Improving the Survival and Targeting of Genetically Modified T Lymphocytes for Adoptive Cancer Immunotherapy

A thesis submitted to the University of Manchester for the degree of PhD in the Faculty of Medicine, Dentistry, Nursing and Pharmacy

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Abstract

Ex-vivo retroviral genetic modification provides a strategy to improve the efficacy of adoptively transferred immune effector cells in cancer immunotherapy. Two novel approaches in this field were investigated.

The prolonged *in vivo* survival of genetically modified effector cells is crucial to the success of gene-modified adoptive cellular immunotherapy. However, in cancer clinical trials to date, detection of circulating gene-modified T cells has required highly sensitive techniques, suggesting that the transduced cells do not survive *in vivo* in large numbers for significant lengths of time.

In this thesis, retroviral vectors containing two critical regulators of T cell apoptosis, Bcl-X_L and $cFLIP_S$, were constructed and used to transduce primary human T cells and T cell lines. *In vitro* assays confirmed that over-expression of Bcl-X_L promoted the survival of human T lymphocytes cultured in pro-apoptotic conditions but did not increase proliferation. T cell lines over-expressing $cFLIP_S$ were also protected from anti-Fas antibody-induced apoptosis, but poor transduction efficiencies impeded the further assessment of over-expression of this gene in human lymphocytes.

These results indicated that co-expression of Bcl-X_L in donor lymphocyte infusions or in conjunction with a therapeutic gene might enable the long-term survival and persistence of transduced cells *in vivo* thereby potentially enhancing the clinical outcome of gene-modified adoptive cellular therapy.

In further *in vitro* studies, the feasibility of co-expressing Bcl-X_L in a bicistronic construct with a chimeric T cell receptor (scFv-CD3ζ fusion) gene (that would modify the T lymphocyte to target and become specifically activated upon contact with tumour cells) was tested. However, in the experiments performed, IRES-dependent expression of Bcl-X_L did not result in a significant pro-survival effect.

Further investigation of this strategy in syngeneic *in vivo* murine models was hindered by a failure to adequately optimise the retroviral transduction of fresh murine T cells. An antigen-specific primary murine T cell line with specificity against a B cell lymphoma idiotype was successfully gene-modified to over-express Bcl-X_L. Transduced cells underwent a limited expansion and retained *in vitro* cytolytic activity in a ^{51}Cr-release assay. Unfortunately, in a subsequent *in vivo* lymphoma model adoptively transferred transduced T cells failed to demonstrate anti-tumour activity. A

number of deficiencies in this single experiment however make it difficult to conclude on the basis of this alone that there is definitely no *in vivo* effect of Bcl-X_L over-expression in murine T lymphocytes and further studies are planned.

In the final part of this thesis, an antibody scFv fragment (CGS-1) specific for an antigen found in and around tumour neovasculature (ED-B protein) was utilised in an attempt to target gene-modified human peripheral blood lymphocytes against these vessels. The high affinity of the CGS-1 antibody for ED-B was confirmed and a retroviral vector was constructed that resulted in cell surface expression of CGS-1. Jurkat T-cells expressing CGS-1 bound to plates coated with recombinant ED-B in *in vitro* assays demonstrating the potential for such an approach. However, efforts to confirm this in primary human lymphocytes were limited by low levels of transgene expression and the uncertain nature of the antigen. Variable results were obtained when chimeric CGS-1.CD3ζ-modified lymphocytes were targeted against ED-B.

Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning except for the study illustrated in Figure 6.1 performed by Dr Anne Armstrong that has been submitted as part of her PhD thesis to the University of Manchester.

Signed..... Dated.....

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Abbreviations

BSA	Bovine serum albumin
BP	Base Pair
DNA	Deoxyribose nucleic acid
ED-B	Extra domain B
ELISA	Enzyme linked immunosorbant assay
GALV	Gibbon ape leukaemia virus
GFP	Green fluorescent protein
hFc	Human constant region antibody fragment
hIFN	Human Interferon
HRP	Horse radish peroxidase
HsTK	Herpes simplex-thymidine kinase
IL-2	Interleukin-2
iCD3/iCD28	Immobilised anti-CD3 and anti-CD28 antibodies
LAK	Lymphokine-activated killer
MHC	Major histocompatibility complex
MTM	Murine transmembrane domain
OM	Oncostatin M leader sequence
PBS	Phosphate-buffered saline
PBL	Peripheral blood lymphocyte
PCR	Polymerase chain reaction
scFv	Single chain antibody fragment
TIL	Tumour infiltrating lymphocyte
TNF	Tumour necrosis factor
TCR	T cell receptor

Dedication

This thesis is dedicated to

Fiona, Ben and Hannah

1 Introduction

In the United Kingdom cancer is diagnosed each year in one in every 250 men and one in every 300 women (Souhami and Tobias 2003). Although early stage disease can often be treated successfully by surgery or radiotherapy, disseminated metastatic disease and relapse post surgery present difficult management problems to cancer physicians. Despite many advances in recent years, as a cause of mortality it is second only to cardiovascular disease. Novel, effective treatments are urgently needed.

The importance of the interaction between the immune system and cancer cells was first recognised over a century ago when in the 1890s William Coley used streptococcal bacterial toxins in an attempt to activate general immunity against the tumour burden resulting in clinical responses (Coley 1893). More recently evidence has come from the demonstration of tumour infiltrating lymphocytes which are able to lyse tumour cells *in vitro* (Rosenberg, Spiess et al. 1986) and from the phenomenon of continual surveillance of virally transformed cells which breaks down in immunosuppressed patients leading to the development of lymphoproliferative disease (Aguilar, Rooney et al. 1999). In addition, administration of high-dose interleukin-2, a cytokine known to activate and expand T-lymphocytes has been shown to result in objective tumour responses (Fyfe, Fisher et al. 1995; Atkins, Lotze et al. 1999) in certain disease groups.

The observation that tumour cells express antigens that can, in certain situations, lead to an anti-tumour immune response has led to many approaches aimed at improving this immune response to cancer. This modulation of the immune system has become known as immunotherapy. In active immunotherapy, the patients' own immune system is stimulated to respond to the tumour. Passive immunotherapy involves the adoptive transfer to the tumour-bearing host of active components of the immune system, such as lymphocytes, which can mediate, either directly or indirectly, regression of the cancer.

To date, adoptive cancer immunotherapy has shown efficacy in only a limited number of patients and disease groups. Effective immunity against cancer frequently fails to develop due to a number of different mechanisms that will be discussed in more detail later in this chapter.

The field of gene therapy can be defined as 'the introduction of new genetic material into cells with a therapeutic intent' (Devita Jr 2001). There are many potential

approaches currently under investigation aimed at utilising gene therapy in the treatment of cancer.

In recent years, ex-vivo gene transfer methods have been developed that allow the efficient genetic modification of effector cells of the immune system such as T lymphocytes and may well provide a means of significantly improving the efficacy of adoptively transferred immune effector T cells in cancer immunotherapy approaches.

In this thesis two novel approaches involving the genetic modification of T lymphocytes were investigated.

For any form of cellular gene-modified immunotherapy to be effective, the prolonged survival of genetically modified cells *in vivo* is of crucial importance. In chapters 3-6, a potential approach to preventing the *in vivo* apoptosis of gene-modified lymphocytes (and consequently improving the efficacy of these cells) through the over-expression of two anti-apoptotic proteins, Bcl-X_L and cFLIP$_S$ was studied.

In chapter 7, a possible method of targeting genetically modified lymphocytes to tumour neovasculature was investigated. Such gene-modified lymphocytes could be engineered to either become specifically activated upon contact with tumour vessels, or to secrete a therapeutic protein at locally high concentrations thereby providing an excellent system of *in vivo* protein delivery. In this latter approach, the lymphocytes could potentially be acting as 'cellular vectors' in order to deliver the desired gene expression to the target tissue (in this case tumour blood vessels).

1.1 Adoptive lymphocyte immunotherapy

The transfer of immune effector cells with anti-tumour activity is known as 'adoptive cellular therapy'. As early as the 1960's studies of tumour immunity have demonstrated that it is possible to protect normal mice from developing experimental tumours by the injection of donor lymphocytes obtained from the spleens of previously vaccinated mice (Klein 1960).

Additional evidence for the potential of lymphocytes to cause regression of human cancers came from subsequent studies involving the use of interleukin-2 in patients with metastatic renal cell tumours and melanoma. IL-2 is a cytokine produced by (mainly CD4+ve T helper) lymphocytes following exposure to antigen. It is a major growth factor and results in the expansion of activated T lymphocytes during the early phase of

the immune response. In large non-randomised studies of patients with metastatic melanoma and kidney cancer, treatment with recombinant IL-2 resulted in objective remissions in 15-20% of cases (Fyfe, Fisher et al. 1995; Atkins, Lotze et al. 1999). In one case series, 8% of patients experienced a complete response to treatment and most of these responses have been durable with patients remaining in remission for several years (Rosenberg, Yang et al. 1998). Most studies have failed to show any direct effect of IL-2 on tumour cells *in-vitro* and consequently the mechanism by which these clinical remissions occur is considered to be through T lymphocyte activation (Vlasveld and Rankin 1994).

A variety of adoptive cellular immunotherapy approaches have been tested in cancer patients and illustrate that the approach can be therapeutic. These techniques generally involve the removal of cells from an individual followed by a period of manipulation and growth *in vitro* prior to re-infusion back into the patient.

1.1.1 Allogeneic T Lymphocyte therapy

Following allogeneic stem cell transplantation for haemopoietic malignancies the eradication of leukaemic cells leading to cure is thought to be mediated primarily via a donor-derived T-cell dependent immune reaction. Sixty to eighty percent of chronic myeloid leukaemia patients who relapse following allogeneic bone marrow transplantation have been shown to go into remission following infusion of allogeneic donor T cells (Drobyski, Keever et al. 1993). Recent reports suggest that following nonmyeloablative allogeneic transplantation a graft versus tumour response may also be successfully induced against solid tumours such as renal cell carcinoma (Childs, Chernoff et al. 2000) and more recently against breast cancer (Bregni, Dodero et al. 2002).

Infusions of donor T cells have been shown to be effective in the treatment of the EBV-associated post-transplant lymphoproliferative disease (PTLD) which is seen after allogeneic bone marrow transplantation (Rooney, Smith et al. 1998). More recently, partly HLA-matched allogeneic cytotoxic T cells (derived from a frozen bank of cells obtained from healthy volunteers) have also shown efficacy in the treatment of EBV-associated PTLD (Haque, Wilkie et al. 2002).

In addition, adoptively transferred cytomegalovirus-specific T cells have previously been shown to prevent CMV infections in patients after allogeneic bone marrow transplantation (Walter, Greenberg et al. 1995).

1.1.2 Autologous T lymphocyte therapy

In solid tumour therapy an early approach was to use lymphokine-activated killer (LAK) cells (Rosenberg, Lotze et al. 1993). These are autologous lymphocytes isolated from peripheral blood and subsequently activated with high doses of IL-2 prior to re-infusion. In the laboratory LAK cells were able to selectively lyse tumour target cells in a MHC-unrestricted fashion and were subsequently shown to produce sustained remissions of metastatic tumours in some patients with either melanoma or renal cell carcinoma. A subsequent randomised trial comparing IL-2 alone or in combination with LAK cells demonstrated a trend towards improved survival rates with combination treatment (Rosenberg, Lotze et al. 1993).

With advances in cellular manipulation it subsequently became possible to isolate tumour-infiltrating lymphocytes (TIL) from within tumour samples. These could be expanded to large numbers in the presence of IL-2 and irradiated tumour cells prior to reinfusion. In contrast to the non-specific LAK cell cytotoxicity, TILs showed a greater propensity for specific tumour cell killing in pre-clinical studies (Rosenberg, Spiess et al. 1986). In a clinical study objective responses were seen in 34% of patients treated with a combination of TILs and IL-2 (Rosenberg, Yannelli et al. 1994) including some patients who had progressed through previous treatment with IL-2 alone.

Auto-lymphocyte therapy (Osband, Lavin et al. 1990) refers to adoptive immunotherapy with autologous non-specifically activated memory T lymphocytes that have been expanded and activated *ex-vivo* using an anti-CD3 monoclonal antibody. The theoretical basis for this is that memory T cells previously exposed *in vivo* to tumour antigens have the potential for mediating tumour regression following non-specific activation.

More recently, Dudley and colleagues have reported the adoptive transfer to patients with metastatic melanoma of highly selected autologous tumour-reactive T cells (derived from TIL cultures), directed against overexpressed self-derived differentiation antigens (eg MART-1), following a nonmyeloablative chemotherapy conditioning regimen. This approach resulted in persistent clonal repopulation of T cells in the cancer

patients and, encouragingly, six out of the thirteen patients treated experienced objective clinical responses (Dudley, Wunderlich et al. 2002).

These approaches clearly demonstrate the potential of adoptive cellular therapy in highly immunogenic cancers such as renal cell carcinoma or melanoma. It is important to note however that, notwithstanding these recent positive results, to date the administration of autologous immune effector cells has not clearly improved patient survival rates substantially in these malignancies when compared with immuno-modulation using high dose intravenous IL-2 alone.

Equally, although these examples are encouraging, in the more common but less immunogenic epithelial malignancies such as lung and colorectal cancer responses to any adoptive immune manipulations are rare.

1.1.3 Mechanisms of Tumour Escape

There are thought to be several possible explanations why tumours 'escape' from immune control and may be poorly responsive to these forms of immunotherapy.

Optimal activation of T cells requires the ligation of various co-stimulatory receptors on the cell surface (Sperling and Bluestone 1996). The molecules known to bind to these receptors (so called co-stimulatory molecules such as B7.1) are expressed on professional antigen presenting cells and on a variety of other cells after exposure to inflammatory cytokines but are not present on the surface of tumour cells. Upon recognition of antigen on tumour cells, lymphocytes activated via the T cell receptor in the absence of sufficient 'co-stimulation' may be rendered anergic or die by apoptosis (see section 1.3).

Tumour cells have unstable genomes. This can result in loss, or mutation of, key elements required for effective antigen presentation such as MHC class I molecules, β_2-microglobulin, or the TAP (transporter associated with antigen processing) transporters, rendering them unable to present tumour antigen peptides to $CD8^+$ cytotoxic T cells (Marincola, Jaffee et al. 2000). In addition, tumour cell variants can evolve that lack the actual tumour antigen being targeted (Riker, Cormier et al. 1999).

The local tumour microenvironment may well be hostile to immune effector cells. Tumours can secrete cytokines capable of interfering with the immune response. An example of this is the production of transforming growth factor-ß (TGF- ß). Normally

produced by certain immune and other somatic cells it is known to have immunosuppressive activity (Stearns, Garcia et al. 1999). Transgenic mice engineered to be insensitive to TGF-β signalling have been shown to be more effective in eradicating tumours when challenged with tumour cells (Gorelik and Flavell 2001).

Molecules found on the surface of tumour cells, such as Fas ligand (FasL, CD95), can also induce lymphocyte apoptosis and be an explanation for tumour escape (Hahne, Rimoldi et al. 1996).

Other groups have proposed the existence of regulatory 'suppressor' T cells that are thought to maintain peripheral tolerance by preventing the activation of autoreactive T cells recognizing tissue-specific antigens (Thornton and Shevach 1998; Piccirillo, Letterio et al. 2002). Since most cancer immunotherapy approaches are directed against self-antigens such cells could potentially prevent the development of an anti-tumour immune response.

1.2 Genetic modification of T lymphocytes

With current vector technology, access to and efficiency of target cell transduction *in vivo* remains very difficult. Lymphocytes have several features that make them attractive as targets for *ex-vivo* genetic modification.

They are readily available from the peripheral blood of patients (either by venesection or apheresis) and are easily manipulated *in vitro*. Using current retroviral techniques, gene transfer efficiencies of up to 95% without the need for further *in vitro* selection have been reported (Movassagh, Boyer et al. 2000). Following transduction lymphocyte numbers can be rapidly expanded in tissue culture and this allows time to test for transgene expression and other properties of the genetically modified cells prior to their reinfusion to the patient. Another potential advantage is that peripheral blood lymphocytes are more fully differentiated than bone marrow precursor cells and thus inserted genes might not be as susceptible to inactivation as they would be in cells going through many steps of differentiation (Culver, Cornetta et al. 1991).

In addition, certain populations of unmodified memory T cells are very long-lived and if such cells could be gene-modified the effect of this gene transfer has the potential to be quite prolonged *in vivo*.

Clearly crucial to the success of gene-modified lymphocyte strategies is the efficiency of lymphocyte transduction and subsequent transgene expression.

1.2.1 Methods of gene transfer

The choice of method of gene transfer is determined by the specific requirements of the gene therapy approach under investigation. The most straightforward method, transfection, refers to gene transfer by (non-viral) processes that make a cell transiently permeable to naked DNA. Examples of transfection methods include calcium phosphate co-precipitation (see 2.2.6.2.1), liposomal transfection (see 2.2.6.2.2) and electroporation. Unfortunately, when used to transfer genes into human T lymphocytes the efficiency of gene transfer is very low and generally transient. Nevertheless, very low level stable transgene insertion can occur into the host genome using non-viral methods and electroporation is currently being used in at least one clinical trial of gene-modified lymphocyte therapy (Jensen, Clarke et al. 2000).

Most current clinical lymphocyte gene therapy protocols however use retroviral vectors. As discussed above, high efficiency gene transfer into lymphocytes can be achieved using retroviral vectors and stable integration of genetic material results in long-term transgene expression. Gene-modified cells do not subsequently express viral proteins resulting (theoretically) in a low immunogenicity of reinfused cells. Disadvantages of current retroviral vectors include relatively low viral titres (when compared with adenoviruses) and the requirement for target cells to be replicating for gene insertion to occur.

