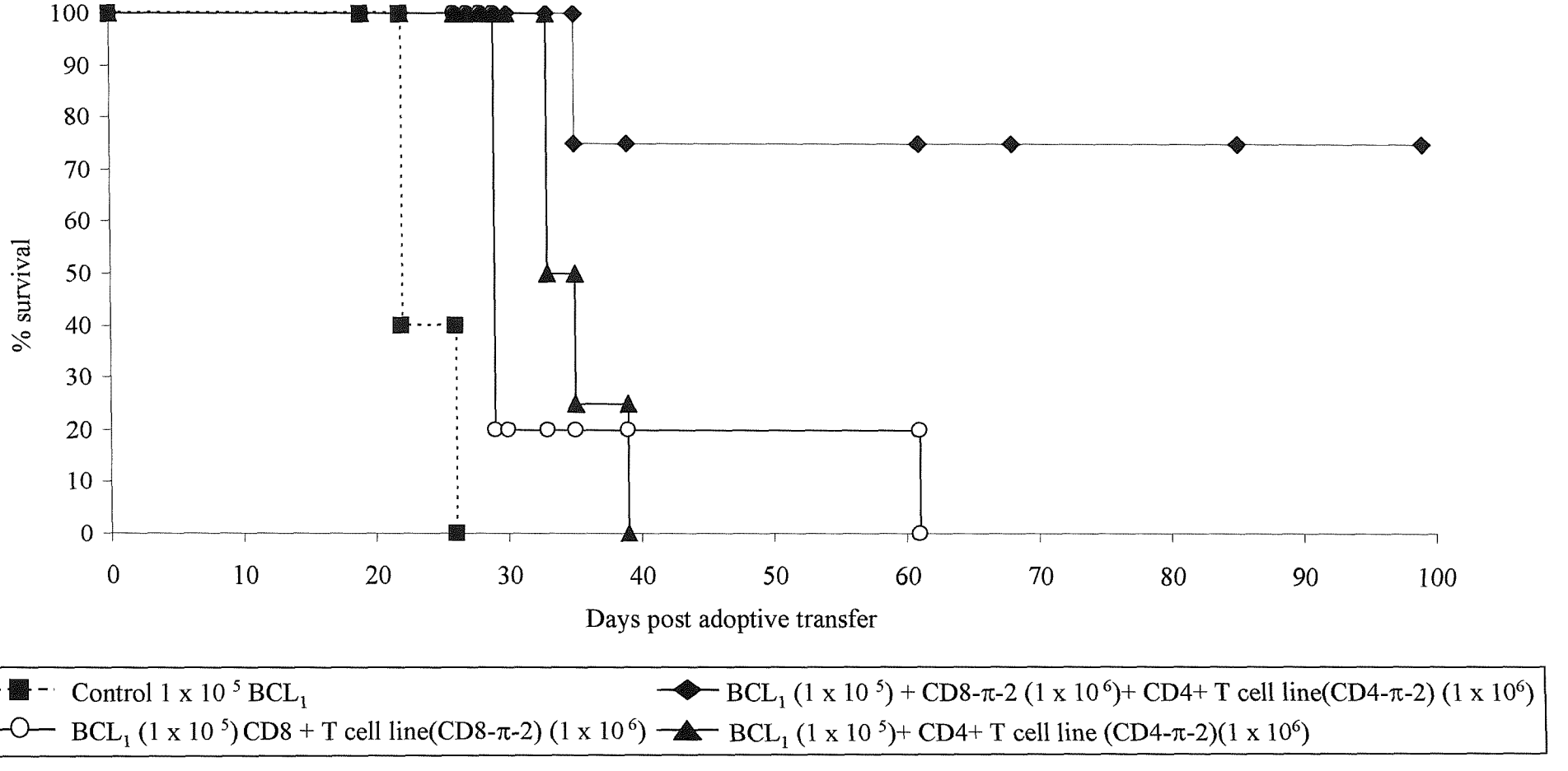


Figure 7.3.10) Effect of CD4+ T cell help. Adoptive therapy of BCL_1 using *in vitro* generated CD8+ CTL with and without the administration of CD4-π-2, a cytotoxic CD4+ T cell line (two weeks culture). *In vitro* generated CD8+ CTL (CD8 line 2) were transferred into recipient mice after 2 weeks co-culture with πBCL_1 at an E:T ratio of 10:1 (CD8: BCL_1) with or without the administration of anti-π-2 CD4+ T cells which had also been cultured with πBCL_1 for 2 weeks. All mice received 1 x 10^5 BCL_1.

Figure 7.3.11) Effect of CD4+ T cell help. Adoptive therapy of BCL_1 using *in vitro* generated CD8+ CTL with and without the administration of naive CD4+ T cells and anti-CD4 mAb.