1.2.2 Retroviral vectors

1.2.2.1 Retroviral Life Cycle

Retroviruses are RNA viruses (originally isolated from rodents or chickens) that are capable of stably integrating up to approximately eight kilobases of DNA into the host cell genome. The life cycle of a retrovirus begins when viral envelope glycoproteins bind to specific receptors on the cell surface (*Figure 1.1*). Viral RNA then enters the cell and is reverse transcribed to DNA by the virally encoded reverse transcriptase. The viral DNA is transported to the nucleus where it integrates into the host chromosome. Transcription of the integrated viral DNA, or provirus is directed from the viral long terminal repeats. The host cell then translates spliced and unspliced viral transcripts to form viral structural proteins. The presence of a packaging signal (Ψ) results in some of

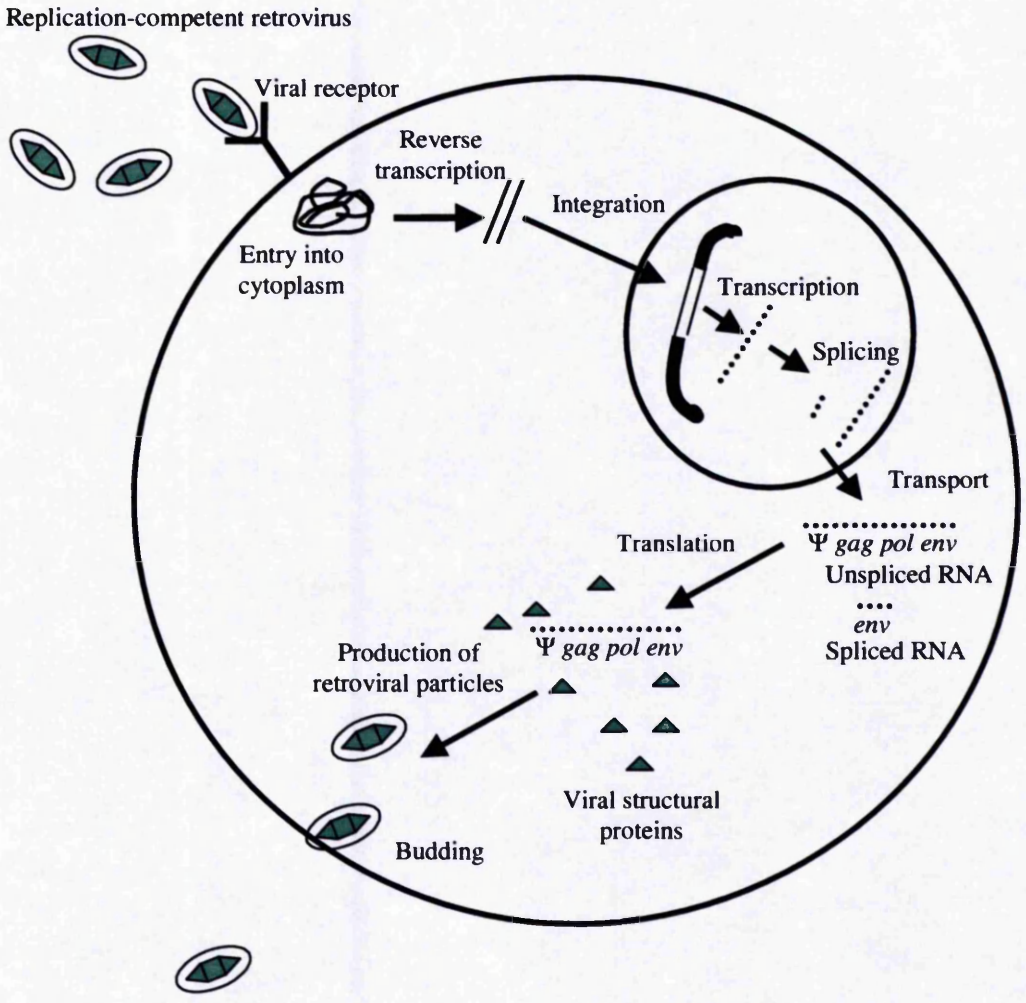


Figure 1.1 Life cycle of a replication-competent retrovirus – All retroviruses infect host cells through an interaction on the host cell surface (*Figure 1.1*). Viral RNA then enters the cell and is reverse transcribed to DNA by the virally encoded reverse transcriptase. The viral DNA is transported to the nucleus where it integrates into the host chromosome. Transcription of the integrated viral DNA, or provirus is directed from the viral long terminal repeats. The host cell then translates spliced (*env*) and unspliced viral transcripts to form viral structural proteins. The presence of a packaging signal (Ψ) results in some of the unspliced viral RNA transcripts being packaged to form new viral particles, which are released from the cell by a process known as budding.

the unspliced viral transcripts being packaged to form new viral particles, which are released from the cell by a process known as budding.

1.2.2.2 Production of Retroviral vectors

Retroviral vectors for gene transfer are produced by removing the viral coding regions (*gag, pol* and *env*) of retroviruses and replacing them with the therapeutic gene of interest. This makes the vector unable to replicate, or 'replication incompetent'. To produce infectious viral particles the retroviral vector is then transfected into 'packaging' cell lines that already contain the structural viral protein genes in *trans* (ie from another site in the packaging cell genome) (*Figure 1.2*). Because the vector still contains the packaging ψ signal (and also the signals for reverse transcription and integration), it is encapsidated into a mature virus, which is capable of infecting target cells but incapable of replication. In contrast to the retroviral vector, the protein coding genes are not packaged because of the absence of a ψ signal.

In this way, stable 'retroviral producer' cell lines can be constructed. Stable producer cell lines contain integrated provirus DNA and are capable of long-term production of specific retroviral supernatants.

Unfortunately, the preparation of high titre retroviral producer cell lines for a specific vector is a laborious and time-consuming process, especially when comparing multiple different vectors (see section 3.6). In addition, with some retroviral constructs it has proven difficult to obtain high titre retroviral supernatants. One possible explanation for this is the outgrowth of clones expressing low levels of retrovirus during the prolonged selection processes needed to create such lines (Ausubel, Brent et al. 1995).

In an attempt to overcome some of these issues, several groups have developed alternative strategies based on transient retroviral production (Landau and Littman 1992; Finer, Dull et al. 1994). In these approaches the retroviral vector plasmid is co-transfected into either COS or 293/293T cells at the same time as another plasmid encoding viral packaging genes. Using these systems, high titre retroviral supernatants can be harvested and be ready for use 48 and 72 hours post transfection, without the need to construct stable producer lines, allowing the rapid testing of newly prepared constructs. One such transient retroviral packaging system, *kat* (Finer, Dull et al. 1994) was used to make much of the retrovirus tested in this thesis and a schematic

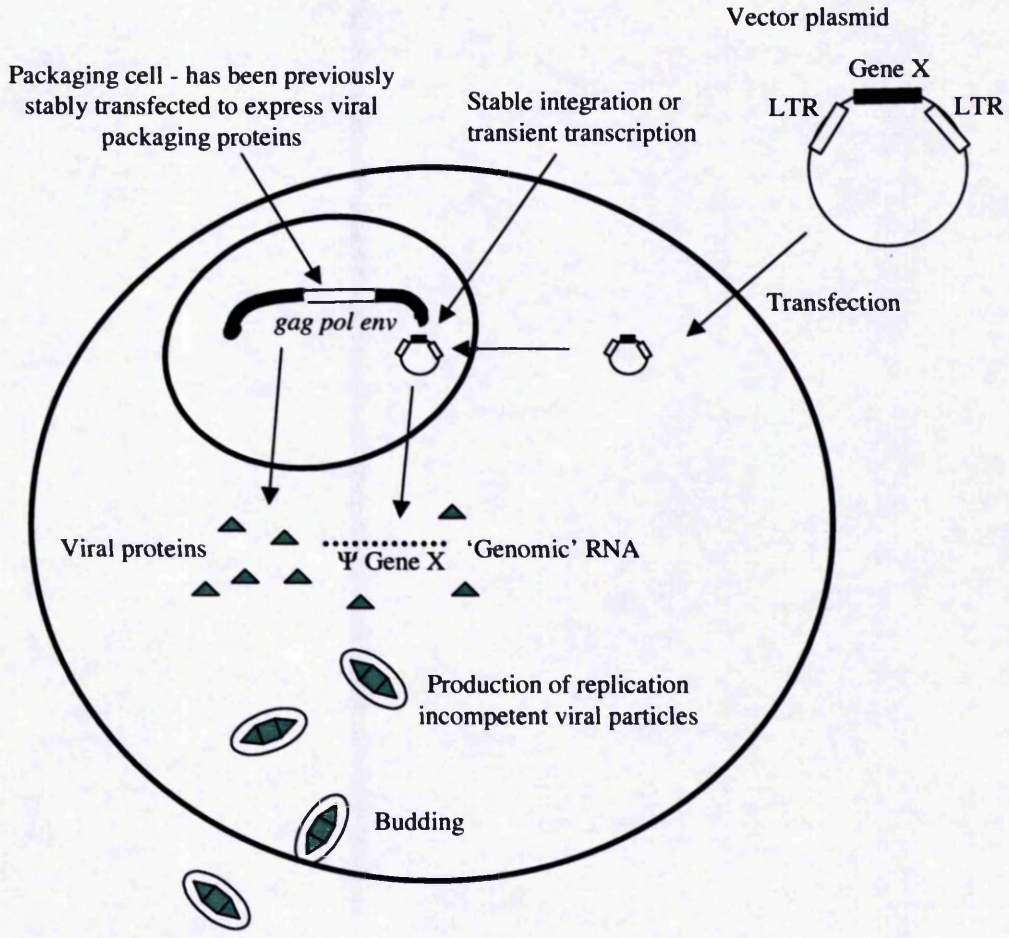


Figure 1.2 Production of replication-incompetent retroviral particles using packaging cells – To produce infectious viral particles the retroviral vector containing the gene of interest is transfected into 'packaging' cell lines that have already been stably transfected to express the structural viral protein genes in *trans*. The vector DNA is then transcribed either transiently for several days post transfection from non-integrated plasmids, or stably, from integrated molecules. Because the vector still contains the packaging ψ signal (and also the signals for reverse transcription and integration), it is encapsidated into a mature virus, which is capable of infecting target cells but incapable of replication. In contrast to the retroviral vector, the protein coding genes are not packaged because of the absence of a ψ signal.

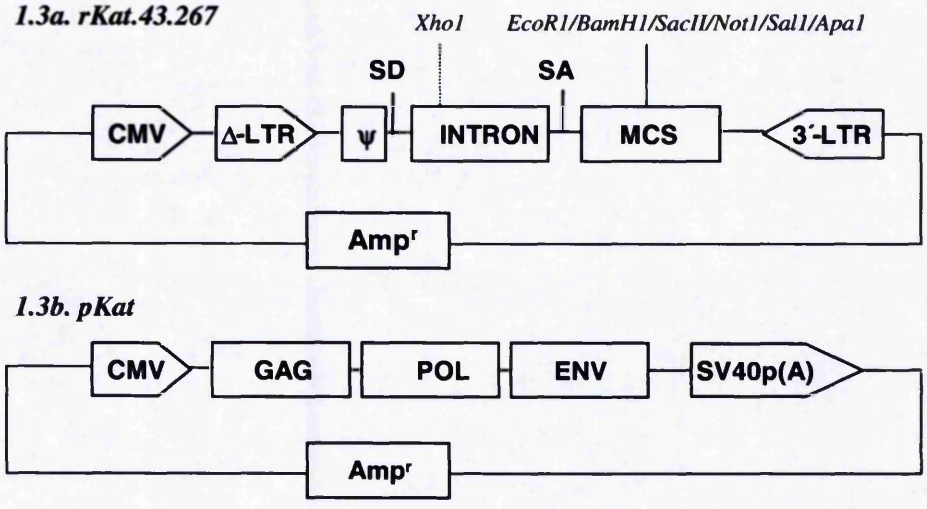


Figure 1.3 Schematic representation of basic rKat and pKat vectors – The *Kat* retroviral packaging system was purchased from Cell Genesys, California, USA. Briefly, following co-transfection into 293T cells (by a calcium phosphate co-precipitation method – section 2.2.6.2), the human cytomegalovirus (CMV) promotor controls transcription of viral packaging genes *gag, pol* and the amphotropic *env* gene from the packaging plasmid, *pKat*, and the moloney murine leukaemia virus-based *rKat* vector. The transgene of interest can be cloned into the multiple cloning site (MCS), which is preceded by the retroviral packaging signal, ψ , and the splice donor/splice acceptor sequences (splicing vectors express the gene of interest from a spliced message as though it were the *env* gene. Following insertion into the target cell, transgene expression is controlled by promotors within the 3'-LTR. The ampicillin resistance gene, Amp^r allows for selection in bacterial cultures.

representation of the basic vector plasmid, *rKat* and the packaging plasmid, *pKat* (containing *gag, pol* and an amphotropic *env* gene) is shown in *Figure 1.3.*

Clearly, such systems have the disadvantage that retrovirus production is transient and consequently (unlike stable packaging lines) repeat transfections are required each time retrovirus is needed. In addition, viral titre will vary to a certain degree between batches of cells transfected.

1.2.2.3 Retroviral Pseudotyping with envelope proteins

As with naturally occurring retroviruses, packaged retrovirus vectors enter the target cell through an interaction between the viral envelope glycoprotein and a specific receptor on the host cell surface.

Retroviruses have several classes of envelope glycoprotein that interact with different host cell receptors (see *Table 1.1*). Retroviral packaging cells can use a variety of these different envelope genes from other viruses. Because the envelope protein is the main determinant of the host range of the retrovirus (see *Table 1.1*), the specific envelope (or 'pseudotype') used can have a major effect on transduction efficiency.

The development of packaging cell lines that use different envelope genes has led to significant advances in the ability to transduce primary human lymphocytes. Retroviral vectors produced from the PG 13 packaging cell line, which use the gibbon ape leukaemia virus (GALV) envelope (Miller, Garcia et al. 1991) have been shown to transduce lymphocytes with a several fold higher efficiency compared with those derived from earlier amphotropic packaging cell lines (Lam, Reeves et al. 1996). When PG 13-packaged vectors and a cell centrifugation protocol were combined a T cell transduction efficiency of 95% has recently been reported (Movassagh, Boyer et al. 2000).

Rather than interacting with specific cell surface receptors, the vesicular stomatitis virus G glycoprotein (VSV-G) interacts with a phospholipid component of the cell membrane to mediate entry by membrane fusion. Unlike other envelope proteins, VSV-G confers enhanced physical stability to retroviral particles, allowing viral supernatant to be concentrated by ultracentrifugation to titres of 10^9 or higher (Burns, Friedmann et al. 1993). VSV-G pseudotyped vectors have also been used successfully to transduce human T lymphocytes (Gallardo, Tan et al. 1997).

Table 1.1 Host Range of Retroviral Envelopes (adapted from(Ausubel, Brent et al. 1995))

Vector Pseudotype	Cells that can be transduced	
	Murine	**Human**
Ecotropic	Yes	No
Amphotropic	Yes	Yes
GALV	No	Yes
VSVG	Yes	Yes
10A1*	Yes	Yes

GALV – gibbon ape leukaemia virus, VSVG – vesicular stomatitis virus G. *See section 6.1.

1.2.3 Optimising conditions for retroviral lymphocyte transduction

Much research has been carried out studying the optimal vector type, conditions for and methods of *ex vivo* retroviral lymphocyte transduction. Most retroviral vectors currently used in clinical trials are originally derived from the Moloney murine leukaemia virus, although frequently (as in the case of the *Kat* vector (Finer, Dull et al. 1994)) various regulatory elements within the long terminal repeats (LTRs) of the vector are modified, or replaced with elements from other viruses in an attempt to improve transduction and expression.

Clearly, as discussed in the last section, the retroviral envelope is a major factor in determining the transduction efficiency of a given retrovirus into a specific target cell. However, a number of other factors will have an effect on the success of the transduction process, not least of which is the method of transduction.

Initially, co-cultivation of retrovirus packaging cells with lymphocytes gave high levels of gene transfer into human lymphocytes. However, safety concerns (about the risk of re-injecting packaging cells) and worries about the reproducibility of co-cultivation on a large scale made this infection method unattractive for clinical gene therapy protocols when compared with using viral supernatant alone.

Many factors have been shown to affect the efficiency of gene transfer using retroviral supernatant. Retroviruses require cells to be mitotically active for insertion to occur. It is logical therefore that the method of pre-activating the lymphocyte will affect the gene transfer efficiency. Pollok et al (Pollok, Hanenberg et al. 1998) demonstrated that activation of lymphocytes on immobilised CD3 and CD28 antibodies (iCD3/iCD28) resulted in more efficient gene transfer than various other lymphocyte activation conditions such as using phytohaemagglutinin (PHA). Other workers have shown that different T cell subsets require different pre-activation conditions for optimal transduction efficiency (Movassagh, Boyer et al. 2000).

Increasing the proximity of T lymphocytes and retrovirus using polycations such as Polybrene (Davis, Morgan et al. 2002), or co-localisation on recombinant fibronectin fragments (Retronectin®) (Hanenberg, Xiao et al. 1996; Pollok, Hanenberg et al. 1998) has also been shown to improve efficiency of gene transfer.

Several groups have also demonstrated that retrovirus mediated gene transfer into lymphocytes can be enhanced by incorporating a centrifugation step in the transduction

process (Bahnson, Dunigan et al. 1995; Bunnell, Muul et al. 1995; Kuhlcke, Fehse et al. 2002), although the mechanism by which this occurs remains unclear.

A further observation has been that reducing the temperature at which lymphocytes and retroviral supernatant is incubated from 37°C to 32°C can increase transduction efficiency, presumably by increasing the half-life of the retrovirus (Bunnell, Muul et al. 1995).

1.2.4 Clinical trials of T lymphocyte gene transfer

The first study involving gene transfer into T lymphocytes to be performed in humans was published in 1990 (Rosenberg, Aebersold et al. 1990) and involved the use of tumour infiltrating lymphocytes (TIL) modified to express a marker gene, neomycin phosphotransferase (an enzyme that inactivates the antibiotic neomycin), in patients with metastatic melanoma. Gene-modified lymphocytes were found to be safe, and because the TILs contained a foreign gene, for the first time it was possible to determine the survival and distribution of the infused cells through polymerase chain reaction (PCR) assays on DNA from blood samples obtained from the patients.

Since then a number of T-lymphocyte marking studies have been performed using TILs (Favrot and Philip 1992), bone marrow stem cells (Brenner, Rill et al. 1993), HIV-specific cytotoxic T lymphocytes (CTL) (Riddell, Elliott et al. 1996) and Epstein-Barr virus (EBV)-specific CTLs (Heslop, Ng et al. 1996). In the latter study, gene-marked EBV-specific CTLs given to (immunosuppressed) patients following allogeneic bone marrow transplantation were detected for up to six years following infusion with accumulation of marked cells at disease sites. In the TIL studies, marked cells were detected for several months after infusion. These early studies established the feasibility and safety of using gene-modified lymphocytes in humans.

In an alternative field of medicine, the potential of this approach was further confirmed when *ex-vivo* retrovirus-mediated transfer of the adenosine deaminase (ADA) gene into the T lymphocytes of children with severe combined immunodeficiency was shown to result in successful immune reconstitution in these patients (Blaese, Culver et al. 1995; Aiuti, Vai et al. 2002).

To date the best example of gene transfer modifying cancer immunotherapy is in the field of allogeneic bone marrow transplantation for haematological malignancies. As discussed in section 1.1.1, patients with CML who relapse or patients with EBV-related

lymphoproliferation following bone marrow transplantation have been shown to go into remission following infusion of donor T cells (Drobyski, Keever et al. 1993; Rooney, Smith et al. 1995).

Unfortunately this treatment may be complicated by the development of the potentially life-threatening graft versus host disease, whereby donor T cells recognise histocompatibility antigens with wide tissue distribution resulting in immune attack against host tissues in the skin, liver and gut. Two studies have now been published (Bonini, Ferrari et al. 1997; Tiberghien, Ferrand et al. 2001) using donor lymphocytes genetically-modified to express the herpes simplex-thymidine kinase (hsTK) 'suicide' gene which specifically sensitises cells to the anti-viral agent ganciclovir.

In the study by Bonini et al (Bonini, Ferrari et al. 1997) three out of eight patients who received transduced donor lymphocytes developed acute or chronic graft versus host disease. In these patients, subsequent ganciclovir administration significantly diminished the number of circulating hsTK-transduced cells within 24 hours, resulting in complete or partial remissions of the disease.

1.2.5 Redirecting T lymphocytes with chimeric T cell receptors

For the majority of common malignancies tumour-reactive T lymphocytes remain difficult to isolate. One major approach by which gene transfer is being utilised to modify the immune system is by attempting to improve the way in which lymphocytes recognise tumour cells.

First described by Eshhar and colleagues, fusion of the antigen-recognition domains of specific anti-tumour antibodies with intracellular T cell receptor signalling chains to form 'chimeric' T cell receptors represents a potential solution to this problem (Eshhar, Waks et al. 1993).

Upon contact with tumour antigen, cytotoxic T lymphocytes genetically modified to express such receptors are specifically activated without the need for tumour MHC expression. Such an approach potentially allows the production of large numbers of specific T cells against any antigen for which a monoclonal antibody exists and effectively bypasses several of the common mechanisms of tumour escape (eg downregulation of HLA molecules and defects in antigen processing).

Although a variety of antigen-recognition domains could be used, most chimeric receptors use single chain antibody fragments (scFv). These consist of the two antigen-binding variable regions of an antibody (V_H and V_L) joined by a short peptide linker (see *Figure 1.4*). Single chain Fv fragments are simple and can be encoded in a retroviral vector by a single gene. Chimeric receptors have now been described that target a variety of tumour antigens expressed by many different malignancies (Pule, Bollard et al. 2002; Sadelain, Riviere et al. 2003). Alternative potential targets such as the tumour vasculature are also under investigation (Niederman, Ghogawala et al. 2002) (see chapter 7). In the work described in chapters 4, 6 and 7 a chimeric receptor expressing a scFv (C23 (Chester, Begent et al. 1994)) specific for the carcinoembryonic antigen (over-expressed on colorectal and other gastrointestinal malignancies) was used (Gilham, O'Neil et al. 2002).

The antigen-recognition domains of chimeric receptors are joined to intracytoplasmic signalling domains via a transmembrane region (20 to 30 exclusively hydrophobic amino acids that form an α-helix). The type of activatory signal generated by the chimeric receptor following binding of the antigen-recognition domain to its respective ligand is dependent on the intracytoplasmic signalling chain used. In the work described in this thesis the cytoplasmic segment of the CD3ζ chain (Gilham, O'Neil et al. 2002; Niederman, Ghogawala et al. 2002; Rossig, Bollard et al. 2002) was used, although some researchers have used the FcεRIγ and CD3ε chains (Kershaw, Westwood et al. 2000). A recent publication suggests that, in *in vivo* murine lymphocytes, chimeric receptors containing the CD3ζ chain give superior signalling efficacy compared to those containing FcεRIγ (Haynes, Snook et al. 2001).

Ligand binding by the chimeric receptor leads to phosphorylation of CD3ζ immunoreceptor tyrosine-based activation motifs (ITAMs) that in turn causes phosphorylation of the tyrosine kinase ZAP 70 and an activatory signalling cascade resulting in cytolysis, cytokine secretion and T cell proliferation (see *Figure 1.4*).

By substituting CD3ζ with other cytoplasmic signalling domains, chimeric receptors have also been designed that provide the lymphocyte with other 'costimulatory' signals such as CD28 after encountering the tumour cell (Alvarez-Vallina and Hawkins 1996; Hombach, Wieczarkowiecz et al. 2001). More recently, single chimeric receptors transmitting both CD3ζ and CD28 signals have been reported to give improved results

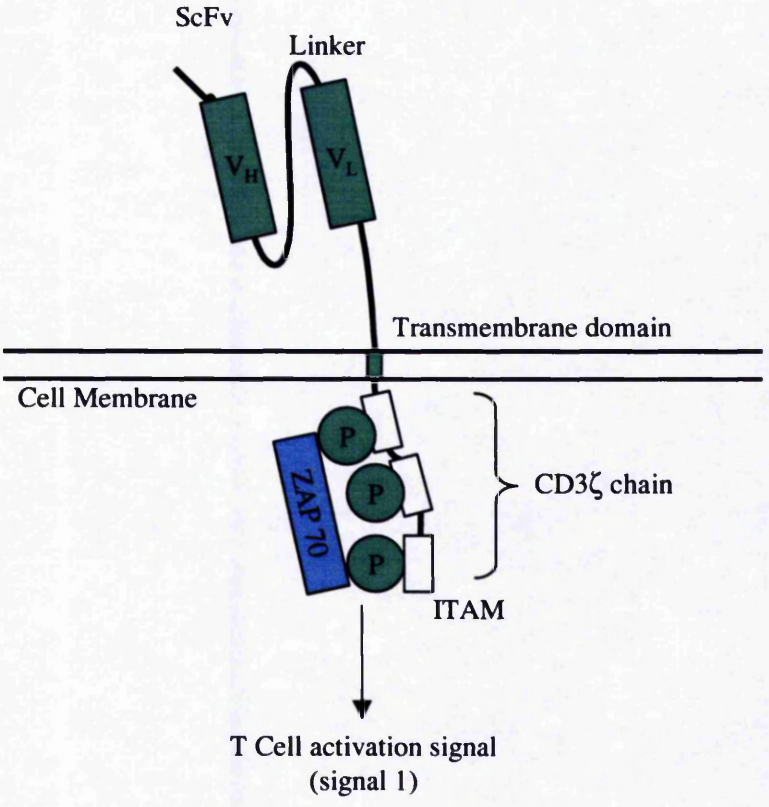


Fig 1.4 Chimeric T Cell Receptor (adapted from Pule, Bollard et al. 2002) – Chimeric T cell receptors combine single-chain antibody variable regions with T cell signalling chains. A single chain antibody variable (scFv) fragment derived from the variable regions of a monoclonal antibody is illustrated linked to the CD3ζ T cell receptor signalling chain. Upon ligand binding by the chimeric receptor leads to phosphorylation of of the ITAMs within the ζ chain cytoplasmic domain with a resultant T cell activatory signalling cascade.

in both *in vitro* and murine *in vivo* experiments (Haynes, Trapani et al. 2002; Maher, Brentjens et al. 2002).

In vitro chimeric receptor mediated cytotoxicity has now clearly been demonstrated in both mouse and human T cells (Sadelain, Riviere et al. 2003). Whatsmore, *in vivo* efficacy of murine chimeric receptor modified T cells has also now been demonstrated against a number of tumour targets (Hwu, Yang et al. 1995; Darcy, Haynes et al. 2000) and in another recent publication, human T cells targeted against CD19 using chimeric receptors were shown to eliminate established Burkitt's lymphomas in immunodeficient mice (Brentjens, Latouche et al. 2003).

1.3 T lymphocyte activation and survival

1.3.1 The Importance of 'Co-stimulation'

In 1970 Bretscher and Cohn first proposed a 'two-signal model' for antigen-specific lymphocyte activation and this continues as the prevailing hypothesis to explain how T cells discriminate self from non-self (Bretscher and Cohn 1970).

In this model two signals are required for T cells to become fully activated. Initiation of T cell activation and proliferation occurs following ligation of the T cell receptor (TCR)-CD3 complex with peptides bound to MHC molecules on the surface of antigen-presenting cells. The downstream intracellular effects of this primary interaction are known as Signal 1 and result in an antigen-specific cellular immune response (*Figure 1.5*) (Jenkins 1994).

However, TCR signalling alone is insufficient for stimulating IL-2 production, proliferation and differentiation of naïve lymphocytes and can in fact result in activation-induced apoptosis, or a tolerant state known as 'anergy' (Sperling and Bluestone 1996). In addition to Signal 1 many other, antigen independent, interactions occur between the two cells. These 'co-stimulatory' signals are thought to be essential for full activation and are known as Signal 2 (*Figure 1.5*).

The best studied of these co-stimuli is the interaction between the CD28 receptor found on the surface of T cells and its natural ligands, B7.1 and B7.2, on the surface of antigen presenting cells (Sperling and Bluestone 1996). CD28 is a homodimeric transmembrane glycoprotein found on the majority of human peripheral T cells. CD28 signalling in the

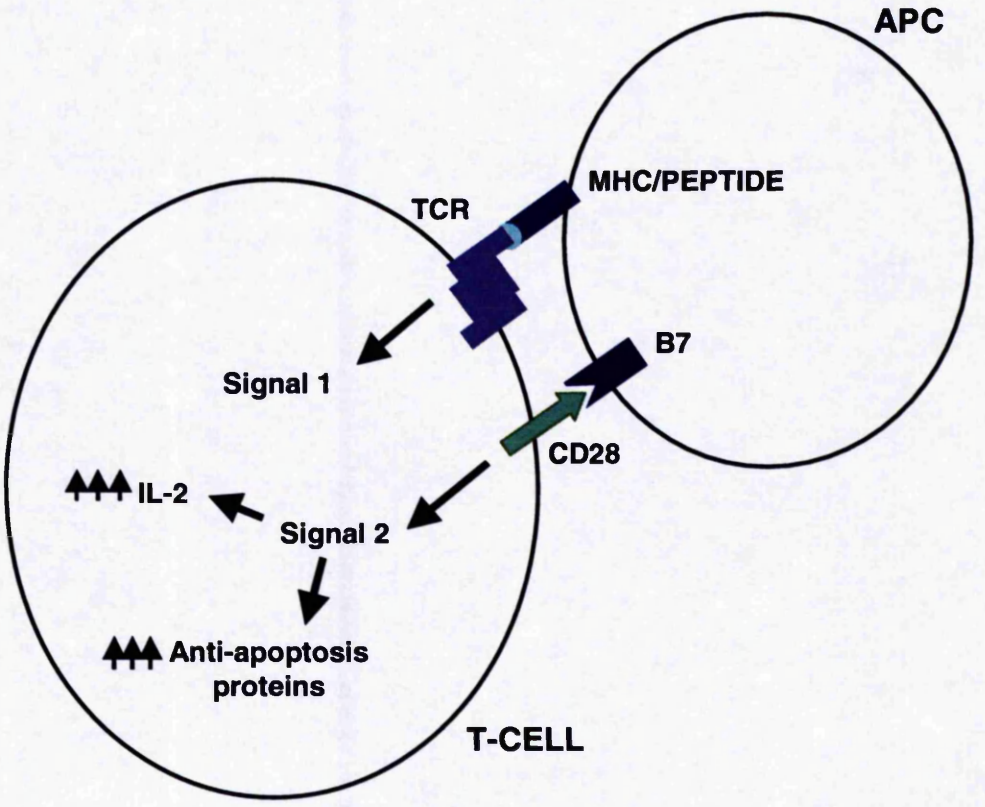


Fig 1.5 T-Cell Activation and Survival - "Costimulation" - Antigen-specific T cell activation occurs following ligation of the T cell receptor (TCR)-CD3 complex with peptide antigens bound to MHC molecules on the surface of antigen-presenting cells (APC). CD28 co-stimulation results in increased production of the T cell growth factor, IL-2 and up-regulation of certain anti-apoptotic proteins resulting in a marked increase in cell survival and proliferation.

absence of a Signal 1 can lead to T cell apoptosis (Collette, Benziane et al. 1998), however CD28 co-stimulation has the opposite effect and has been shown to promote both T cell proliferation and survival. Co-stimulation has also been shown to significantly lower the degree of TCR-Ag signal required to initiate proliferation (Gimmi, Freeman et al. 1991).

Transgenic mouse models (Sperling, Auger et al. 1996) have shown that, although not essential for early proliferative responses, co-stimulation dramatically influences late proliferation and improves long-term T cell survival. Co-stimulation also makes lymphocytes more resistant to apoptosis induced by γ-irradiation and decreases the apoptosis seen when lymphocytes are stimulated through CD3 alone (Boise, Minn et al. 1995).

By what mechanisms does this occur? The downstream signalling pathways of CD28 co-stimulation have been extensively studied.

CD28 co-stimulation causes prolonged IL-2 production by both increasing gene transcription (Fraser, Irving et al. 1991) and, as a result of post-transcriptional modifications, by increasing the half-life of its mRNA (Lindstein, June et al. 1989). Interleukin-2 is the major growth factor for T cells. Produced following lymphocyte activation, it stimulates both cell proliferation and survival and is likely to be the main mechanism by which co-stimulation improves proliferation.

Sperling et al (Sperling, Auger et al. 1996) however, demonstrated that the increased cell survival following CD28 ligation was not due to increased IL-2 production alone. As had been suggested earlier (Boise, Minn et al. 1995), their work suggest that this improved survival may in fact mainly due to the increased expression of certain genes whose products appear to protect against apoptosis, in particular Bcl-X_L.

1.3.2 Bcl-X_L – A Dominant Regulator of Apoptotic Cell Death

Multicellular organisms have a highly developed and organised system of programmed cell death. This is known as apoptosis, derived from the Greek word meaning 'falling off' of leaves from trees (Kerr, Wyllie et al. 1972). Many genes are known to be involved in the regulation of this process; in particular the Bcl-2 gene family, which has been extensively studied.

The t(14:18) translocation is the most common translocation in human lymphoid malignancies. Studies of this translocation demonstrated that it resulted in the abnormally high level of expression of the Bcl-2 oncogene resulting from placement of the gene under the influence of the highly expressed immunoglobulin heavy chain promotor.

Rather than stimulating proliferation, the main action of the Bcl-2 gene has since been shown to be the enhancement of lymphoid cell survival by prevention of apoptosis. A whole family of Bcl-2 related proteins have now been identified that are either positive or negative regulators of cell death (Cory and Adams 2002).

Using low stringency hybridisation with a murine Bcl-2 cDNA probe, Boise et al (Boise, Gonzalez-Garcia et al. 1993) identified a gene that displayed 44% amino acid homology with Bcl-2 that he named Bcl-X. Using chicken Bcl-X, two distinct cDNAs were isolated from the human Bcl-X gene, which were shown to arise from differential usage of two 5' splice sites. Bcl-X_L was identified as a 233 amino acid protein, with similar domains to Bcl-2.

In the same paper, Bcl-X_L over-expression was shown to render a murine IL-3-dependent lymphocytic cell line resistant to apoptotic cell death upon growth factor deprivation. Expression of Bcl-X_L was also shown to be restricted to long-lived cells such as mature neural cells leading to the hypothesis of a role for Bcl-X_L in long-term cell viability.

Further work, by the same group, studied Bcl-2 and Bcl-X_L expression in resting and co-stimulated human lymphocytes (Boise, Minn et al. 1995). They showed that Bcl-2 was constitutively expressed in both resting and activated T cells and the level of expression remained unchanged. In contrast, no Bcl-X_L protein was found in resting or CD28 only-stimulated T cells. TCR stimulation resulted in expression of Bcl-X_L after six hours and this expression was greatly enhanced by CD28 co-stimulation (levels after 24 hours being similar to those obtained using a lysate of a Bcl-X_L-transfected tumour cell line). Bcl-X_L expression in co-stimulated cells was shown to peak at 24-48 hours and decline thereafter and this correlated with a resistance to Fas antibody-induced cell death.

Another study (Sperling, Auger et al. 1996) has shown that survival of T cells from Bcl-X_L transgenic mice is not inhibited by blocking CD28 ligation; suggesting CD28

induced T cell survival is regulated by expression of Bcl-X_L. These results are consistent with the hypothesis that specific induction of Bcl-X_L following CD28 co-stimulation results in enhanced activated T cell survival.

Dahl et al (Dahl, Klein et al. 2000) strengthened this hypothesis in a recent paper. Using retrovirus-mediated gene transfer they expressed Bcl-X_L in murine T cells that were CD28-deficient but expressed a TCR recognising a specific antigen. They showed that, in the absence of CD28, Bcl-X_L expression prolonged lymphocyte survival but did not restore normal proliferation or effector cell development.

1.3.3 cFLIP$_S$ and Death Receptor Induced T cell Apoptosis

Aside from increased Bcl-X_L expression, it has become apparent more recently that CD28 co-stimulation can reduce the T cell apoptosis seen following TCR activation ('signal 1') by other mechanisms. These predominently affect the sensitivity of the T cell to stimulation by what are known as 'death receptor' pathways. The prototypic and best studied of these being the CD95 (Fas) receptor that binds with its natural ligand CD95L culminating in the death of lymphocytes by apoptosis (Krammer 2000).

These pathways, and the implications for lymphocyte apoptosis are described in more detail in the discussion section of Chapter 3 (3.14) and the introduction section to Chapter 5 (5.1), however, one of these mechanisms involves the strong upregulation of another anti-apoptotic protein, cFLIP (Van Parijs, Refaeli et al. 1999; Kirchhoff, Muller et al. 2000).

1.4 Aims

The focus of this thesis was to study two distinct strategies by which the efficacy of T lymphocytes in adoptive cancer immunotherapy could be improved through retroviral genetic modification.

1.4.1 Prevention of Lymphocyte Apoptosis

The aim of this work was to construct retroviral vectors containing the two anti-apoptotic proteins, Bcl-X_L and cFLIP, upregulation of which are considered to be predominently responsible for the improved survival seen in CD28 co-stimulated T cells. Such vectors would be used to genetically modify T cells and the effects of over-expression of these proteins studied, both *in vitro* and *in vivo*. The ultimate aim was to

incorporate such genes into other vectors being used for T cell modification in an attempt to improve *in vivo* survival and function of these gene-modified cells in adoptive immunotherapy.