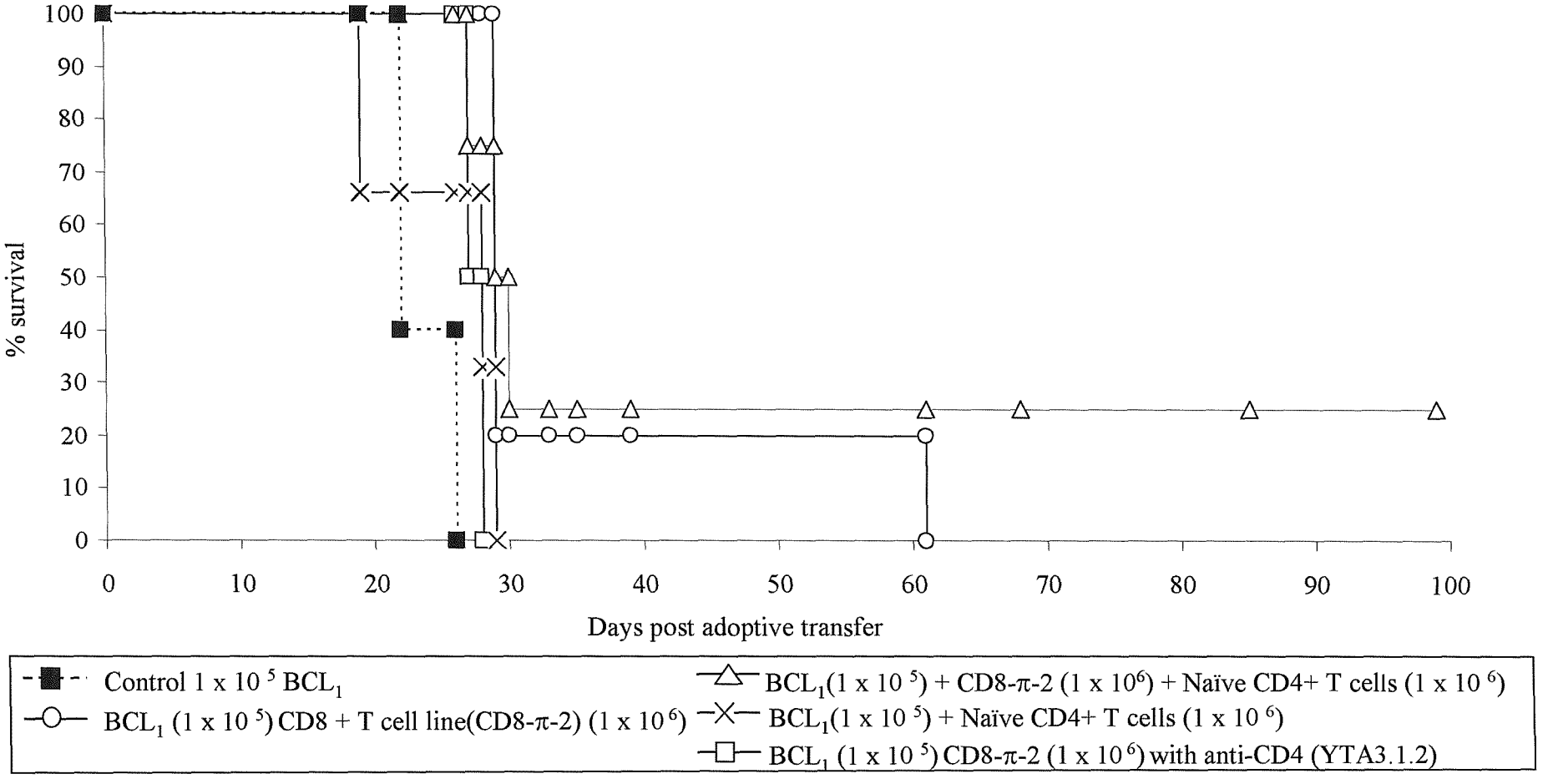


Figure 7.3.11) Effect of naïve CD4+ T cell help. Adoptive therapy of BCL_1 using *in vitro* generated CD8+ CTL with and without the administration of naïve CD4+ T cells and administration of anti-CD4 mAb. *In vitro* generated CD8+ CTL (CD8-π-2) were transferred into recipient mice after 2 weeks co-culture with πBCL_1 at an E:T ratio of 10:1 (CD8: BCL_1) with or without 1 x 10^6 naïve CD4+ T cells. The CTL line was also transferred in to mice which had been depleted of CD4+ T cells by administration of anti-CD4 mAb 300μg per day days -1,0,1 and 2. All mice received 1 x 10^5 BCL_1.

CD4 and CD8-π-2 all succumbed to tumour within two days after control mice, however, this was not significantly different from mice which received CD8-π-2 alone. Thus naïve CD4+ T cells had no role in this adoptive therapy.

7.4 Discussion.

Results from this chapter show that a limited amount of protection from BCL_1 can be achieved by adoptively transferring CTL taken from donor mice during the peak CTL response. These *in vivo* generated CTL are cytotoxic against BCL_1 as established by standard ^{51}Cr release assays and are therefore an obvious choice for adoptive transfer studies. In all adoptive transfer studies using these CTL the tumour was administered i.p. at the same time as the CTL, ensuring both were in the same location and the CTL did not need to migrate to the site of the tumour. In addition to this, 100 times more CTL, which could lyse 50% of tumour at an E:T ratio of 10:1, were injected than tumour. Even in this situation, where it would be expected the best therapy would be seen, only 11 days protection was achieved and, disappointingly, no mice were cured. Even if the CTL killed the majority of BCL_1 i.p. as they were expected to do they may have not killed 100% before perhaps dying themselves? Indeed, the fact that no cure was achieved despite the CTL seemingly being sufficiently cytotoxic would suggest that the CTL did not persist in the peritoneum for long enough to kill all BCL_1 present. Alternatively, the tumour could have migrated out of this site. This data is supported by experiments where *in vivo* generated CTL were CFSE labelled and placed with and without BCL_1 i.p. in to recipient mice to observe the proliferative response *in vivo* to tumour. After 24 hours neither CTL nor tumour could be detected i.p.(data not shown) although identically treated littermates succumbed to BCL_1 suggesting that the CTL didn't persist for long enough to ensure that all BCL_1 was killed, leaving the small numbers (undetectable by flow cytometry) of remaining cells to proliferate and kill the mice. This is speculation and future work would aim to prove whether these CTL persisted by tracking numbers of CTL and BCL_1 found in the peritoneum, spleen and draining lymph nodes of recipient mice over the first 24-48 hours after transfer.