In Chapter 3 the aim was to utilise a cDNA encoding for the anti-apoptotic protein Bcl-X_L and to introduce this into retroviral vectors used for gene therapy. The activity of this Bcl-X_L cDNA would be then tested by functional *in vitro* assay in primary human T cells and T cell lines in order to determine whether over-expression of Bcl-X_L resulted in protection from apoptosis in conditions prone to inducing this.

The purpose of the subsequent work described in Chapter 4, was to construct a vector that expressed Bcl-X_L as the second (IRES-dependent) gene in a bicistronic construct (ie expressing two transgenes). The relative expression of Bcl-X_L in cells transduced using this vector when compared with cells transduced using a vector in which Bcl-X_L was the first gene in the construct could then be studied, and further functional *in vitro* assays performed. The ultimate aim of this work was to examine the feasibility of combining the expression of a pro-survival gene in conjunction with a chimeric T cell receptor gene (Eshhar, Waks et al. 1993) (see section 1.2.5) in a single retroviral vector that could be used for genetic modification of T cells.

In a similar way to the work presented in Chapter 3 using Bcl-X_L, the aim of the work in Chapter 5 was to obtain cDNAs encoding the cFLIP proteins (long and short) and to incorporate these genes into vectors used for cancer gene therapy. The functional effect of cFLIP over-expression could then be tested with the hypothesis being that cFLIP over-expressing lymphocytes might be protected from activation-induced apoptosis.

1.4.2 Targeting Lymphocytes to Tumour Neovasculature

The aim of this work was to study a possible method of targeting *ex-vivo* genetically modified lymphocytes to tumour neovasculature. Upon contact with tumour vessels, the lymphocytes could be engineered to either become specifically activated (through the use of chimeric T cell receptors (see section 1.2.5), or to secrete a therapeutic second protein at locally high concentrations thereby providing a system of *in vivo* protein delivery.

These aims would be achieved through the construction of retroviral vectors that, when used to transduce T cells resulted in the cell surface expression of an antibody scFv fragment (CGS-1) (either alone, or as part of a chimeric T cell receptor) specific for an

antigen found in and around tumour neovasculature (ED-B protein). These vectors would then be used to genetically modify initially T cell lines and subsequently primary cells and *in vitro* and *in vivo* assays testing the targeting ability of these gene-modified cells would be developed. This work is described in Chapter 7.

1.4.3 Retroviral genetic modification of murine lymphocytes

A further aim of this thesis was to optimise the retroviral genetic modification of fresh murine lymphocytes and murine antigen-specific T cell lines. This was with a view to examining both of the above strategies in *in vivo* murine tumour models. The results of this work are summarised in Chapter 6.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

2.1.1.1 Molecular Biology

Antibodies	R&D Systems, Abingdon, UK, Pharmingen, Oxford, UK or Sigma, Poole, UK, unless otherwise stated.
30% Acrylamide Solution	Roche Biochemicals Ltd, UK
Bacterial Strain – E.Coli GM 2163	New England Biolabs, Hertfordshire, UK
Big Dye Terminator Sequencing Mix	Perkin Elmer, Shelton, USA
BM Blue POD Substrate	Roche Biochemicals Ltd, UK
Chemical Compounds	BDH Laboratory Supplies, UK, Sigma, Poole, UK or Roche Biochemicals Ltd, UK, unless otherwise stated
Complete™ Mini Protease Inhibitor	Roche Biochemicals Ltd, UK
DNA Restriction Enzymes	Roche Biochemicals Ltd, UK or New England Biolabs, Hertfordshire, UK
dNTP's	Promega, Southampton, UK
ECL™ Chemiluminescence Kit	Amersham Pharmacia Biotech, Buckinghamshire, UK
1 Kb DNA Ladder	New England Biolabs, Hertforshire, UK
Nitrocellulose Membrane – Hybond C Extra	Amersham Life Science, Buckinghamshire, UK
Oligonucleotide Primers	Gibco BRL, Paisley, Scotland
PCR Purification/Gel Extraction/RNA Extraction/Mini-prep and Maxi-prep Plasmid Purification Kits	Qiagen Ltd, Crawley, West Sussex, UK

Protein Estimation Kit	Bio-Rad, CA, USA
Rainbow™ Coloured Protein Molecular Weight Marker	Amersham Pharmacia Biotech, Buckinghamshire, UK
RNAsin	Promega, Southampton, UK
Taq DNA Polymerase/Klenow Polymerase/T4 DNA ligase/Expand Reverse Transcriptase	Roche Biochemicals Ltd, Roche, UK
Vectors – TOPO TA®	Invitrogen, Paisley, UK
PLNCX and pIRES	Clontech, CA, USA
rKat 43.267 and rKat.GFP	Cell Genesys, Inc, CA, USA

2.1.1.2 Cell Culture

Cell lines - *293T*	ATCC (CRL-11268)
PG 13	ATCC (CRL-10686)
EcoPak, PT67	Clontech, CA, USA
HeLa	ATCC (CCL 243)
3T3, Gp+e86	ATCC (CRL-1658 and 9642, respectively) obtained from Dr Lez Fairbairn, PICR, Manchester, UK
Jurkat	ATCC (TIB-152) obtained from Dr Nigel Smith, PICR, Manchester, UK
Dulbecco's MEM with Glutamax	Gibco BRL, Paisley, Scotland
10% Fetal Calf Serum	Imperial UK, UK
1M Hepes Buffer	Sigma, Poole, UK
Histopaque®-1077	Sigma, Poole, UK
IL-2 (Proleukin)	Chiron, Amsterdam, Netherlands
2-Mercaptoethanol	Sigma Chemicals, Poole, UK
1% Penicillin,Streptomycin,L-Glutamine	Sigma Chemicals, Poole, UK
Plastic ware	Falcon® - Becton Dickinson (Fred Baker

	Scientific, Runcorn, UK)
Polybrene	Sigma Chemicals, Poole, UK
Retronectin®	Takara Biomedicals, Japan
RPMI-1640 Medium	Gibco BRL, Paisley, Scotland
Transfast™ transfection system	Promega, Southampton, UK
Trypan Blue	Sigma Chemicals, Poole, UK
Trypsin	Sigma Chemicals, Poole, Dorset, UK
WST-1 Proliferation Assay Kit	Roche Biochemicals Ltd, Roche, UK

2.1.2 Composition of Buffers, Media and Solutions

1% BSA/PBS	1g Bovine Serum Albumin in 100ml PBS
Blot Buffer	24ml Water, 8ml Methanol and 8ml 5xTris-glycine electrophoresis buffer
Borate Buffer	100mM boric acid, 150mM NaCl, pH 8.5
Cell Freezing Media	90% Fetal Calf Serum, 10% DMSO
DNA Gel Loading Buffer	0.25% Bromophenol Blue, 0.25% Xylene Cynol and 25% Ficoll
L-Broth Medium	25g Yeast Extract, 25g Tryptone and 25g Sodium Chloride in 5 Litres Water
L-Broth/Agar	Add 8g Bactoagar to 300ml L-Broth
Nitrocellulose Membrane Stripping Buffer	31.25ml 1M Tris pH 6.7, 100ml 10% SDS and 3.5ml 2-mercaptoetanol made up to 500ml with ddH_2O
Phosphate-buffered Saline (PBS)	8g Sodium Chloride, 0.2g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 in 800ml ddH_2O. pH to 7.4. Add H_2O to 1000ml
Psi Broth	5g Bactoyeast extract, 20g Bactotryptone and 5g Magnesium Sulphate per litre. pH to 7.6 with potassium hydroxide

RIPA Buffer	150mM Sodium Chloride, 1% Triton®X-100, 0.5% Sodium Deoxycholate, 0.1% SDS and 50mM Tris pH 8.0
10% Sodium Dodecylsulphate (SDS)	100g SDS in 900ml H_2O. Heat to 68°C to dissolve. pH to 7.2. Adjust volume to 1000ml with H_2O
12% SDS Polyacrylamide Resolving Gel	To make 20ml – 6.6ml H_2O, 8ml 30% Acrylamide mix, 5ml 1.5M Tris (pH8.8), 0.2ml 10% SDS, 0.2ml 10% APS and 0.008ml TEMED
5% SDS Polyacrylamide Stacking Gel	To make 10ml – 6.8ml H_2O, 1.7ml 30% Acrylamide mix, 1.25ml 1.0M Tris (pH 6.8), 0.1ml 10% SDS, 0.1ml 10% APS, 0.01ml TEMED
1xSDS Gel Loading Buffer	50mM Tris Cl (pH6.8), 100mM Dithiothreitol, 2%SDS, 0.1% Bromophenol Blue and 10% Glycerol
Sequencing Dilution Buffer	5mM $MgCl_2$ and 200mM TrisCl (pH 9)
SOC Medium	2% bacto-tryptone, 0.5% bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM$MgSO_4$, 20mM glucose (autoclaved before use)
10xTAE	48.4g Tris Base, 11.4ml Glacial Acetic Acid and 20ml 0.5M EDTA (pH8.0) in 1000ml ddH_2O
T Cell Media	RPMI-1640 Medium supplemented with 10% fetal calf serum (filtered through a 0.45mn filter), 1% L-glutamine, 1% penicillin-streptomycin, 50mM 2-mercaptoethanol, 20mM HEPES
TδbI	30mM Potassium acetate, 100mM Rubidium chloride, 10mM Calcium

	Chloride, 50mM Manganese Chloride and 15%v/v Glycerol. pH to 5.8
TδbII	10mM MOPS, 75mM Calcium chloride, 10mM Rubidium chloride and 15%v/v Glycerol. pH to 6.5 with dil NaOH
1M Tris (pH6.8)	121.1g Tris Base in 800ml ddH_2O. pH to 6.8. Allow to cool to room temperature. Make up to 1000ml with ddH_2O
Tris-buffered saline (TBS)	25mM Tris, 8g NaCl, 0.2g KCl and 3g Tris Base in 800ml ddH_2O. Add 0.015g phenol red and pH to 7.4. Add ddH_2O to 1000ml
Tris-buffered saline-Tween (TBST)	1xTBS and 0.05% Tween 20
Blotto	1xTBS/T and 5% Non-fat dried milk (eg marvel)
Tris-glycine Electrophoresis Buffer	25mM Tris Base, 250mM Glycine (pH 8.3) and 0.1% SDS
Wash Buffer	0.05% Tween in PBS

2.1.3 Oligonucleotide Primers (5' to 3')

CGS-1-F	CTG TTT CCA AGC ATG GCA TCG ATG CAG GTG CAG CTG GTG GAG TCT GGG GGA GGC
CGS-1-R	CGA CGA ACC TTT GTC TTC TGC GGC CGC GCC TAG GAC GGT CAG CTT GGT CCC
PVAC FOR 562	CAA TAA ACG CCA TTT GAC C
MLM REV 1441	GGT CGC CAT GTT GGA GAC AG
CGS-1 PLASMID-FOR1	TCA CCT TTA GCA GCT ATG CC
CGS-1 PLASMID-FOR2	CGT CTG AGC TGA CTC AGG AC
CGS-1 PLASMID-REV1	CGG TCA GCT TGG TCC CTC C
CGS-1 PLASMID-REV2	CAA GGC CAC AGA CAC AGC
BCL-XL-FWD	TTC TCT AGA CGA AGA ATT CAA ATG TCT CAG AGC AAC CGG GAG CT
BCL-XL-REVR	CAA CAG CTG GGG TTG CGC AAA TCA TTT CCG ACT GAA GAG TGA GCC CA
BCL-XL-REV2	CAA GTC GAC GGG TAC GCG TAA TCA TTT CCG ACT GAA GAG TGA GCC CA
T7	GTA ATA CGA CTC ACT ATA GGG C
M13R	CAG GAA ACA GCT ATG AC
CFLIPSH-FOR	TGT GAC CTT CCC GAA TTC TAG AGT AGG ATG TCT GCT G
CFLIPSH-REV	GAT TAA GTA GAG GCA GGT CGA CGC GTA TCA CAT GGA ACA ATT TC
CFLIPLONG-FOR	CTT CCA TCT AGA CAT GTC TGC TGA AGT CAT CCA TCA G
CFLIPLONG-REV	CTT CCT GTC GAC GCT ACG CGT TTT ATG TGT AGG AGA GGA TAA G
LINK 1	GGC CGC GAG GAG ATG GAT GGG CC
LINK 2	GGC CGG CCC ATC GAT CTC CTC GC

2.2 Methods

2.2.1 Bacterial Plasmid Preparation

TOP-10 bacteria (Invitrogen, San Diego, USA) (Genotype: *mcrA, Δ(mmr-hsdRMS-mcrBC), ϕ80ΔlacZΔlacM15, lacX74, deoR, recA1, araΔ139, Δ(ara,leu), 7697, galU, galK, λ-, rpsL, endA1, nupG)* a derivative strain from *Escherichia coli* were chosen for plasmid transformation.

2.2.1.1 Preparation of LB Agar Plates

Agar plates were prepared by melting L-broth/agar in a microwave until fully dissolved. After cooling, carbenicillin (0.1mg/ml L-Broth) was added to liquid L-Broth/agar prior to pouring into petri dish and allowing to set.

2.2.1.2 Preparation of Chemically Competent Bacteria

The rubidium chloride method was used to make chemically competent bacteria. Briefly, a 2 ml starter culture was inoculated with the appropriate strain of *E.Coli* and grown up overnight. Psi Broth was then inoculated with the starter culture (1ml/100ml broth) and incubated at 37°C with aeration to A_{550}=0.48. Cells were then cooled on ice for 15 minutes prior to harvesting by centrifugation at 6000 rpm for 10 minutes. After discarding supernatant, bacteria were resuspended in 0.4 volume TδbI and left on ice for 15 minutes. Cells were then pelleted again and resuspended in 0.04 volume TδbII and again left on ice for 15 minutes. Aliquots of 200µl were then snap frozen to –70°C.

2.2.1.3 Chemical Transformation

50µl of an appropriate strain of chemically competent bacteria was mixed with 15µl of pre-chilled ligation mixture and incubated on ice for 30 minutes in a large eppendorf tube. The tube was heat shocked at 42°C for 90 seconds and then put back on ice for 2 minutes. One ml L-Broth was added and the mixture was incubated at 37°C for one hour with shaking. Finally, 200µl of this bacterial cell mixture was plated out on to LB agar with 0.1mg/ml carbenicillin and incubated overnight at 37°C.

2.2.1.4 Mini prep Plasmid Purification

A single bacterial transformant was picked, inoculated into 2ml of L-Broth with 0.1mg/ml of carbenicillin and incubated overnight at 37°C with shaking. Bacteria from 1 ml of the starter culture was harvested by centrifugation at 10 000 rpm for 60 seconds and the supernatant was removed.

Qiaprep mini prep kits (Qiagen®) were then used for plasmid DNA purification. These kits use the modified alkaline lysis method of Birnboim and Doly (Birnboim and Doly 1979). Bacteria are lysed under alkaline conditions, the lysate is neutralised, adjusted to high salt binding conditions and subsequently purified on silica gel membranes.

2.2.1.5 Maxi prep Plasmid Purification

For preparation of larger amounts of plasmid DNA the selected plasmid clone was inoculated into 400ml L-Broth with 0.1mg/ml carbenicillin and incubated overnight at 37°C with shaking. Bacterial cells were then harvested by centrifugation at 6000 rpm (4°C) for 15 minutes and the supernatant was discarded.

Qiagen plasmid maxi prep kits (Qiagen®) were then used for plasmid DNA purification. As above, these kits are based on a modified alkaline lysis procedure followed by binding of plasmid DNA to an anion-exchange resin under appropriate low salt and pH conditions. Plasmid DNA is then eluted in a high salt buffer, concentrated, and desalted by isopropanol precipitation.

2.2.1.6 Preparation of Glycerol Stock

Prior to maxi prep plasmid purification, 3ml of bacterial culture was mixed gently with 750µl 80% glycerol. After making 1ml aliquots, this mixture was immediately placed on dry ice and subsequently stored in liquid nitrogen.

2.2.2 General Molecular DNA Techniques

2.2.2.1 Restriction Digestion

Analytical restriction digestions were generally carried out in a volume of 20µl with $1/10^{th}$ the volume of the appropriate 10x enzyme buffer and restriction enzyme at 1 unit/µg DNA digested. Digestions were incubated at 37°C for 2-4 hrs. $1/6^{th}$ volume of

DNA gel loading buffer was added and samples were loaded on to a 1.2% agarose gel (with ethidium bromide). Samples were run with a 1Kb DNA marker in 1xTAE at 60-100V for 15-40 minutes.

Large-scale digestion prior to fragment isolation was generally carried out in 50µl. Samples were again run on 1.2% agarose gels prior to the required fragment being excised under UV light. DNA fragments were subsequently extracted and purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen®) according to the manufacturers' instructions.

2.2.2.2 Blunting DNA Fragment Ends

Typically following gel extraction DNA was eluted in 52µl ddH_20. This was made up to 70µl by adding $1/10^{th}$ Volume (ie 7µl) enzyme buffer, 7µl dNTPs (each at 2.5mM) and 8u Klenow DNA polymerase. The sample was then incubated at 37°C for 1 hour. DNA was then purified using the QIAquick PCR Purification Kit (Qiagen®) according to the manufacturers' instructions.

2.2.2.3 Dephosphorylation of DNA Fragment Ends

DNA vector fragments were dephosphorylated by adding 1u Calf intestine alkaline phosphatase and $1/10^{th}$ volume enzyme buffer to the sample and incubating at 37°C for 15 minutes. DNA was then purified using the QIAquick PCR Purification Kit (Qiagen®) according to the manufacturers' instructions.

2.2.2.4 DNA Ligation

Ligations were performed generally using 500ng vector and at several different vector to insert molar ratios in a volume of 30µl using 2u T4 DNA ligase and $1/10^{th}$ volume ligase buffer. Ligations of DNA fragments with overhanging ends were performed overnight at 4°C, whereas blunt ended ligations were performed at 16°C overnight. As described in 2.2.1.3, typically 15µl of ligation mix was then used to transform into chemically competent bacteria.

2.2.2.5 DNA Sequencing

Sequencing reactions were carried out according to the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer) based on the established dideoxy chain termination method of Sanger (Sanger, Nicklen et al. 1977).