Despite disappointing results using *in vivo* generated CTL, attempts were made to elucidate whether CD4+ T cells contained within the transferred total splenocyte population contributed to the therapy seen. We however failed to establish a role for CD4+ T cells transferred from donor mice with CTL in the total splenocyes. These T cells were taken from donor mice treatment D9, the day at which the CD8+CTL response was at its peak. However, we have already

established from previous experiments that the CD4+ T cell peak of response is D7 (Chapter 3). This being the case, the CD4+ T cells transferred would be less activated and lower in number than at D7. Therefore, a more conclusive experiment would be to take CD4+ T cells from donor mice D7 and adoptively transfer them with CTL from D9 the point at which both CD4+ and CD8+T cells would be at their respective peak.

The removal of enodogenous CD4+ T cells from recipient mice by administration of anti-CD4 did not reduce the level of protection form tumour to that of control mice and protection was not significantly different from the group without endogenous CD4+ T cell depletion. This suggests that the recipient animals' naïve CD4 were not providing help to the CTL in the peritoneum. This is perhaps entirely expected as in order for the endogenous CD4+ T cells to aid with survival and anti-tumour activity of the transferred CTL they would have to track to the peritoneum and become primed against tumour antigen possibly by macrophages, mature into effector T_H cells and contribute to a constructive cytokine milieu possibly by producing IL-2 to aid with the survival of the transferred CTL. This process would have to happen within 24 hours as after this CTL were not observed in the peritoneal cavity (data not shown). Presumably, by the time naïve CD4+ T cells responded, if they responded at all (without the administration of anti-CD40) the CTL may well have perished.

The fact that the E:T ratio transferred into recipient mice had no bearing on the efficacy of therapy was surprising as we would expect that more CTL available should kill more tumour. Indeed, in standard ^{51}Cr release assays this is usually the case (a quench is however seen when too many cells in a well stearically hinder the cytotoxic activity but this should not be the case i.p). However it was observed that there was no more protection against tumour when an 100:1 E:T ratio was used compared to when a 10:1 ratio was used. Even if not all the CD8+ T cells transferred were tumour-specific CTL there would still be 10 x the amount of tumour-specific CTL in the 100:1 group compared to the 10:1 group thus we still have to consider that 10 x the tumour will be killed in the 100:1 E:T group. Perhaps the observation the E:T ratio had no effect on therapy can be explained by the CTL persistence. Even with higher numbers of CTL

available i.p. at the time of transfer if they don't persist at the site of tumour then no matter how few BCL_1 remain they will go on to proliferate and migrate to other sites in the animals and the animals will eventually die of tumour. The shorter the persistence of the CTL in the peritoneum the less the difference in therapy seen between the groups given differing titrations of CTL.

An attempt to improve the persistence of transferred CTL by the administration of
IL-2 in the experimental group given CTL:BCL1 at a 10:1 ratio may have actually lead to the shortening of the life of the CTL through activation induced cell death (AICD) as the concentration of IL-2 may have been too high. This was suggested by the lack of therapy in these mice.

The activation status as assessed by CD62L expression on *in vivo* generated CTL had an affect on the efficacy of adoptive therapy. Here the CTL which were activated, that is $CD62L^{low}$, were so in response to BCL_1 and anti-CD40 in the donor mouse and thus would be expected to be the tumour-specific population of the CTL. The $CD62L^{low}$ CTL would probably kill considerably more BCL_1 when placed with the tumour i.p. at an early time-point of the adoptive therapy. This would explain the significant difference between the therapy in the $CD62L^{low}$ and $CD62L^{hi}$ groups. In the $CD62L^{hi}$ group very few BCL_1 may have been killed because presumably the CD8+ T cell transferred would not have been tumour-specific (in future work the efficacy of all groups could be investigated in ^{51}Cr release assays). The mixed population, $CD62L^{hi}$ and $CD61L^{low}$, when transferred together gave protection from tumour which was slightly better than the $CD62L^{low}$ group as expected.

Adoptive therapies using *in vitro* generated CTL (lines CD8-π-1 and CD8-π-2) were more successful than those using *in vivo* generated CTL both at an E:T ratio of 100:1 and in direct comparison (using CD8-π-1) at 10:1. Why should the *in vitro* CTL perform better than their *in vivo* counterparts? Both sets of CTL were comparably cytotoxic against BCL_1 at the time that adoptive therapy was carried out so again perhaps the persistence of the CTL at the site of tumour

is of importance. IL-2 administration with CD8-π-1 rendered it inactive in adoptive therapy studies which is suggestive of AICD. Perhaps the difference between the *in vivo* and *in vitro* CTL is that the *in vivo* CTL are terminal effectors which is suggested by the fact that CTL from the peak of the anti-CD40 response are unable to be effectively cultured *in vitro* (chapter 3) whereas the *in vitro* lines used were cultured weekly on BCL_1, those which did not die *in vitro* had proliferated and created a progressively more cytotoxic and tumour-specific CTL line and when used for adoptive transfers were rested (day 7). CD8-π-1 may have been sustainable i.p. for just long enough to kill enough BCL_1 to ensure the survival of two out of five mice and delay onset of disease in the other three.

CD4-π-1 the non-cytotoxic CD4+ T cell line seemed to augment the therapy elicited by CD8-π-1 in an adoptive therapy study although the difference is not highly significant ($p>0.3$). Importantly, the non-cytotoxic CD4-π-1 was not therapeutic without CD8-π-1 suggesting the CD4-π-1 cells provided help for the CD8-π-1 cells.

We cannot rule out the fact that CD4-π-1 could have been providing IL-2 for the CD8-π-1 cells as even though with IL-2 administration, CD8-π-1 was not as effective, a titration of IL-2 was not carried out and more optimal doses of IL-2 may have been provided by the CD4-π-1 CD4+ T cell line than were administered i.p. Future studies would aim to block IL-2 from CD4+ T cell lines using anti-IL-2 mAbs and investigating the effect of this block on therapy. Future studies should also investigate whether this help is required at higher E:T ratios using *in vitro* generated CTL lines. In many studies in transgenic mice help is not required at all as the numbers of tumour specific CTL are very high it would be interesting to find the cut off point in this model where tumour-specific CTL numbers are high enough not to need help.

In contrast to the effect of CD4-π-1 CD4+ T cells alone, CD4-π-2 CD4+ T cells elicited a therapeutic effect in adoptive transfer studies without the addition of CD8-π-2. This effect was attributed to their cytotoxic activity. Furthermore CD4-π-2 CD4+ T cells significantly increased therapy of BCL_1 from therapy

with CD8-π-2 alone and therapy with CD8-π-2 with naïve CD4+ T cells ($p<0.01$). This data indicates a role for tumour-specific CD4+ T cell lines in adoptive therapy of BCL_1. Considering CD4-π-2 cells were cytotoxic and produced T_H1 cytokines they may have contributed to anti-tumour effects in this experiment by direct tumour killing through Fas mechanisms or by production of IFNγ which could have enhanced peritoneal macrophage activity and increased MHC class I and II expression on the tumour cells themselves in addition to production of IL-2 to support the persistence of the CTL. In the case of direct killing, it could be argued that because the CD4-π-2 were cytotoxic then mice survived due to the combined cytotoxic activity of CD4-π-2 and CD8-π-2. However the cytotoxic activity after two weeks in culture when the adoptive therapy was carried out was low for both CD4-π-2 and CD8-π-2 (Chapters 4 and 5) lower in fact than both *in vivo* CTL previously used at 100:1 and the *in vitro* line CD8-π-1. This evidence again supports the idea that the *in vitro* lines tested were able to survive longer *in vivo*, long enough to ensure BCL_1 transferred at the same time were eradicated.

In all of the adoptive therapies CTL and tumour were injected together at the same site, thus killing could have taken place before tumour had any time to grow. This almost prophylactic protection from tumour does not take into account that in a clinical setting tumour would be already established in the host before therapy would take place. CTL would therefore have to track to tumour sites and would need to persist in the tumour site long enough to effect its destruction. Future work would, therefore, include adoptive therapies where CTL lines were injected after BCL_1 administration or injected at a different site from tumour, for example CTL could be injected i.v.

8 Discussion

To destroy tumour cells and fight most cancers, an effective CTL response is required from a small number of CTL precursors (CTLp). It is now accepted that this process, which involves the priming of naïve CD8+ T cells is initiated and regulated by professional APC which capture and display antigen. These APC are also activated by a growing list of inflammatory stimuli inducing them to increase migration to lymphoid organs and acquire the capacity to prime naïve T cells. In the absence of such stimuli, APC take on a non-stimulatory role and can even tolerise T cells (265), (266), (267). The capacity of an APC to obtain antigen and migrate to T cell areas can therefore be considered as a regulatory control during CTL priming. In most cases, CTL priming also requires the active participation of CD4+ T_H cells which recognise tumour antigens presented on the same APC recognised by the CTLp. As detailed in Chapter 1 the emerging paradigm of CTL activation is that CD4+ T_H cells act to further functionally mature APC, through CD40:CD40L interactions allowing potent activation of CTLp.

Experiments performed with the murine lymphoma model, BCL_1, suggest that anti-CD40 mAb acts to provide immunotherapy by bolstering a pre-existing anti-tumour T_H1 response (258). This may occur through augmentation of the tumour initiated DC licensing which would allow the effective presentation of tumour antigens in the context of MHC class I. Enhanced presentation could occur via the up-regulation of MHC class I on DCs or indeed CD40+ B cell lymphomas, the improvement of processing of peptide onto MHC class I, or importantly, via the up-regulation of both B7-like and TNF family member co-stimulatory molecules upon the APC.