200-500ng double-stranded DNA or 30-90ng PCR product DNA was mixed with 3.2pmol primer, 4µl Big Dye Terminator Sequencing mix, 4µl dilution buffer and made up to 20µl with sterile H_2O. The reaction was placed in a GeneAmp 9600 thermal cycler and run on the following programme:

Rapid thermal ramp to 96°C

25 Cycles: 96°C for 10 seconds

50°C for 5 seconds

60°C for 4 minutes

Rapid ramp to 4°C

Extension products were purified by mixing reaction with 2µl 3M sodium acetate, pH 5.5 and 50µl 95% ethanol, leaving on ice for 15minutes, pelleting, removing supernatant, rinsing in 70% ethanol and vacuum drying pellet.

Sequencing reactions were then run on an ABI 310 automated DNA sequencer.

2.2.3 Polymerase Chain Reaction

2.2.3.1 RNA Isolation and cDNA Synthesis

Total RNA was isolated from peripheral blood lymphocytes using the RNeasy® mini kit (Qiagen®). Briefly, samples were first lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate-containing buffer that inactivates Rnases. Ethanol was added to provide appropriate binding conditions and the sample was then applied to a silica gel–based membrane where the total RNA binds to the membrane and contaminants are subsequently washed away. RNA is then eluted in water.

To synthesise copy DNA 10µg RNA was incubated at 65°C for 3 minutes and then transferred to ice. $1/5^{th}$ enzyme buffer, 10µl 2.5mM dNTPs, 4µl Rnase inhibitor, 2.5µg oligothymydlic acid primer and 10u reverse transcriptase was added to this reaction mix which was made up to 100µl with ddH_2O. Following reverse transcription at 42°C for

one hour, the mixture was incubated at 98°C for 5 minutes to denature any protein contaminant and centrifuged at 13000 rpm for one minute. The resulting supernatant, containing cDNA, was either used immediately or stored at –20°C.

2.2.3.2 Polymerase Chain Reaction

Oligonucleotide primers (Gibco BRL) were initially reconstituted in 200µl ddH_2O. Primer concentration was then adjusted to 1µg/µl following UV spectrophotometry. Typically, polymerase chain reactions were carried out in 100µl with $1/10^{th}$ enzyme buffer (containing magnesium), 10µl 2.5mM dNTPs, 5µl cDNA, 1µl Taq Polmerase, 100ng forward primer, 100ng reverse primer and 70µl ddH_2O.

The reaction was placed in a Pro Gene Techne thermal cycler and run on the following programme:

25 Cycles: 94°C 60 seconds

55°C 60 seconds

72°C 60 seconds

72°C 7 minutes

Rapid ramp to 4°C

PCR DNA was then purified using the QIAquick PCR Purification Kit (Qiagen®) according to the manufacturers' instructions.

2.2.3.3 TOPO TA Cloning®

PCR Products were cloned directly into a plasmid vector using the TOPO TA Cloning® Kit (Invitrogen) according to the manufacturers' instructions.

2.2.4 Enzyme-Linked Immunosorbent Assay

2.2.4.1 Indirect ELISA to detect CGS-1hFc antibody

A 48-well ELISA microtitre plate was coated with ED-B protein (obtained from D Neri, University of Cambridge, UK) (10µg/ml) and left overnight at room temperature. Plates were rinsed over a sink by filling wells with wash buffer (0.05% Tween/PBS) using a squeezy bottle. This was then flicked into the sink and wells were washed twice more.

Residual binding capacity of plate was blocked by adding 1% BSA/PBS (300µl/well) for one hour and the wells were again rinsed three times.

Test supernatant containing antibody was filtered through a 0.45µm filter and added to the wells at several dilutions (diluted in DMEM). After allowing antibody to bind overnight at room temperature, non-bound antibody was washed away by rinsing the plate with wash buffer three times as above.

100µl Horse radish peroxidase (HRP)-conjugated anti-human Fc antibody diluted 1:1000 (in 1% PBS/BSA) was added to each well and incubated at room temperature for one hour. Unbound antibody was again washed away by rinsing wells three times with wash buffer and 100µl BM blue POD substrate was added to each well. After 30 minutes the hydrolysis reaction was stopped with 100µl 0.18M H_2SO_4.

Degree of hydrolysis was then measured by absorbance using a multi-well spectrophotometer at a wavelength of 450nm.

2.2.4.2 Human Interferon-γ Assay (antibody sandwich ELISA)

A 96-well flexible microtitre plate (Falcon©) was coated with mouse anti-human interferon-γ (hIFN-γ) antibody (R&D, MA-B285, diluted in borate buffer to a concentration of 1µg/ml, 100µl/well) and left overnight at 4°C. The plate was washed 3 times with wash buffer and residual binding capacity was blocked with 1% BSA for 2 hours. After a further 3 washes, the test sample was applied to the plate in quadruplicate (100µl/well) at an appropriate dilution. In order to calculate quantitatively the concentration of hIFN-γ present in the sample, recombinant hIFN-γ standards were also plated out, in triplicate, in dilutions from 1000 to 16 pg/ml. The samples were left to bind to the coating antibody overnight at 4°C. The following day, after a further 3 washes, biotinylated mouse anti-human interferon-γ antibody (R&D, diluted in 1% BSA/PBS to a concentration of 100ng/ml, 100µl/well) was added and incubated at room temperature for 2 hours. Unbound antibody was again washed away and 100µl of streptavidin-POD conjugate (1:10000 dilution) was added to each well. Following a final 3 washes, 100µl BM blue POD substrate was added to each well. After 30 minutes the hydrolysis reaction was stopped with 100µl 0.18M H_2SO_4.

The degree of hydrolysis was again measured by absorbance using a multi-well spectrophotometer at a wavelength of 450nm. Data from the limiting dilution standards

was used to create a standard curve and this equation was then used to estimate the concentration of interferon-γ within each sample.

2.2.4.3 Murine Interferon γ Assay

The assay to detect murine interferon-γ was perfomed in an identical way to above with anti-mouse antibodies substituted for anti-human. Purified rat anti-mouse IFN-γ antibody (BD PharMingen, R4-6A2) was used for ELISA capture and biotinylated rat anti-mouse IFN-γ antibody (BD PharMingen, XMG1.2) was used for detection.

2.2.5 Western Blotting

2.2.5.1 Cell Lysate Preparation

RIPA buffer (see 2.1.2) was prepared by adding protease inhibitors (1 tablet per 10ml RIPA). Cells were washed in PBS, pelleted at 4°C and resuspended in RIPA buffer (100µl RIPA per 10^7 cells for lymphocytes). After incubating on ice for 30minutes, cells were homogenised by passing through a 21 gauge needle attached to a 1ml syringe. After a further 30 minute incubation on ice, the cell debris was pelleted by centrifugation at 13000 rpm for 10 minutes at 4°C and the protein lysate (ie supernatant) was harvested and stored at –20°C.

2.2.5.2 Protein Concentration Estimation

Protein concentration of cell lysates was determined using the Bio-Rad DC Protein Assay (Bio-Rad) according to the manufacturers' instructions. This is a colorimetric assay that is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent and is similar to that described by Lowry (Lowry 1951).

2.2.5.3 SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by electrophoresis in a polyacrylamide gel (Lamelli 1970). A 12% SDS resolving gel was poured followed by a 5% SDS stacking gel. Whilst the stacking gel was polymerising, protein lysates were denatured by heating them to 100°C for 3 minutes in an equal volume of 2x SDS gel-loading buffer.

Equal amounts of protein were then loaded into wells and a voltage of 150V was applied to the gel for 60 minutes or until the bromophenol blue reached the base of the gel. Rainbow® coloured molecular weight markers (Amersham Pharmacia Biotech) were also run alongside the lysate samples.

2.2.5.4 Western Blotting

In order to transfer proteins on to a nitrocellulose membrane, the SDS-polyacrylamide gel was first soaked in blot buffer for 20 minutes.

Whatman 3MM paper and nitrocellulose (Hybond C Extra) was soaked in blot buffer for a few seconds and the polyacrylamide gel was then placed in a sandwich within a semi-dry electroblotting apparatus as follows:

-ve electrode

2 sheets W3MM

polyacrylamide gel

nitrocellulose

2 sheets W3MM

+ve electrode

Proteins were then transferred by electrophoresing at 200mA, 25V for 60 minutes.

Non-specific binding was then blocked by incubating the membrane overnight at 4°C in blotto. Bcl-X_L was detected by incubating the membrane in the presence of a mouse monoclonal anti-Bcl-X antibody (Clone 2H12, Pharmingen) diluted 1 in 250 with blotto for 2 hours on a shaker. Non-bound antibody was removed by washing the membrane three times in TBST (10 minutes per wash). The membrane was subsequently incubated with an HRP-conjugated anti-rabbit secondary antibody (Sigma) at a dilution of 1 in 2000 in blotto. After 3 further washes, the membrane was developed using an enhanced chemiluminescence kit (ECL™, Amersham Pharmacia Biotech) according to the manufacturers' instructions.

2.2.5.5 Nitrocellulose Membrane Stripping Procedure

In order to probe the same nitrocellulose membrane using different antibodies, stripping buffer was preheated to 50°C. Developed membranes were then washed twice in

stripping buffer at 50°C (30 minutes per wash) and the western procedure was restarted from the blocking non-specific binding stage.

2.2.6 Cell Culture

2.2.6.1 General Cell Culture

2.2.6.1.1 Cell Lines

293T, HeLa, 3T3, EcoPak, PT67, F9, PG13, Gpe86, XS-52 and A20 tumour cell lines were cultured in Dulbecco's MEM with Glutamax supplemented with 10% fetal calf serum (filtered through a 0.45μm filter). DC2.4 cells were also cultured in DMEM with Glutamax, but with 5% fetal calf serum (filtered through a 0.45μm filter). The Jurkat tumour cell line was cultured in RPMI-1640 Medium also supplemented with 10% fetal calf serum (filtered through a 0.45μm filter).

Media for the XS-52 cell line was also supplemented with recombinant murine GM-CSF at 20u/ml (R&D Systems) and supernatant harvested from confluent cultures of the syngeneic fibroblast cell line, NS47 (5% v/v). The XS-52 and NS47 cell lines were kindly provided by A Takashima, University of Texas, Southwestern Medical Center, Dallas, USA.

Cell lines were cultured in T25-T175 sealed tissue culture flasks gassed to 5% CO_2 in air, incubated at 37°C and sub-cultured approximately every 72 hrs or when confluent. Flasks used to culture the F9 tumour cell line were also pre-coated for 2 hours with 0.1% gelatin (in PBS).

Adherent cells were removed from tissue culture flasks using either Trypsin Edta solution (Sigma) or cell scrapers.

2.2.6.1.2 Primary Cells

Primary human lymphocytes, primary murine lymphocytes and the A20 idiotype-specific murine T cell lines were cultured in RPMI-1640 Medium supplemented with 10% fetal calf serum (filtered through a 0.45μm filter), 1% L-glutamine, 1% penicillin-streptomycin, 50mM 2-mercaptoethanol, 20mM HEPES (Sigma) and 100u/ml Interleukin-2 (IL-2) (50u/ml for T cell lines). Human lymphocytes were cultured at a

concentration of between $5x10^5$ to 10^6 cells per ml with fresh media and IL-2 added every 2-3 days. Murine lymphocytes were cultured at a concentration of 10^6 cells/ml. The A20 idiotype-specific murine T cell lines were maintained by weekly restimulation with irradiated (3000 rad) XS52.A1.A20 dendritic cells at an effector:stimulator ratio of 1:10.

2.2.6.1.3 General Techniques

Cell concentration was measured by mixing 10µl cell suspension with 10µl 0.4% trypan blue solution and counting cells using a Neubauer double cell haematocytometer (Weber Scientific International). Cells that had taken up trypan blue were considered to be non-viable.

To freeze cells they were harvested by centrifugation at 800G for 5 minutes and resuspended in cell freezing media at a concentration of 2 to $5x10^6$/ml. One ml aliquots were pipetted into cell freezing vials and incubated at –80°C for 24 hrs in a polystyrene box (to ensure slow freezing), prior to transfer into liquid nitrogen.

Cells were thawed from liquid nitrogen by incubating frozen vials at 37°C in a water bath until melted and then immediately re-suspending them in 10ml growth media. Cells were then centrifuged at 800G for 5 minutes in order to remove traces of DMSO and resuspended in fresh growth media.

2.2.6.2 Transfection of Cell Lines

2.2.6.2.1 Calcium Phosphate Co-precipitation

This method was used for transient transfection of 293T and EcoPak cell lines. Cells were split such that they were 60-80% confluent on a 10cm plate on the day of transfection. 13.2µg retroviral vector (rKat) DNA and 6.8µg pKat vector (encoding retroviral packaging genes – not needed for EcoPak transfection) DNA were mixed with 950µl DMEM with 25mM Hepes at a pH of 7.1. Fifty microlitres 1M calcium chloride was added and the mixture was left at room temperature for 30 minutes. After washing the 293T cells with serum-free DMEM, 4ml DMEM with 10% FCS and 25mM hepes at a pH of 7.9 was added to the cells. The transfection mix was then added drop wise on to the plate. This procedure leads to the formation of a DNA/calcium phosphate precipitate that is then dispersed on to the cells and taken up by endocytosis or phagocytosis. After

incubating plates at 37°C for 10 to 20 hrs, buffered media was removed and replaced with usual media.

2.2.6.2.2 Liposome-mediated

This method was used for transfection of the PG 13 cell line. The Transfast™ cationic lipid transfection system (Promega) was used according to the manufacturers' instructions. Incubation of cationic lipid –containing liposomes with DNA results in quick association and a compaction of the DNA. Entry of the liposome complex into the cell is thought to occur by endocytosis, or fusion with the plasma membrane via the lipid moieties of the liposome. It is not known precisely how the transfected DNA or liposome/DNA complex gains entry to the nucleus.

The liposome reagent was mixed with DNA in serum-free media (at a 3:1 charge ratio of reagent to DNA) and briefly vortexed. Fifteen minutes later growth media was removed from the PG 13 cells and replaced with the transfection mixture. After an incubation period of one hour complete growth media was added to the cells.

2.2.6.3 Retrovirus Production

2.2.6.3.1 Transient Transfection of 293T or EcoPak cells

Twenty-four hours after transient transfection of the 293T or EcoPak cell line with the rKat retroviral vector system (see section 2.2.6.2.1), growth media was replaced with T cell media (5ml/10cm plate). After a further 24hrs (48hrs post transfection) viral supernatant was harvested from the cells and replaced with another 5ml of T cell media, with more viral supernatant harvested at 72 hrs post transfection. Supernatant was then filtered through a 0.45μm filter (which had been pre-wet with media containing serum in order to block protein binding sites on the filter so as to reduce the loss of viral particles) and either used fresh, or snap frozen to –80°C in liquid nitrogen.

2.2.6.3.2 Stable Retrovirus Producing Cell Lines

To prepare viral supernatant from stable retrovirus producing cell lines (PG13, PT67 or Gpe86) cells were grown to confluence, washed with PBS and then incubated overnight at 37°C with sufficient T cell growth media to just cover the surface of the plate/flask.

Supernatant was subsequently harvested and filtered through a 0.45μm filter as above prior to use fresh or freezing for later use.

2.2.6.3.3 Determination of Retroviral Titre

3T3 or Hela target cells ($3x10^4$/well) were plated out in a 24 well tissue culture plate. Twenty-four hours later media was removed from the target cells and they were washed in PBS. Fresh viral supernatant harvested from the retrovirus producing cells being tested was then placed on to the cells (200μl/well), plus polybrene (4μg/ml). Serial dilutions of viral supernatant in DMEM (ranging from neat to 1:5000) were added to different wells. Cells were then cultured at 37°C overnight and the following morning the viral supernatant was replaced with DMEM (one ml per well).

Target cells were then analysed for marker transgene expression (ie GFP) by flow cytometry 5 days later. An estimate of viral titre could then be determined using the formula – Viral Titre (cfu/ml) = % Cells GFP positive x (number of cells plated x 2) x dilution factor x (1000/volume of viral supernatant in μl) (using a dilution of viral supernatant which resulted in a population of target cells less than 30% transgene positive; statistically this reduces the chance of double infection of the same cell resulting in a falsely low estimate of titre).

2.2.6.4 Human Peripheral Blood Lymphocyte Isolation and Activation

Human peripheral blood lymphocytes (PBL) were obtained from healthy donors by centrifugation on Histopaque (density, 1.077g/ml; Sigma) at 600G for 20 min at 20°C and were then washed twice with PBS. Monocytes were eliminated by allowing them to adhere to a tissue culture flask at 37°C for one hour. The resulting lymphocyte population was cultured in T cell media (RPMI-1640 (GIBCO-BRL) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% penicillin-streptomycin, 50mM 2-mercaptoethanol and 20mM HEPES (Sigma)). Prior to retroviral transduction lymphocytes were activated for 48 to 72 hrs on immobilised anti-human CD3ε (iCD3) (OKT3, Orthoclone) and anti-human CD28 (iCD28) monoclonal antibodies (R+D Systems) with IL-2 (30u/ml) (Chiron).

For the immobilisation of antibodies, 6-well non-tissue culture treated plates were coated with antibody (1μg/ml in PBS, 2ml/well) for 2hrs at 37°C. Coated plates were then blocked with 2% BSA/PBS for 20 min at 37°C and washed once with PBS.

2.2.6.5 Murine Splenocyte Isolation and Activation

One to two freshly removed spleens were placed on a 100μm filter. Using a circular motion, the plunger of a 5ml syringe was used to expel splenocytes from the splenic capsule until only fibrous tissue remained. The splenocytes were then flushed through the filter using 50ml serum-free RPMI into a 50ml Falcon tube. After centrifugation at 200xg for 10 minutes the supernatant was discarded and the resulting cell pellet resuspended in 20ml T cell media.

In initial experiments splenocytes were obtained by centrifugation on a Lymphoprep gradient at 600xg for 20 min at 20°C and were then washed twice with PBS. This resulted in a significant amount of red cell contamination remaining however and in view of this splenocytes were subsequently depleted of red cells by hypotonic lysis with ammonium chloride (see 6.3.1). The cell pellet obtained was resuspended in 5ml Pharmalyse™ ammonium chloride lysing reagent (10x, diluted in ddH_20, PharMingen) and incubated for 5mins at room temperature (with occasional shaking). Serum-free T cell media was added to fill the tube and cells were then pelleted at 200G for 10 minutes. After a further wash in serum-free media, cells were resuspended in warm T cell media.