The administration of anti-CD40 mAb leads to the enhanced differentiation of tumour-reactive CTLp into effective CTL. The proliferation and accumulation of large, anti-CD40 augmented, numbers of these tumour-reactive CTL are enough to produce tumour eradication as shown in Chapter 3.

We can therefore consider the tumour-associated antigens(s) presented by BCL_1, like many other tumours, to be weakly immunogenic (131). Anti-CD40 mAb administration may affect tumour eradication by ways detailed above; thereby allowing low-affinity anti-tumour T cells to adequately differentiate in

response to these antigens (268, 269). However, this premise also raises the possibility that anti-CD40 may induce autoimmunity by allowing the differentiation of low-affinity autoreactive T cell into effector cells. Preclinical *in vivo* data shows that administration of 200μg anti-CD40 mAb to BALB/c mice caused minor histopathological abnormailies in both the kidneys and livers of treated animals. In animals receiving 1 mg or more 3/23, splenic changes were also noted. Although, in further toxilogical studies it was demonstrated that these changes were fully reversible, this may represent an important hurdle of using anti-CD40 mAb *in vivo* in the clinical setting (T Geldart, personal communication). Therefore, we wished to establish an alternative method of targeting lymphoma by use of tumour-specific T cells. Adoptive transfer therapy for patients with cancer requires isolating tumour-reactive T cells, activating and expanding them *ex vivo* and transferring them back into the autologous host. The systemic administration of anti-CD40 would, due to the broad expression profile of CD40, target many cells as opposed to adoptive therapy where the tumour should be specifically targeted. This is of importance since therapies should strive to reduce the level of concomitant damage to healthy tissue. Theoretically this approach overcomes many obstacles to successful immune based therapies as described in Chapter 1 allowing the selection expansion and activation of tumour-reactive cells as well as the manipulation of the host environment.

Animal studies have documented that adoptive immunotherapy has been effective against established tumours (270, 271). In our model, unlike in many of the clinical settings outlined in the literature, TAA(s) have not been identified. However, the T cell response against BCL_1 has been established.

Anti-tumour effector CTL can be isolated during anti-CD40 therapy, typically on day 9, from the spleen of BALB/c mice. These CTL are cytotoxic against πBCL_1, have an activated phenotype ($CD62L^{low}$, $41BB^{hi}$, $CD44^{hi}$, $CD49d^{hi}$, $LFA\text{-}1^{hi}$, $ICAM\text{-}1^{hi}$) and are polyclonal. Their absolute requirement for the eradication of tumour in the primary response has been confirmed previously by CD8+ T cell depletion in recipient mice (258).

Protection from tumour re-challenge was also dependent upon CD8+ T cells (250). This suggests the persistence of memory CD8+ T cells which were able

to respond to tumour upon re-challenge without the administration of anti-CD40.

Data presented in Chapter 3 and Chapter 4 show that CD8+ T cells from mice cured of BCL_1 by administration of anti-CD40 mAb and harvested from the spleen day 20-65 of therapy were the only T cell pool from which anti-tumour CTL *in vitro* could be generated.

There were three available pools of CD8+ T splenocytes which could potentially be used to generate tumour-specific CTL lines for adoptive therapy: naïve cells from non immunised mice, effector cells from the peak of response during anti-CD40 mAb immunotherapy and those taken from mice which were cured of tumour with anti-CD40 mAb. These T cell pools were assessed using the method of co-culture with irradiated lymphoma outlined in the aims of the project and similar methods were used to expand CD4+ T cell lines.

We were not able to successfully expand CD8+ T cells from naïve mice in co-culture systems. In the *in vivo* setting, in the primary response to BCL_1, TAA(s) would be presented to naïve CTLp in the context of MHC class I with a concurrent helper T cell or anti-CD40 induced up-regulation of co-stimulation on APC. This APC could be the lymphoma itself. *In vitro* co-culture systems using πBCL_1 and naïve CD8+ T cells could never, in our hands, mimic the response seen *in vivo*. Thus CTL lines could not be generated in this way from naïve CD8+ splenocytes.

During anti-CD40 mAb therapy high numbers of tumour-specific CTL are generated and these are sufficient for successful tumour clearance. However, both direct adoptive therapy with these cells and *ex vivo* co-culture with irradiated lymphoma using these cells was reasonably unsuccessful. When used in co-culture with πBCL_1 peak effector cells died *in vitro* typically 1-3 days after being isolated. The same was assumed but not confirmed for their fate in adoptive therapy as peak cells were never seen to cure any recipient mice in our hands. Preliminary observations, tracking CFSE labelled CTL injected in to recipient mice with lymphoma, indicate that these cells may have been taken up by macrophages within 24 hours of transfer (data not shown). Presumably these peak effector cells were terminal effectors which died via AICD. However, the

transferred peak CTL could also have simply migrated away from the tumour site causing lack of efficiency. This was not examined.

CD8+ T cells from cured mice were successfully used in co-culture with πBCL_1 to generate anti-tumour lines. The success with this T cell pool may have been due, firstly to them being present in the spleen of cured mice in larger numbers than potentially tumour-specific CTLp in naïve mice. Secondly, memory CD8+ T cells having encountered TAA(s) once, can respond to antigen at an increased rate upon second encounter. Thirdly, memory CD8+ T cells differ for naïve T cells in their requirement for co-stimulation/signal 2 (Reviewed in (272)).

Re-challenge experiments showed the protection from tumour did not require additional anti-CD40 mAb dependent help, nor did it require the presence of CD4+ T cells indicating that that it was a memory response. The secondary response could occur without the bolstering of the immune system as there was no requirement for signal 2. It may have been due to this fact that memory cells were able to be used to generate tumour-specific CTL lines upon co-culture with irradiated πBCL_1 and with minimal cytokines (IL-7 and IL-2) *in vitro*.

Both BCL_1 and its *in vitro* line πBCL_1 can be classed as functional APC thus allowing the presentation of their own TAA(s) to T cells in co-culture systems. We chose to use irradiated πBCL_1 in co-culture systems due to its availability *i.e.* it grew in *in vitro* culture, although importantly BCL_1 could also be used in the same manner to generate anti-tumour CTL (personal communication Dr. R. French). In a clinical setting, antigen would have to be provided from the tumour *e.g.* from lymph node biopsy. Importantly, our chosen APC (πBCL_1) differed from many human B cell malignancies as it expressed both B7-1 and B7-2 and adhesion molecules and this expression was sufficient to induce the generation of CTL and CD4+ T cell lines from memory T cells discussed above. Schultze *et. al.* studied a panel of B cell malignancies and although MHC class I and class II were expressed in the majority of cases, adhesion molecules such as LFA-3 and ICAM-1 were absent or had very low expression. Additionally the vast majority also did not express B7 family members or expressed insufficient B7 to induce allogeneic T cells to proliferate *in vitro* (273) indicating that there is a threshold level of B7 expression necessary to

induce productive T cell proliferation and cytokine production (274). Therefore in the clinical setting, presumably up-regulation of co-stimulatory/adhesion molecules would have to be achieved before *in vitro* co-culture with autologous T cells could occur and this could be achieved in several ways. Firstly, B cell lymphomas could be transfected with B7 molecules although, this could require multiple transfections. Secondly, as the major stimuli for the induction of B7-1 and B7-2 in normal and malignant B cells is the cross-linking of CD40 (44), anti-CD40 mAb could therefore be used to induce the expression of these molecules and furthermore to upregulate the expression of both MHC class I and class II molecules (275) as well as adhesion molecules (244). In our hands expression of these molecules could not be significantly increased by the use of either anti-CD40 or CD40L transfected feeder cells although expression of co-stimulatory, adhesion and MHC class I and II molecules were already high on both BCL_1 and πBCL_1. We therefore used irradiated πBCL_1 in co-culture systems without pre-incubation with CD40 cross-linking.

The CTL lines generated (Chapter 4) were all generated *in vitro* from memory CD8+ T splenocytes co-cultured with irradiated πBCL_1. These lines were shown to be cytotoxic against target lymphoma and two such lines were exclusively specific for πBCL_1 when tested for cytotoxicity against a panel of other tumour lines. However, other lines generated showed a broader range of lytic activity against cells such as P815, A20 or WEHI 231 in cytotoxicity assays. This is indicative of the presence of shared antigen(s) *i.e.* the same antigen(s) found on more than one tumour line. This data is also supported by the observation that mice cured of BCL_1 are able to reject other syngeneic lymphoma such as A20, resulting in long term survival (258). The antigens in the BCL_1 model are yet to be identified but data shown here indicates the presence of a CTL response to more than one antigen as the response *in vivo* is polyclonal and *in vitro* TCR skewing was not always to the same TCR (skewing to Vβ10, Vβ8 and Vβ6 were observed). Although it is possible that all of these TCR could bind only one tumour antigen it is, however, improbable. Having established that the TAA within this system could be shared, it is important to discuss the potential source of the antigen. Two types of retrovirus; MMLV and

MLV are evident in BCL_1, and A20; and transcripts encoding the MMLV *env* protein or MLV gp70 have been found by rt-PCR (personal communication, G. Crowther and Dr J. Mann). These viruses and others present in murine tumour cells may have significance for the *in vivo* therapy in terms of providing TAA(s) to which the immune response can be mounted. The *in vitro* lines generated and detailed within this thesis may also be specific for virally derived peptides which have become available for presentation.

The possibility that the tumours studied here present viral epitopes is supported, by the fact that early studies using BCL_1 lymphoma demonstrated that the surface IgM of this lymphoma binds MLV gp70 and, hence, initiates the transformation process (276). Additionally, the cross-protection between the B cell lymphomas BCL_1 and A20, as demonstrated *in vivo* (109) may be mediated by either a shared cancer-testis antigen, viral antigen, or a tissue-differentiation antigen. The origin of tumour antigens has an impact on many immunotherapies as the use of viral epitopes *e.g.* for peptide vaccinations as outlined in Chapter 1 would only be clinically relevant for virally transduced cancers.