In later experiments splenocytes were passed through a nylon wool column to enrich for T cell populations. Following red cell lysis, cells were resuspended in 4ml warm T cell media (maximum $7.5x10^7$ cells/ml) and loaded on to a pre-equilibrated nylon wool column (0.6g teased nylon wool in the barrel of a 20ml syringe, autoclaved, 50ml warm T cell media washed through, then incubated at 37°C for 45mins). A further 2-3ml T cell media was added to the top of the column to wash the cells in and the column was then incubated for 45mins at 37°C. A 23G needle was attached to the bottom of the column and 20ml warm T cell media was washed through. The first 15ml of media to drain from the needle, containing non-adherent T cells, was collected. Cells were then spun at 200xg for 10mins and resuspended in T cell media.

Prior to retroviral transduction murine lymphocytes were either activated for 48-72 hrs on immobilised anti-mouse CD3ε (145-2C11) (iCD3) and anti-mouse CD28(37.51)

(iCD28) monoclonal antibodies (PharMingen) with IL-2 (30u/ml) (Chiron) or activated by adding phytohaemagglutinin (PHA-L) (Sigma) at 5μg/ml.

For the immobilisation of antibodies, 6-well non-tissue culture treated plates were coated with antibody (1μg/ml in PBS, 2ml/well) for 2hrs at 37°C. Coated plates were then blocked with 2% BSA/PBS for 20 min at 37°C and washed once with PBS.

2.2.6.6 Retroviral Transduction

2.2.6.6.1 Cell Lines

PG13, PT67 and Gpe86 cell lines were transduced with the aim of making stable retrovirus producing cell lines. 10^5 cells/well were plated out in a 6 well tissue culture plate. Twenty-four hours later the media was removed from the target cells and they were washed in PBS. Fresh viral supernatant harvested from transiently transfected 293T cells were then placed on to the cells (2ml/well) with polybrene (4μg/ml) and the cells were incubated at 37°C overnight. The following day, fresh viral supernatant/polybrene was placed on to the cells, which were then incubated for a further 24hrs. Viral supernatant was removed and the cells were washed in PBS, before finally adding media. When confluent, cells were split in the usual way and efficiency of transduction was assessed by flow cytometric analysis of marker gene expression after 5 days.

If a high viral titre producer cell line was required, the bulk transduced cell population was cloned by limiting dilution. This was done by trypsinising cells and passing them through a 20G (green) needle in order to create a single cell suspension. After counting, cells were diluted to 1 cell/200μl and plated out into three 96-well plates (200μl/well). Cells were grown up over 2-3 weeks before approximately 20 brightly green (ie highly expressing GFP) colonies were identified by examining plates under ultraviolet (uv) light. These individual clones were transferred to a 24-well plate and separately expanded prior to determining viral titre (see section 2.2.6.3.3).

The Jurkat T cell line was transduced using a spin transduction method similar to the one described in section 2.2.6.6.2. 10^5 cells were pelleted in a 15ml falcon tube. Cells were resuspended in 2ml fresh viral supernatant and an equal volume of T cell media with 4μg/ml polybrene (Sigma) and centrifuged at 1000-1200xg for 2 hrs. Cells were then washed once in PBS, resuspended in 1ml T cell media and expanded.

2.2.6.6.2 Human Peripheral Blood Lymphocytes

Generally, a spin transduction method based on that described by *Costello et al* (Costello, Munoz et al. 2000) was used to transduce PBLs. 10^6 pre-activated PBLs were pelleted in a 15ml falcon tube. Cells were resuspended in 2ml fresh viral supernatant and an equal volume of T cell media with 4µg/ml polybrene (Sigma) and centrifuged at 1000-1200xg for 2-3 hrs. Cells were then washed once in PBS, resuspended in 1ml media and placed on iCD3/iCD28 plates overnight. This transduction process was repeated on two consecutive days.

For fibronectin-mediated lymphocyte transduction, Retronectin® (Takara) was resuspended at 24µg/ml ddH_2O, filtered through a 0.2µm filter and plated on to 3.5cm non-tissue culture plates at 5µg/cm^2. After incubating for 2 hrs at 37°C, the Retronectin® solution was removed and residual protein binding capacity was blocked for 30 minutes with 2% BSA/PBS. Plates were then washed with PBS.

$5x10^5$ lymphocytes in 500µl fresh media were mixed with 1.5ml viral supernatant and IL-2 (100u/ml) and incubated overnight at 37°C on the fibronectin plates. Cells were then harvested by centrifugation at 800G for 5 minutes, resuspended in 500µl fresh media, placed back on to the fibronectin plates with another 1.5ml of fresh viral supernatant and again incubated overnight.

Cells were then either further activated for 72 hrs on iCD3/iCD28 before continued expansion in IL-2 (100u/ml), or cultured straight away in IL-2 alone (100u/ml). When possible efficiency of transduction was assessed by flow cytometric analysis of either transgene expression (eg GFP) or antibody staining of cell surface markers of transduction.

2.2.6.6.3 Murine Splenocytes/T Cell Lines

Several different methods were used to transduce murine splenocytes and murine T cell lines (see Chapter 6). Supernatant-mediated approaches involving either centrifugation or fibronection were identical to the methods used to transduce human lymphocytes as described in section 2.2.6.6.2.

In addition, a co-culture-based method described by *Darcy et al* (Darcy, Haynes et al. 2000) was also used. $2x10^6$ murine T lymphocytes were co-cultivated for 72hrs with $2.5\text{-}10x10^5$ virus-producing packaging cells (either Gpe86 or PT67) in DMEM

supplemented with 4μg/ml polybrene (Sigma), 5μg/ml PHA (Sigma) and 100u/ml IL-2. After removal from packaging cells, T cells were washed in DMEM and cultured in DMEM and IL-2 (100u/ml). Transduction efficiency was analysed by flow cytometry 72hrs after removal of cells from co-culture.

2.2.6.7 Fas-induced Apoptosis Studies

For Fas-induced apoptosis studies, jurkat cells (10^5) were transduced with rKat.Bcl-X_L.IRES.EGFP (and rKat.B1.8.MTM.IRES.EGFP control) retrovirus using the spin transduction method as above (2.2.6.6.1). Transduced cells were suspended in fresh medium ($0.5x10^6$/ml) in a 24-well tissue culture plate. 24 hrs later, the soluble anti-human CD95 (FAS) monoclonal antibody (Clone DX2, R&D Systems) (1μg/ml) and Protein G (Sigma) (1μg/ml) was added to the cells. Control cells were cultured in the presence of an IgG_1 isotype control antibody and Protein G. 24 hrs later, cells were stained with PE-conjugated annexin V (Pharmingen) and the percentage of apoptotic cells within the population was assessed by flow cytometry (All annexin V-positive cells were considered apoptotic).

2.2.6.8 Human Lymphocyte Survival Studies

For lymphocyte survival studies following retroviral transduction T cells were ficolled to remove dead cells, washed twice in serum-free RPMI, counted and put into culture with medium alone or medium and soluble CD3 - OKT3 (Orthobiotech, USA) (1μg/ml). Total viable cell number was measured by trypan blue exclusion. After gating on the live lymphocyte population, the percentage GFP positive live cells was measured by flow cytometry (of aliquots of 10^5 cells) in each group every 72-96 hours.

For tumour co-culture experiments, 293T and HeLa tumour cell lines were split such that they were 70-80% confluent in a six-well tissue culture plate on day 0. Bcl-X_L and control B1.8 transduced T lymphocytes were washed, resuspended at a concentration of $2x10^6$ cells/ml growth media, and co-cultured with the tumour cells for 48 hours in the absence of IL-2. Lymphocytes from each group were then carried over into fresh six well plates (in an attempt to isolate them from the tumour cells) and were cultured in growth media plus IL-2 (100u/ml) for a further 13 days. In fact, due to a significant amount of carry-over of the adherent tumour cells the lymphocytes were effectively cultured with tumour cells for the whole experiment.

In the co-culture experiments, 10^5 HeLa cells were plated out into a six-well plate 24 hours prior to starting the co-culture. Lymphocytes were co-cultured at 10^6 cells/ml either with or without 100u/ml IL-2.

2.2.6.9 Chromium Release Cytotoxicity Assay

The cytolytic activity of the murine idiotype-specific T cell lines and chimeric receptor modified human T cells was tested using an 8 hour Chromium (^{51}Cr) release assay.

Target cells were harvested into a universal tube and pelleted by centrifugation at 800xg for 5 minutes. $5x10^5$ cells were counted and washed in serum-free (SF) RPMI (supplemented with 1%PSG). After pelleting for a further 5 min at 800xg, the cells were resuspended in 50µl media. 100µCi of ^{51}Cr was added to the cells, which were then incubated for 90 to 120 min (cells were resuspended every 30min).

Whilst the target cells were labeling, effector cells were harvested and washed in SF media. Cells were counted and resuspended at a concentration dependent on the required effector:target (E:T) ratio. Triplicate wells containing 200µl of cell suspension were plated and serially diluted across a 96 well plate leaving a final volume of 100µl in each well.

Once labelled, the target cells were washed 3 times in serum free media before finally being resuspended in 10ml culture media to achieve a concentration of 1000 cells /100µl. 100µl of ^{51}Cr loaded target cells was then added to each well of the 96 well plate resulting in a final volume of 200µl/well. Maximum ^{51}Cr release was determined from supernatants of cells that were lysed with 100µl PBS/2% triton x-100. Spontaneous release was obtained by incubating target cells in culture media alone. The plate was incubated at 37°C/5%CO_2 for 8 hours. A total of 100µl of supernatant was collected from each well, transferred to a Luma Plate, air-dried overnight and counted in a Topcount scintillation counter (Packard Instrument, UK). The percentage of lysis was calculated as follows: % lysis = [(specific release – spontaneous release)/(maximum release – spontaneous release)]x100.

2.2.6.10 Flow Cytometry

Generally, cells were washed twice in 1%BSA/PBS and stained with appropriately diluted antibodies according to the manufacturer's recommendations. 10000 cells per sample were analysed on a FACSCalibur flow cytometer (Becton Dickinson).

For sterile-sorting, a FACSVantage flow cytometer (Becton Dickinson) was used. Prior to sorting cells were washed twice in cold PBS and passed through a 30μm filter to avoid clumping.

2.2.7 In Vivo models

Six to 8-week old BALB/c, NOD/SCID and athymic (Nude) mice (BALB/c background) were bred and housed under specified pathogen-free conditions. The Bcl-X_L safety model was undertaken at Covance Laboratories, Harrogate. The A20 and F9 models were performed in the Biological Resources unit, Paterson Institute for Cancer Research, Manchester. Staff members of the BRU performed all animal observations and handling. All procedures were conducted in accordance with Home Office guidelines.

2.2.7.1 Bcl-X_L Safety Model

Bcl-X_L-transduced lymphocytes were washed twice with PBS and adjusted to a cell concentration of approximately $3.75x10^7$ cells/ml in PBS. Five female NOD/SCID mice were dosed intraperitoneally with 200μl of lymphocyte suspension using a 23G needle. Lymphocyte preparation and animal dosings were repeated for 5 sequential days with each animal receiving approximately $3.75x10^7$ lymphocytes in total. Animals were observed regularly for 90 days at which point the animals were killed and examined at post-mortem for evidence of tumour formation or other abnormalities.

2.2.7.2 A20 Model

BALB/c mice (n=21) were injected intravenously, via a tail vein, with 10^6 A20 tumour cells in 200μl PBS. Seventy-two hours later, mice were divided into three groups (n=7/group) and treated with one intravenous dose of 10^6 A20 specific T cells (in 200μl PBS), 10^6 Bcl-X_L-transduced A20 specific T cells (in 200μl PBS) or 200μl PBS control.

Mice were observed daily and weighed/any tumours measured 3 times per week. Mice were sacrificed when first observed to be showing any signs of distress.

2.2.7.3 F9 Model

Groups of athymic (BALB/c) mice (n=4/group) were injected either subcutaneously into the right flank (2 groups) or intravenously (2 groups) with two cell doses, $5x10^5$ or 10^6, F9 tumour cells. Mice were observed daily and weighed/any tumours measured three times per week. Tumour volume was estimated using calipers (length x width^2 x 0.52). Mice were sacrificed when tumour volume>1cm^3 or if mice were showing any signs of distress.

3 Results – Improving lymphocyte survival by over-expression of Bcl-X_L

3.1 Introduction

Over the last decade, since the first genetically modified lymphocytes were tested in humans (Rosenberg, Aebersold et al. 1990), many studies have demonstrated that adoptively transferred gene-modified lymphocytes can be successfully re-infused into patients and subsequently detected *in vivo* (Favrot and Philip 1992; Heslop, Ng et al. 1996). Unfortunately, even in recent studies, the long-term detection of these transduced cells has generally required highly sensitive PCR techniques (Mitsuyasu, Anton et al. 2000). This suggests that the transduced cells do not survive in large numbers for significant lengths of time *in vivo.* The reasons for this are not clear. Certainly, cellular immune responses have been identified against the protein products of some foreign inserted genes (Riddell, Elliott et al. 1996). However, other factors including *ex vivo* T Cell Receptor (TCR) activation, cytokine withdrawal upon re-infusion, inappropriate antigen presentation *in vivo* and a hostile pro-apoptotic tumour microenvironment may all result in a susceptibility to early apoptosis of gene-modified lymphocytes.

CD28 co-stimulation during T cell activation results in increased proliferation (June, Bluestone et al. 1994), decreased activation-induced cell death (AICD) (Radvanyi, Shi et al. 1996) and improved long-term lymphocyte survival (Sperling, Auger et al. 1996).

As discussed in section 1.3.2, these effects are thought to be mediated by a combination of downstream signalling events that result in increased IL-2 production (Fraser, Irving et al. 1991) and the up-regulation of certain anti-apoptotic genes, in particular Bcl-X_L (Boise, Minn et al. 1995; Radvanyi, Shi et al. 1996; Sperling, Auger et al. 1996). Unlike Bcl-2, Bcl-X_L is not expressed in resting T cells. CD28 co-stimulation results in a peak of Bcl-X_L expression at 24-48 hours with a subsequent decline. This has been shown to correlate with resistance to Fas antibody-induced cell death (Boise, Minn et al. 1995).

Another study (Sperling, Auger et al. 1996) has shown that the survival of T cells from transgenic mice over-expressing Bcl-X_L is not inhibited by blocking CD28 ligation, suggesting CD28 induced T cell survival is regulated by the expression of Bcl-X_L.

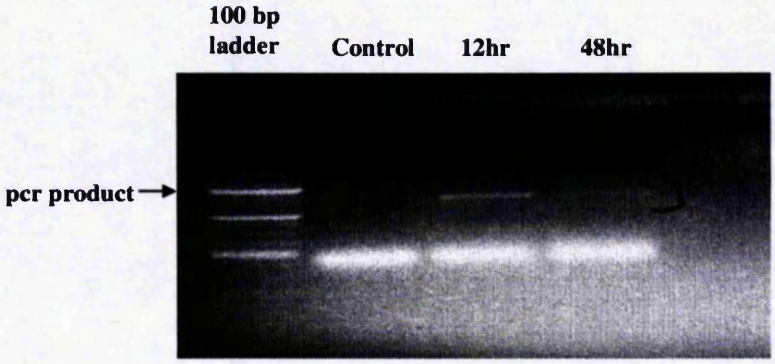


Figure 3.1 Bcl-X_L PCR - Showing approx 750bp pcr product on gel electrophoresis (right hand lanes). Note increased Bcl-X_L cDNA in PCR performed using cDNA prepared from PBL's co-stimulated for 12hrs compared to 48hrs.

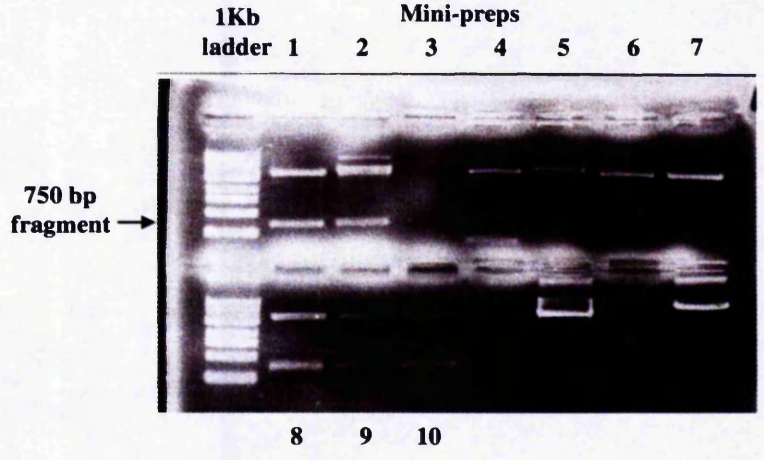


Figure 3.2 TA-Bcl-X_L - TA-Bcl-X_L contains *EcoR1* restriction sites either side of pcr product. Plasmid preps digested with *EcoR1* which contain Bcl-X_L gave pcr product of 750bp (preps 1,2,8,9 and 10).

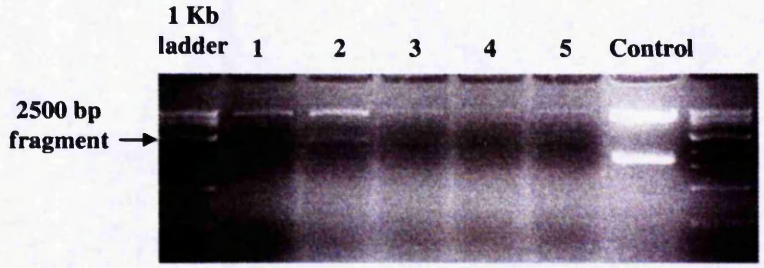


Figure 3.3 - rKat.Bcl-X_L.IRES.EGFP - Plasmid preps digested with *Xba1/EcoR1* with rKat.IRES.EGFP as a control. Following digestion the control vector gives a fragment of 1700bp. Clone 2 (containing Bcl-X_L) gives a fragment of 2500bp.

More recently, Dahl et al (Dahl, Klein et al. 2000) expressed Bcl-X_L in CD28-deficient murine T cells expressing a TCR recognising a specific antigen. They demonstrated that, in the absence of CD28, Bcl-X_L expression prolonged lymphocyte survival but did not restore normal proliferation or effector cell development. This suggested that the proliferative and survival signals generated by ligation of CD28 are separate and remained consistent with the hypothesis that specific induction of Bcl-X_L by CD28 co-stimulation results in enhanced activated T cell survival.

As discussed in section 1.4, the aim of this work was to utilise a cDNA encoding for the anti-apoptotic protein Bcl-X_L and to introduce this into vectors used for gene therapy. The activity of this Bcl-X_L cDNA could then be tested by functional assay in primary T cells and T cell lines in order to determine whether over-expression of Bcl-X_L resulted in protection from apoptosis of gene-modified lymphocytes in conditions prone to inducing this.

3.2 Bcl-X_L PCR

Peripheral blood lymphocytes were isolated from a healthy donor, divided into two groups, and activated on iCD3 and iCD28 antibodies for 12 and 48 hours respectively. RNA was extracted from $5x10^6$ cells using the RNeasy protocol (Qiagen) and cDNA was made by reverse transcription (Methods 2.2.3.1). The human Bcl-X_L gene was cloned from this cDNA by the PCR technique. Primers BCL-XL-FWD and BCL-XL-REVR (Methods 2.1.3) were designed according to the published sequence (GenBank accession no. Z23115). Additional restriction enzyme site sequences were added to enable subsequent subcloning (*EcoR1* in the forward primer and *Fsp1* in the reverse primer). Human Bcl-X_L cDNA PCR product (*Figure 3.1*) was cloned into the TOPO TA™ vector (Invitrogen) according to the manufacturers instructions and a diagnostic restriction enzyme digest was performed (*Figure 3.2*). Prior to further vector construction the Bcl-X_L gene was sequenced (see section 3.4).

3.3 Bcl-X_L Sequencing

Five separate plasmid clones of TA-Bcl-X_L were used to sequence the Bcl-X_L gene (Methods 2.2.2.5) using sequencing primers T7 and M13R (Methods 2.1.3). All five clones contained two identical point mutations when compared with the published sequence (Genbank Accession No. Z23115, *Table 3.1* (Donor 1)) at base pair positions

209 and 210, which resulted in the substitution of an alanine for a glycine at amino acid position 70. Bcl-X_L cDNA was further amplified from cDNA prepared using lymphocytes isolated from a further two different donors with the same mutations being identified on each occasion (*Table 3.1*, Donors 2 and 3) suggesting this to be a common allele.

3.4 Construction of Retroviral Vector rKat.Bcl-X_L.IRES.EGFP

The Moloney murine leukaemia virus vector rKat 43.267bn (Cell GeneSys, CA – see introduction section 1.2.2.2) (*Figure 3.4a*) had previously been modified by Dr Ryan Guest (Dept of Medical Oncology, University of Manchester) to contain the oncostatin M leader sequence and a single chain antibody fragment (ScFv) B1.8 (inserted *blunted EcoR1/Not1* into the multiple cloning site (MCS)). He had also cloned an internal ribosomal entry site (IRES) and the cDNA of enhanced green fluorescent protein (EGFP) into the vector by transferring a *Blunted/Sal1* fragment containing IRES-EGFP from pIRES.EGFP (Clontech, California) into the *Apa1(blunted)/Sal1* MCS of rKat 43.267, making the vector rKat.OM1.B1.8.IRES.GFP (*Figure 3.4b*).

As a first step in making rKat.Bcl-X_L.IRES.EGFP, the vector rKat.MCS.IRES.EGFP (*Figure 3.4c*) was cloned. This was done by taking an *Xho1/Not1* fragment from the original rKat 43.267bn vector (*Figure 3.4a)* and inserting it into rKat.OM1.B1.8 IRES.GFP also cut *Xho1/Not1* (ie removing the oncostatin M and B1.8 ScFv sequence). In other words the original multiple cloning site was reconstituted.

The human Bcl-X_L cDNA was then inserted into *blunted Not1/EcoR1* sites in front of the IRES as an *EcoR1/blunted Fsp1* fragment (cut from TA-Bcl-X_L), generating the vector rKat.Bcl-X_L.IRES.EGFP (*Figure 3.4d*). Using this vector, a bicistronic mRNA encompassing the Bcl-X_L and EGFP cDNA was generated under the control of the LTR promoter. A diagnostic restriction enzyme digest was performed (*Figure 3.3*) and vector construction confirmed.

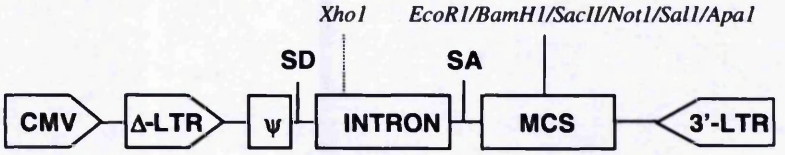
A control vector rKat.B1.8.MTM.IRES.EGFP containing the cDNA of an irrelevant cell surface expressed single chain antibody in front of the IRES, was also constructed by Miss Alison O'Neil (Department of Medical Oncology, University of Manchester) (*Figure 3.4e*).

Table 3.1 – Bcl-X_L Gene/Amino Acid Sequence

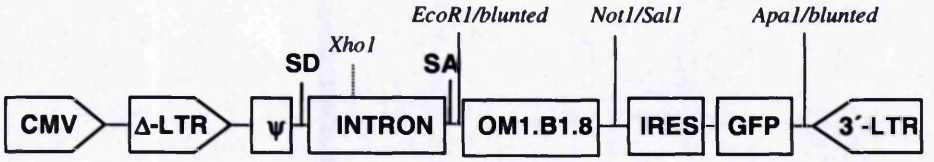
```
P - ATGTCTCAGAGCAACCGGGAGCTGGTGGTTGACTTTCTCTCCTACAAGCTTTCCCAGAAAGGATACAGCTGGAGTCAGT 79
1 - ATGTCTCAGAGCAACCGGGAGCTGGTGGTTGACTTTCTCTCCTACAAGCTTTCCCAGAAAGGATACAGCTGGAGTCAGT 79
2 ----------------------------GGTTGACTTTCTCTCCTACAAGCTTTCCCAGAAAGGATACAGCTGGAGTCAGT 53
3 ------------------------------------------------------TTCCCAGAAAGGATACAGCTGGAGTCAGT 29
AA- M  S  Q  S  N  R  E  L  V  V  D  F  L  S  Y  K  L  S  Q  K  G  Y  S  W  S  Q  F

P - TTAGTGATGTGGAAGAGAACAGGACTGAGGCCCCAGAAGGGACTGAATCGGAGATGGAGACCCCCAGTGCCATCAATGG 158
1 - TTAGTGATGTGGAAGAGAACAGGACTGAGGCCCCAGAAGGGACTGAATCGGAGATGGAGACCCCCAGTGCCATCAATGG 158
2 - TTAGTGATGTGGAAGAGAACAGGACTGAGGCCCCAGAAGGGACTGAATCGGAGATGGAGACCCCCAGTGCCATCAATGG 132
3 - TTAGTGATGTGGAAGAGAACAGGACTGAGGCCCCAGAAGGGACTGAATCGGAGATGGAGACCCCCAGTGCCATCAATGG 108
AA-   S  D  V  E  E  N  R  T  E  A  P  E  G  T  E  S  E  M  E  T  P  S  A  I  N  G
                                                        **
P - CAACCCATCCTGGCACCTGGCAGACAGCCCCGCGGTGAATGGAGCCACTGCGCACAGCAGCAGTTTGGATGCCCGGGAG 237
1 - CAACCCATCCTGGCACCTGGCAGACAGCCCCGCGGTGAATGGAGCCACTGGCCACAGCAGCAGTTTGGATGCCCGGGAG 237
2 - CAACCCATCCTGGCACCTGGCAGACAGCCCCGCGGTGAATGGAGCCACTGGCCACAGCAGCAGTTTGGATGCCCGGGAG 211
3 - CAACCCATCCTGGCACCTGGCAGACAGCCCCGCGGTGAATGGAGCCACTGGCCACAGCAGCAGTTTGGATGCCCGGGAG 187
AA-  N  P  S  W  H  L  A  D  S  P  A  V  N  G  A  T  G  H  S  S  S  L  D  A  R  E
                                                     (A)
P - GTGATCCCCATGGCAGCAGTAAAGCAAGCGCTGAGGGAGGCAGGCGACGAGTTTGAACTGCGGTACCGGCGGGCATTCA 316
1 - GTGATCCCCATGGCAGCAGTAAAGCAAGCGCTGAGGGAGGCAGGCGACGAGTTTGAACTGCGGTACCGGCGGGCATTCA 316
2 - GTGATCCCCATGGCAGCAGTAAAGCAAGCGCTGAGGGAGGCAGGCGACGAGTTTGAACTGCGGTACCGGCGGGCATTCA 290
3 - GTGATCCCCATGGCAGCAGTAAAGCAAGCGCTGAGGGAGGCAGGCGACGAGTTTGAACTGCGGTACCGGCGGGCATTCA 266
AA- V  I  P  M  A  A  V  K  Q  A  L  R  E  A  G  D  E  F  E  L  R  Y  R  R  A  F  S


P - GTGACCTGACATCCCAGCTCCACATCACCCCAGGGACAGCATATCAGAGCTTTGAACAGGTAGTGAATGAACTCTTCCG 395
1 - GTGACCTGACATCCCAGCTCCACATCACCCCAGGGACAGCATATCAGAGCTTTGAACAGGTAGTGAATGAACTCTTCCG 395
2 - GTGACCTGACATCCCAGCTCCACATCACCCCAGGGACAGCATATCAGAGCTTTGAACAGGTAGTGAATGAACTCTTCCG 369
3 - GTGACCTGACATCCCAGCTCCACATCACCCCAGGGACAGCATATCAGAGCTTTGAACAGGTAGTGAATGAACTCTTCCG 345
AA-   D  L  T  S  Q  L  H  I  T  P  G  T  A  Y  Q  S  F  E  Q  V  V  N  E  L  F  R

P - GGATGGGGTAAACTGGGGTCGCATTGTGGCCTTTTTCTCCTTCGGCGGGGCACTGTGCGTGGAAAGCGTAGACAAGGAG 474
1 - GGATGGGGTAAACTGGGGTCGCATTGTGGCCTTTTTCTCCTTCGGCGGGGCACTGTGCGTGGAAAGCGTAGACAAGGAG 474
2 - GGATGGGGTAAACTGGGGTCGCATTGTGGCCTTTTTCTCCTTCGGCGGGGCACTGTGCGTGGAAAGCGTAGACAAGGAG 448
3 - GGATGGGGTAAACTGGGGTCGCATTGTGGCCTTTTTCTCCTTCGGCGGGGCACTGTGCGTGGAAAGCGTAGACAAGGAG 424
AA-  D  G  V  N  W  G  R  I  V  A  F  F  S  F  G  G  A  L  C  V  E  S  V  D  K  E


P - ATGCAGGTATTGGTGAGTCGGATCGCAGCTTGGATGGCCACTTACCTGAATGACCACCTAGAGCCTTGGATCCAGGAGA 553
1 - ATGCAGGTATTGGTGAGTCGGATCGCAGCTTGGATGGCCACTTACCTGAATGACCACCTAGAGCCTTGGATCCAGGAGA 553
2 - ATGCAGGTATTGGTGAGTCGGATCGCAGCTTGGATGGCCACTTACCTGAATGACCACCTAGAGCCTTGGATCCAGGAGA 527
3 - ATGCAGGTATTGGTGAGTCGGATCGCAGCTTGGATGGCCACTTACCTGAATGACCACCTAGAGCCTTGGATCCAGGAGA 503
AA- M  Q  V  L  V  S  R  I  A  A  W  M  A  T  Y  L  N  D  H  L  E  P  W  I  Q  E  N

P - ACGGCGGCTGGGATACTTTTGTGGAACTCTATGGGAACAATGCAGCAGCCGAGAGCCGAAAGGGCCAGGAACGCTTCAA 632
1 - ACGGCGGCTGGGATACTTTTGTGGAACTCTATGGGAACAATGCAGCAGCCGAGAGCCGAAAGGGCCAGGAACGCTTCAA 632
2 - ACGGCGGCTGGGATACTTTTGTGGAACTCTATGGGAACAATGCAGCAGCCGAGAGCCGAAAGGGCCAGGAACGCTTCAA 606
3 - ACGGCGGCTGGGATACTTTTGTGGAACTCTATGGGAACAATGCAGCAGCCGAGAGCCGAAAGGGCCAGGAACGCTTCAA 582
AA-   G  G  W  D  T  F  V  E  L  Y  G  N  N  A  A  A  E  S  R  K  G  Q  E  R  F  N


P - CCGCTGGTTCCTGACGGGCATGACTGTGGCCGGCGTGGTTCTGCTGGGCTCACTCTTCAGTCGGAAATGA        - 700
1 - CCGCTGGTTCCTGACGGGCATGACTGTGGCCGGCGTGGTTCTGCTGGGCTCACTCTTCAGTCGGAAATGA        - 700
2 - CCGCTGGTTCCTGACGGGCATGACTGTGGCCGGCGTGGTTCTGC---------------------------------- 650
3 - CCGCTGGTTCCTGACGGGCATGACTGTGGCCGGCGTGGTT------------------------------------- 622
AA-  R  W  F  L  T  G  M  T  V  A  G  V  V  L  L  G  S  L  F  S  R  K
```

Mutations highlighted in **bold.** P – Published genbank sequence; 1,2+3 – Sequence from Donors 1,2+3 (Donor 1 sequence is in construct); AA – Amino Acid Sequence.

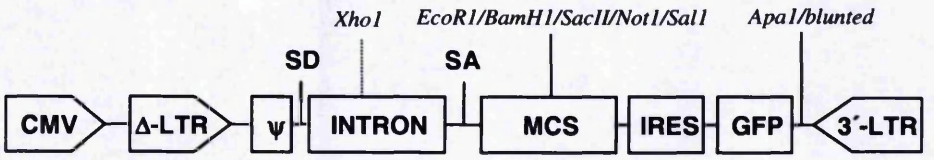
3.4a. rKat.43.267bn



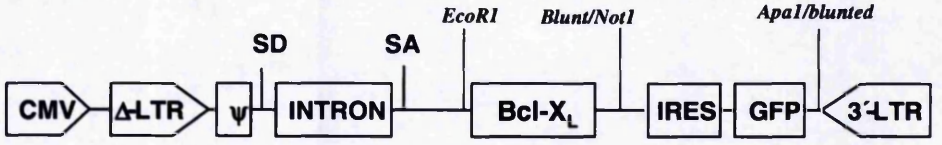
3.4b rKat.OM1.B1.8.IRES.GFP



3.4c rKat.MCS.IRES.GFP



3.4d rKat.Bcl-X_L.IRES.GFP



3.4e rKat.B1.8MTM.IRES.GFP

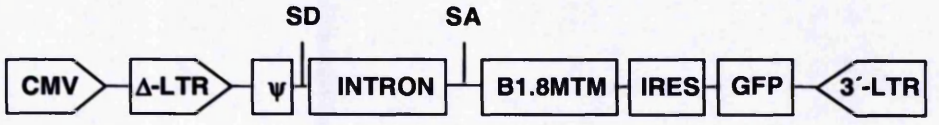


Figure 3.4 - Schematic representation of retroviral constructs – a) Basic rKat 43.267 vector, b) rKat vector containing ScFv and GFP, c) rKat vector containing multiple cloning site and GFP, d) rKat vector expressing Bcl-X_L and GFP, e) Control rKat vector expressing surface bound single chain antibody B1.8 and GFP. ψ = packaging signal, SD, SA are splice donor and splice acceptor sites respectively. MTM is murine MHC class I transmembrane region. MCS, multiple cloning site.

3.5 Western Blot for Bcl-X_L Expression in Bcl-X_L transfected 293T Cells

This experiment was performed in order to confirm that the Bcl-X_L cDNA within the rKat.Bcl-X_L.IRES.GFP vector was functional and led to Bcl-X_L protein expression within transfected cells. Recombinant retrovirus used in subsequent lymphocyte and packaging cell line transduction experiments was produced by a transient calcium phosphate transfection method of the 293T tumour cell line (materials and methods 2.2.6.2.1). In this experiment, 293T cells were transfected using the retroviral vectors rKat.GFP and rKat.Bcl-X_L.IRES.GFP. Forty-eight hours post transfection 293T cells were lysed, separated by SDS/PAGE (12% gel, 300µg/lane) and the expression of Bcl-X_L was assessed by western blot (*Figure 3.5*) using a mouse monoclonal anti-Bcl-X antibody (Clone 2H12, Pharmingen) (methods 3.2.5).

Figure 3.5 clearly shows anti-Bcl-X_L antibody detected immunoreactive bands at the expected molecular weight of wild-type Bcl-X_L (28kD). Overexpression of Bcl-X_L can be seen in Bcl-X_L-transfected 293T cells with no evidence of expression in the GFP-transfected or non-transfected 293T cells. For comparison lysates of the Bcl-X_L over-expressing K562 and Jurkat cell lines, and co-stimulated and naïve lymphocytes are also shown.

3.6 Creation of a PG13 rKat.Bcl-X_L.IRES.GFP stable packaging cell line

For the transduction experiments described in this chapter amphotropic retrovirus made by transient transfection of 293T cells was used (materials and methods 2.2.6.2.1 and 2.2.6.3.1). This approach is convenient for testing several different constructs and has the significant advantage that reasonable titre retrovirus can be obtained in under a week. However, production of large amounts of retrovirus is time-consuming and there can be significant variation between batches depending on the efficiency of the particular transfection procedure. In addition, human T lymphocytes express higher levels of GLVR-1 mRNA (encoding the GALV amphotropic envelope receptor) compared to GLVR-2 mRNA (encoding amphotropic envelope receptor), thus PG 13-packaged vectors (GALV-pseudotyped) have been shown to result in an improved T cell transduction efficiency when compared with amphotropic (ie 293T) retrovirus (see introduction section 1.2.2.2) (Bunnell, Muul et al. 1995).