The origin of the TAA is not central to this thesis as the tumour itself is being used as an APC, presenting its own presumably multiple TAA(s). The lack of known antigens within our system however did lead to restrictions in how the T cells were expanded. The two most available methods were by using the lymphoma itself as an APC which was our method of choice or by pulsing DCs with apoptotic lymphoma cells. However, although limited, this method closely reflects the clinical situation where the lack of known antigens would be commonplace. In Chapter 4 of this thesis, we have shown that the CTL lines used for adoptive therapy were polyclonal suggesting that more than one TAA was being targeted; however, two CTL lines CD8-π-1 and CD8-π-2 were unable to lyse other tumour cells in standard ^{51}Cr release assays indicating that these CTL were not targeting shared antigens. Furthermore all recipient mice described in this study remained healthy, although failure to exhibit symptoms of ill health cannot be considered as direct evidence that autoimmunity is not induced. However, it could be argued that other CTL lines generated, such as those which were also cytotoxic against P815 and WEHI 231, may have a wider antigen specificity and therefore if used in adoptive therapies would affect host

tissue. This speculation does not however rule out the use of CTL such as line CD8-π-4, a cross-resctive CTL line, in adoptive therapies as self-antigens involved in tumour rejection may be over expressed on tumour but expressed at too low levels on normal tissue for recognition by T cells. This thesis also suggests that there are MHC class II shared TAA(s) shown by both cytotoxicity data and cytokine profiles. For example, CD4-π-2 was able to lyse πBCL_1, A20 and P815, and produced IFNγ in response to both πBCL_1 and A20.

Both the *in vitro* generated CTL lines and the *in vivo* generated CTL used in adoptive therapy in this thesis were polyclonal *i.e.* they expressed a range of Vβ TCR chains. The polyclonal nature of these lines may improve their efficacy *in vivo* by limiting tumour escape as may occur with clonal T cells. This is obviously an important point when considering using cloned T cells or when generating CTL using a single known TAA e.g. MART-1.

We have shown that there is both a CD4+ and CD8+ response to tumour within the BCL_1 model of lymphoma. Data in Chapter 3 confirms previously published data concerning a CD8+ response and shows a modest CD4+ T cell response to tumour. Furthermore Chapters 4 and 5 show that both CD8+ and CD4+ T cell lines could be generated *in vitro* upon co-culture with irradiated lymphoma.

Many CD4+ T cell epitopes have been identified in human malignancies detailed in Chapter 1 and thus in a clinical setting could be used to isolate CD4+ T cells in a similar way to tumour-specific CD8+ T cells. In our model, CD4+ T cell lines were generated in the same way as CTL lines indicating a memory pool of CD4+ T cells was present in the spleen of cured mice. In the primary response to tumour with the addition of anti-CD40 mAb, CD4+ T cells are not required. Without the administration of anti-CD40 the CD4+ T cells present are presumed not able to prime CTLp effectively. This failure to elicit an effective anti tumour response could be due to the fact that by the time an antigen-specific response has occurred the tumour burden may be too large. Despite this, Chapter 3 shows that a modest CD4+ response did occur in response to tumour and was augmented by anti-CD40 mAb. This may have been of some significance as the CD4+ T cells used in generating CD4+ T cell lines were

taken from cured mice and due to the effect of anti-CD40 may have been present in greater numbers than naïve CD4+ T cells in the spleen.

In effective adoptive therapy studies in animals(277), (270, 271) and in clinical applications (278), (160) it has been shown that the success of adoptive immunotherapy is directly linked with both the quantity and immunologic reactivity of the cells transferred. The inability to generate large enough numbers of tumour-reactive T cells is a major impediment to therapy and often therapy is confined to a minority of patients. Methods, by which tumour-specific T cells can be expanded, as detailed in section 1.6.2.2.2, are critical to the development of T cell therapy. We attempted to activate and expand both *ex vivo* and *in vitro* generated tumour-specific CD8+ T cells in the absence of antigen (Chapter 6) by using mAb. The use of mAb against CD3 makes use of the signalling pathway ubiquitous to all T cells. Therefore if T cells expanded in this manner were tumour-specific or tumour primed before expansion, then large quantities of reactive T cells for use in adoptive therapy could potentially be generated (270). Monoclonal antibodies against other co-stimulatory signals *i.e.* CD28 or 4-1BB expansion of the tumour-specific cells could potentially improve this method (204). Many studies have used plate-immobilised mAb or mAb conjugated magnetic beads to expand tumour reactive human T cells (279), (280) and CD4+ T cells resistant to infection by HIV-1(281). In murine models anti-CD3/anti-CD28 conjugated beads have also been recently utilised to expand CD4+ tumour-draining lymph node (TDLN) cells (282). However, in our hands the culture of tumour-specific CTL on mAb conjugated beads, soluble mAb or plate bound mAb could not expand CTL to the numbers required without concurrent initiation of apoptosis. Differences between CD28 receptor ligation have been reported between CD4+ and CD8+ T cells (283, 284). Proliferation of CD4+ T cells is more pronounced upon CD28 ligation compared to CD8+ T cells and they have greater longevity. This may explain why in the literature especially in murine studies CD4+ T cells and not CD8+ T cells have been used. A promising antigen independent system for the expansion of human CD8+ T cells which has also been used to expand CD4+ T cells is the cell based artificial APC described by Maus *et al* which is detailed in section 1.6.2.2.2. This system utilises both mAb and 4-1BB ligand. We

attempted to increase survival of our CTL upon expansion with anti-CD3 by using anti-4-1BB mAb. Results were disappointing as survival of sufficient CTL numbers could not be achieved. The lack of survival could be due for the requirement for the physiological ligand (4-1BBL) within this system (216) which could be addressed in future studies by transfection of 4-1BB ligand into feeder cells.

The inability to expand T cells sufficiently was a limiting step to the work in this thesis. However, cultures of T cells were generated using the lymphoma co-culture system allowing a modest number of adoptive therapies. We transferred peak CTL directly *ex vivo* from mice undergoing anti-CD40 mAb therapy and compared these *ex vivo* CTL to *in vitro* generated CTL lines. We found that the activational status of the peak CTL affected therapy; activated, $CD62^{low}$, CTL were able to provide better protection from concurrent tumour cell administration. However, *in vitro* generated CTL were more effective at eradication tumour than *ex vivo* CTL in all adoptive therapies carried out and numbers of cells transferred had a bearing on anti-tumour efficacy *in vivo*. We studied the effect of both IL-2 administration and concurrent CD4 transfer on the efficacy of transferred CTL and found only transferred CD4+ T cells improved immunotherapy.

When used in adoptive therapy alongside *in vitro* generated CTL lines both cytotoxic and non cytotoxic tumour-specific CD4+ T cells improved the efficacy of therapy. CD4+ T cells have been shown to have paramount importance in the provision of IL-2 at the CTL priming stage (144). In adoptive therapy tumour-specific CD4+ T cells would need to provide help at the CTL effector stage as prolonged anti-tumour immunity due to the persistence of CTL is postulated to correlate with increased tumour regression (231, 237). In our hands *in vitro* generated tumour-specific CD4+ T cell lines improved efficacy of adoptive therapy which we presumed to be due to the secretion of soluble factors such as IL-2 in the vicinity of the CTL effectors. However, non-soluble factors may also effect CTL survival. Recent literature also suggests a direct interaction between CD4+ and CD8+ T cells via CD40-CD40L (285). Provision of IL-2 would have allowed CTL to persist for longer at the site of the tumour thus increasing the chances that tumour would be destroyed. Adoptive therapies

using non-cytotoxic CD4+ T cell lines showed that these cells provided little or no therapy against BCL_1 alone. The cytotoxic CD4+ T cell line however did provide a slight therapeutic effect upon adoptive transfer with tumour. This may have been due to direct lysis of target tumour as indicated by their efficacy in ^{51}Cr release assays. From data shown here it is less likely that the anti-tumour effects shown *in vivo* by CD4-π-2 were due to IFN-γ production as the non-cytotoxic line also produced similar amounts of this cytokine. As discussed in Chapter 7 the issue of CTL persistence, which CD4+ T cell lines may have improved *in vivo* could also account for the difference in efficacy between *ex vivo* CTL form the peak of the anti-CD40 mAb response and *in vitro* generated CTL lines. Complete therapy was not achieved using peak CTL even at 100:1 E: T ratios in our hands and these cells were thought to represent terminal effectors, unable to persist for long enough for therapy to be achieved. Complete therapy was achieved using *in vitro* generated CTL at 100:1 E: T suggesting that these cells can induce effective immunotherapy in a dose-dependent manner. In studies where the activated $CD62^{low}$ portion of *in vivo* generated CTL were separated from the non-activated $CD62^{hi}$ portion the activational status was seen to have a positive bearing on therapy. The increased efficacy was postulated to be due to higher numbers of tumour-reactive $CD62^{low}$ cells being transferred. In addition to this is has also recently been discovered that $CD62^{low}$ cells track to anatomical sites which were previously thought to be refractory to treatment (185) although whether this was the reason therapy was more effective in our hands would require further work tracking CTL *in vivo*.

In summary we have achieved successful adoptive therapy of BCL_1 by concurrent injection of *in vitro* generated tumour-specific CTL lines. These lines were generated from CD8+ splenocytes from the spleen of mice cured previously of BCL_1 by anti-CD40 mAb and co-cultured with irradiated lymphoma as detailed in Chapter 3 and Chapter 4. We have also shown that CD4+ splenocytes also from anti CD40 mAb cured mice could be used to generate tumour-specific CD4+ T cell lines which augmented the CTL induced therapy in adoptive transfer studies.

8.1 Further Work

We have described the generation of CTL from memory CD8+ T cells which in adoptive transfer experiments have eradicated tumour. However, to further reflect the clinical setting, we aim to transfer CTL into animals pre-challenged with tumour. Thus, future work would aim to also investigate the trafficking of CTL to the tumour site using either CFSE staining or green fluorescent protein (GFP) tagging, and address their efficacy in a more challenging situation. CTL generated *in vitro* showed a phenotype which could be classed as T_C1, shown to be the most effective phenotype at eradication of tumour in recent studies. However, CD4+ T cells when assessed for T_H1 vs T_H2 cytokines showed a less well defined phenotype. Therefore, future work would also aim to assess whether the *in vitro* cultured CD4+ T cells can be further skewed towards T_H1 by addition of IL-12 to cultures and whether these cells would be more effective in adoptive transfer. In similar experiments, the effect of IL-2 would be further assessed by titration. The effect of other pro-survival factors such as IL-15 and anti-CTLA-4 could be also assessed by measuring CTL survival, persistence at the site of tumour and efficacy against tumour *in vivo*. To address the problem of CTL numbers for effective adoptive therapy, alternative strategies for *in vitro* expansion should be explored. These would include the use of the cell based aAPC described by Maus *et al.* previously detailed. The relative success of expansion of CD4+ T cells in both mouse and human studies using mAb conjugated magnetic beads may implicate a role for this system in the expansion of CD4+ T cell lines within this model.

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