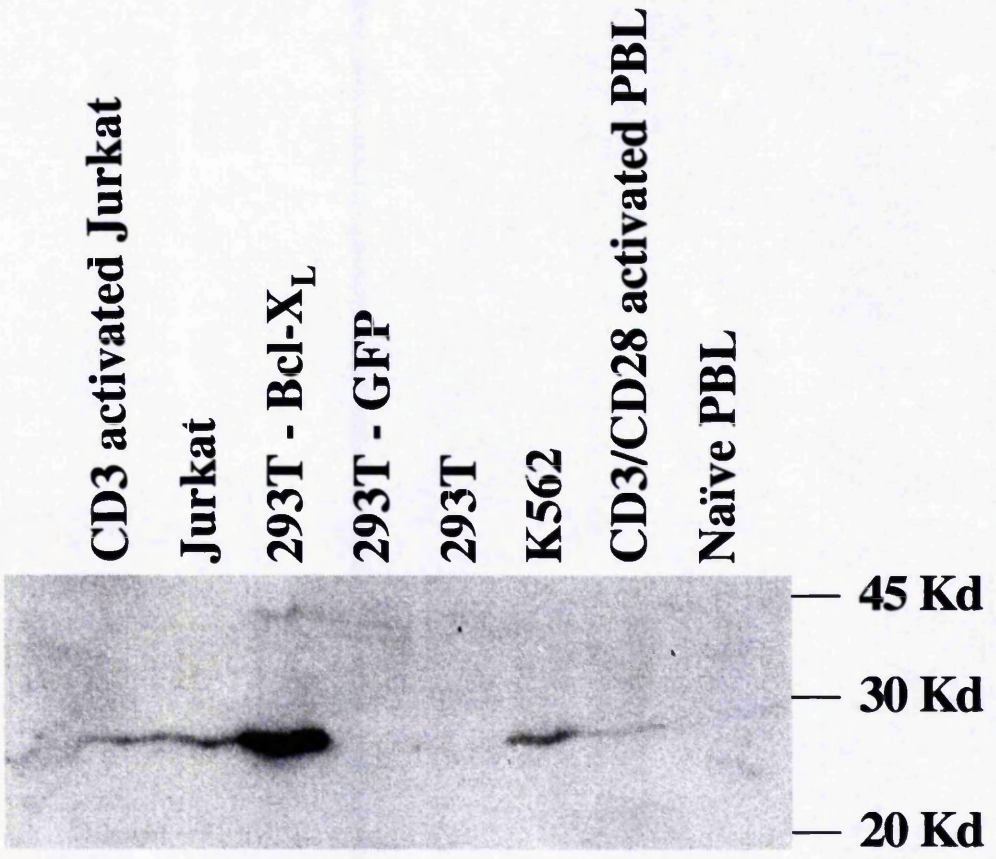


Figure 3.5 Western Blot for Bcl-X_L Expression in Bcl-X_L-transfected 293T cells - 293T cells were transiently transfected by calcium phosphate co-precipitation (materials and methods 2.2.6.2.1) using the retroviral vectors rKat.GFP and rKat.Bcl-X_L.IRES.GFP. 48hrs later cells were lysed, separated by SDS/PAGE (12% gel, 300µg/lane) and expression of Bcl-X_L was assessed by western blot using a mouse monoclonal anti-Bcl-X_L antibody at dilution of 1:250 (Pharmingen) with an anti-mouse HRP secondary antibody (1:2000 dilution). Over-expression of Bcl-X_L can clearly be seen in Bcl-X_L-transfected 293T cells with no evidence of expression in GFP-transfected or non-transfected 293T cells. For comparison lysates of the Bcl-X_L over-expressing K562 and Jurkat cell lines, and also co-stimulated and naïve lymphocytes are also shown.

In view of this, PG13 packaging cells were transduced using amphotropic rKat.Bcl-X_L.IRES.EGFP retrovirus and transduced cells cloned by limiting dilution (materials and methods 2.2.6.1).

Several green cell colonies were picked and viral titre was determined using supernatant from these colonies to infect the human cervical carcinoma HeLa cell line (materials and methods 2.2.6.3.3). *Figure 3.6a* shows representative flow cytometry density plots illustrating percentage GFP expression of HeLa cells transduced using two dilutions of supernatant from clone 9. An approximate viral titre was then calculated using the formula detailed in materials and methods. *Figure 3.6b* shows the viral titres obtained from several clones. Clone 9 was the highest viral titre producer and was used to transduce human lymphocytes in the experiments described in chapter 4.

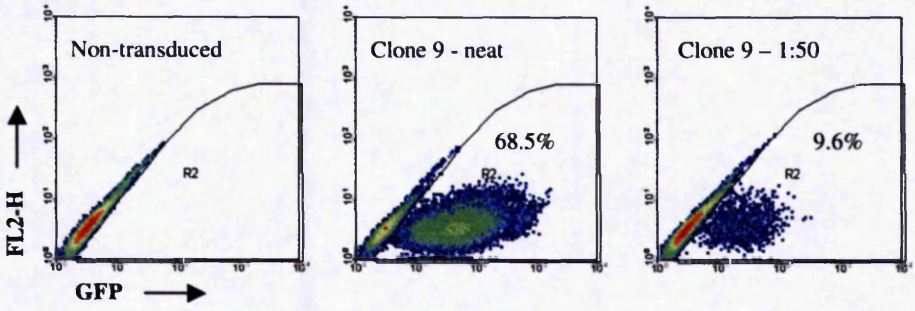
3.7 Bcl-X_L transduced Jurkat Cells are Partially Resistant to anti-Fas antibody Induced Apoptosis

Figure 3.5 clearly demonstrated expression of Bcl-X_L protein following transfection using the rKat.Bcl-X_L.IRES.GFP vector. It did not, however, establish whether the protein was functionally active, nor whether retrovirus made using rKat.Bcl-X_L.IRES.GFP could efficiently transduce cells. In this series of experiments the ability of the Jurkat T cell line to be retrovirally transduced using the rKat.Bcl-X_L.IRES.GFP vector was tested. As discussed in the introduction to this chapter (section 3.1), previous work using Bcl-X_L transfected Jurkat cell clones has shown that Bcl-X_L over-expression can result in resistance to apoptosis induced by anti-Fas antibody (Boise, Minn et al. 1995). In order, therefore, to establish the functionality of the Bcl-X_L cDNA within the vector, in a subsequent assay it was examined whether transduced Jurkat cells were also resistant to anti-Fas antibody-induced apoptosis as determined by Annexin V status.

3.7.1 Concentration of anti-Fas antibody required to induce apoptosis in the Jurkat leukaemia cell line

Jurkat cells were suspended in 2ml fresh medium ($0.5x10^6$cells/ml) in a 24-well tissue culture plate. 24 hrs later, the soluble anti-human CD95 (Fas) monoclonal antibody (R+D Systems) (1μg/ml) and Protein G (Sigma) (1μg/ml) was added to the cells. Control cells were cultured in the presence of an IgG_1 isotype control antibody and Protein G. At 48 hours, cells were stained with PE-conjugated annexin V (Pharmingen)

a



b

Clone	% GFP positive		Viral Titre*
	Neat	1:50	(cfu/ml)
4	24.6	0.7	$2.5x10^4$
5	37.5	2.0	$1x10^5$
6	35.0	0.6	$3x10^4$
7	20.4	0.9	$4.5x10^4$
8	26.9	0.9	$4.5x10^4$
9	68.5	9.6	$4.8x10^5$
11	35.2	1.5	$1.2x10^5$

Figure 3.6 – rKat.Bcl-X_L.IRES.GFP PG13 stable line titring against HeLa cell line - Hela target cells ($3x10^4$/well) were plated out in a 24 well tissue culture plate. Twenty-four hours later, serial dilutions of fresh viral supernatant in DMEM harvested from the green PG13 colonies were placed on to the cells (200µl/well), plus polybrene (4µg/ml). Cells were cultured at 37°C overnight and the following morning the viral supernatant was replaced with DMEM (one ml per well). Target cells were analysed for GFP expression by flow cytometry 5 days later (*Figure 3.6a*). An estimate of viral titre could then be determined using the formula Viral Titre = (%GFP positive cells (as fraction of cell population)) x (number of target cells plated x2) x (1/amount of viral supernatant used in ml) x (dilution factor) (*Figure 3.6b*). *cfu/ml, colony forming units per ml.

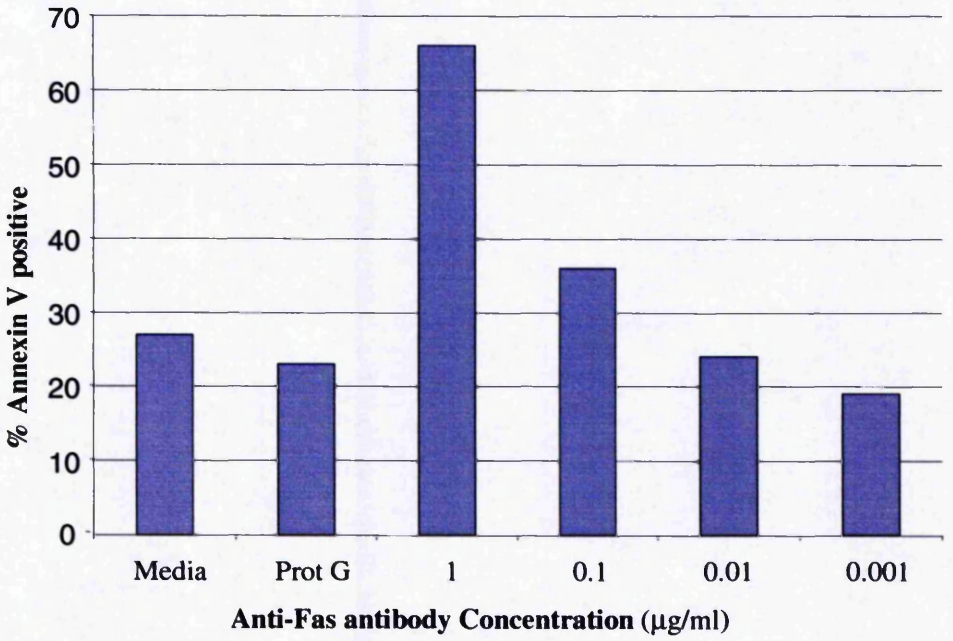


Figure 3.7 - Concentration of Fas antibody required to induce apoptosis in the Jurkat leukaemia cell line - Jurkat cells were suspended in fresh medium ($0.5x10^6$/ml) in a 24-well tissue culture plate. 24 hrs later, the soluble anti-human CD95 (FAS) monoclonal antibody (R+D Systems) (1µg/ml) and Protein G (Sigma) (1µg/ml) was added to the cells. Control cells were cultured in the presence of an IgG_1 isotype control antibody and Protein G. At 48 hours, cells were stained with PE-conjugated annexin V (Pharmingen) and the percentage of apoptotic cells within the population was assessed by flow cytometry (All annexin V-positive cells were considered apoptotic).

and the percentage of annexin V staining cells within the population was assessed by flow cytometry.

Annexin V staining is a method of quantitatively determining the percentage of cells within a population that are actively undergoing apoptosis (Vermes, Haanen et al. 1995). It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external environment. Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS. It is therefore an excellent marker of apoptosis. All annexin V-positive cells were considered apoptotic.

In the control cell populations approximately 20% of cells stained annexin V positive indicating significant background levels of apoptosis (*Figure 3.7*). The addition of low concentrations (0.01-0.001μg/ml) of anti-Fas antibody was insufficient to induce further apoptosis. However, at a concentration of 0.1μg/ml a slight increase in annexin V staining was seen and at a concentration of 1μg/ml nearly 70% of Jurkat cells stained annexin V positive after 48 hours, indicating that the majority of cells were undergoing apoptosis.

This result confirmed the ability of anti-Fas antibody to induce the apoptosis of Jurkat T cells and, on this basis, a concentration of 1μg/ml was used in the experiment described in 3.7.2.

3.7.2 Bcl-X_L transduced Jurkat Cells are Partially Resistant to Fas Induced Apoptosis

Jurkat cells (10^5) were transduced with rKat.Bcl-X_L.IRES.EGFP (and rKat.B1.8mtm.IRES.EGFP control) retrovirus using a spin transduction method (materials and methods 2.2.6.6.1). Transduction efficiencies of 35-45% were achieved as assessed by flow cytometry analysis of GFP expression (*Figure 3.8a*). Transduced cells were suspended in fresh medium ($0.5x10^6$/ml) in a 24-well tissue culture plate. 24 hours later, the soluble anti-human CD95 (FAS) monoclonal antibody (R&D Systems) (1μg/ml) and Protein G (Sigma) (1μg/ml) was added to the cells. Control cells were cultured in the presence of an IgG_1 isotype control antibody and Protein G. At 24 hrs, cells were stained with a PE-conjugated annexin V antibody (Pharmingen) and the

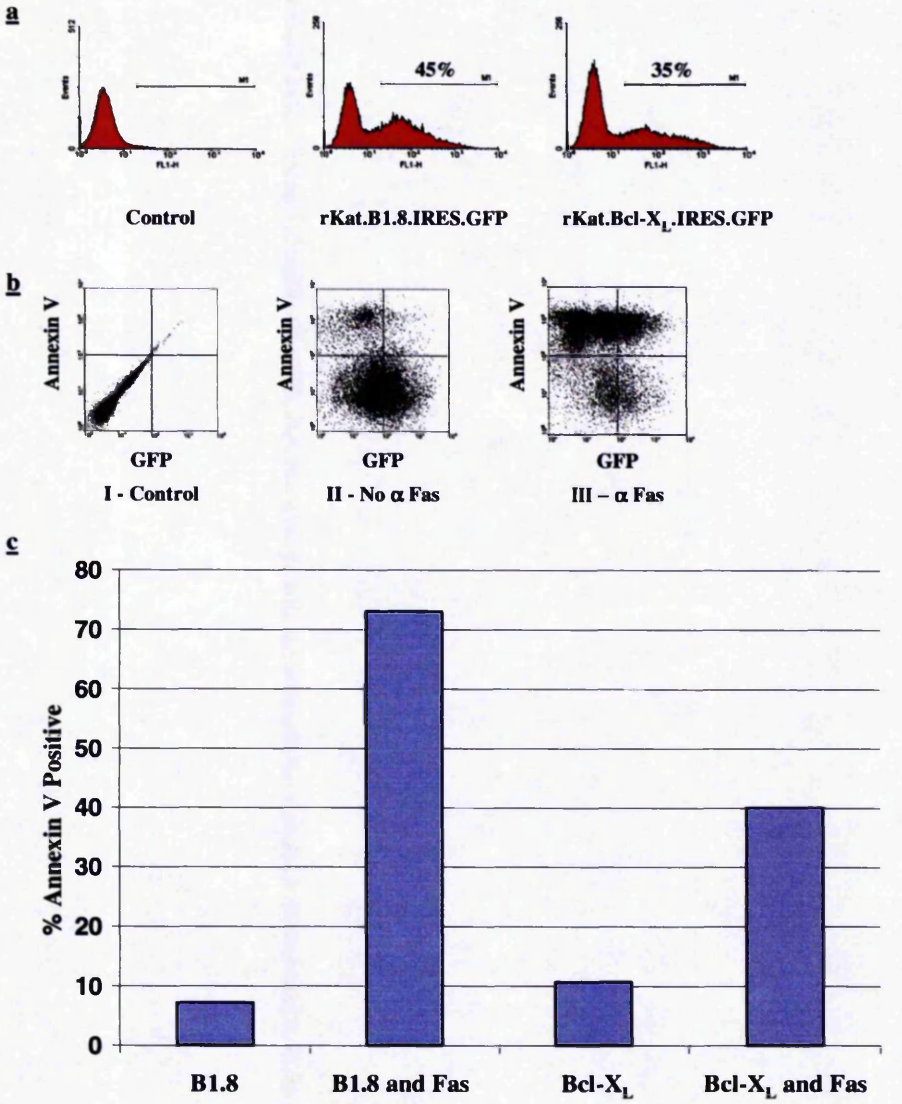


Figure 3.8 - Bcl-X_L transduced jurkat cells are partially resistant to Fas induced apoptosis - Jurkat cells (10^5) were transduced with rKat.Bcl-xl.IRES.EGFP (and rKat.B1.8mtm.IRES.EGFP control) retrovirus using a spin transduction method. Transduction efficiencies of 35-45% were achieved as assessed by flow cytometry analysis of GFP expression (*Figure 3.8a*). Transduced cells were suspended in fresh medium ($0.5x10^6$/ml) in a 24-well tissue culture plate. 24 hours later, the soluble anti-human CD95 (FAS) monoclonal antibody (R+D Systems) (1μg/ml) and Protein G (Sigma) (1μg/ml) was added to the cells. Control cells were cultured in the presence of an IgG_1 isotype control antibody and Protein G. At 24 hrs, cells were stained with a PE-conjugated annexin V antibody (Pharmingen) and the percentage of apoptotic cells within the GFP positive cell population was assessed by two colour flow cytometry (*Figure 3.8b* shows representative plots I – control cells, II – GFP positive cell population stained with annexin V and III – GFP positive cell population stained with annexin V after 24 hours culture with anti-FAS antibody). *Figure 3.8c* illustrates the percentage of GFP positive cells staining for annexin V. Results shown are a representative experiment in single wells. The experiment was repeated four times with similar results.

percentage of apoptotic cells within the GFP positive cell population was assessed by two-colour flow cytometry (*Figure 3.8b*). *Figure 3.8c* illustrates the percentage of GFP positive cells staining for annexin V. All annexin V positive cells were considered apoptotic.

As before, in the absence of anti-Fas antibody, a background percentage of cells (approximately 10%) stained annexin V positive. In the control B1.8 transduced population after a 24 hour period of exposure to anti-Fas antibody, greater than 70% of cells underwent apoptosis. In contrast, only 40% of Bcl-X_L transduced cells were undergoing apoptosis at the same time point. This experiment was repeated 4 times and on each occasion the percentage of Bcl-X_L-transduced cells staining positive for annexin V was approximately 50% that of the control B1.8-transduced cells.

These results indicated that following retroviral insertion into a T cell line the Bcl-X_L cDNA was functional in opposing apoptosis induced by antibody ligation of Fas receptor.

3.8 Human Lymphocyte Transduction with Bcl-X_L Expression Vector

Human peripheral blood lymphocytes (PBL) were obtained from a healthy donor and pre-activated on immobilised anti-CD3 and anti-CD28 monoclonal antibodies with IL-2 (30u/ml) for 48hrs (materials and methods 2.2.6.4). rKat.Bcl-X_L.IRES.EGFP retrovirus was again made by a transient transfection method and control virus containing packaging plasmid, pKat, alone and an rKat vector containing an irrelevant ScFv, rKat.B1.8.IRES.EGFP was also prepared. A spin transduction method (materials and methods 2.2.6.6.2) was then used to transduce lymphocytes. Following transduction, cells were further expanded on iCD3/iCD28 antibodies for 72 hours. An aliquot of 10^5 cells was then labelled with a PE-conjugated anti-human CD3 antibody (Pharmingen) and analysed by flow cytometry analysis for GFP and PE expression. *Figure 3.9a* illustrates that following this isolation, activation and transduction protocol the vast majority of cells were CD3 positive indicating a population composed mainly of T lymphocytes. In both Bcl-X_L and B1.8 groups transduction efficiencies of approximately 17-18% were achieved. The remaining lymphocytes were lysed in RIPA buffer (materials and methods 2.2.5.1) and stored at –20°C for protein analysis by western blotting.

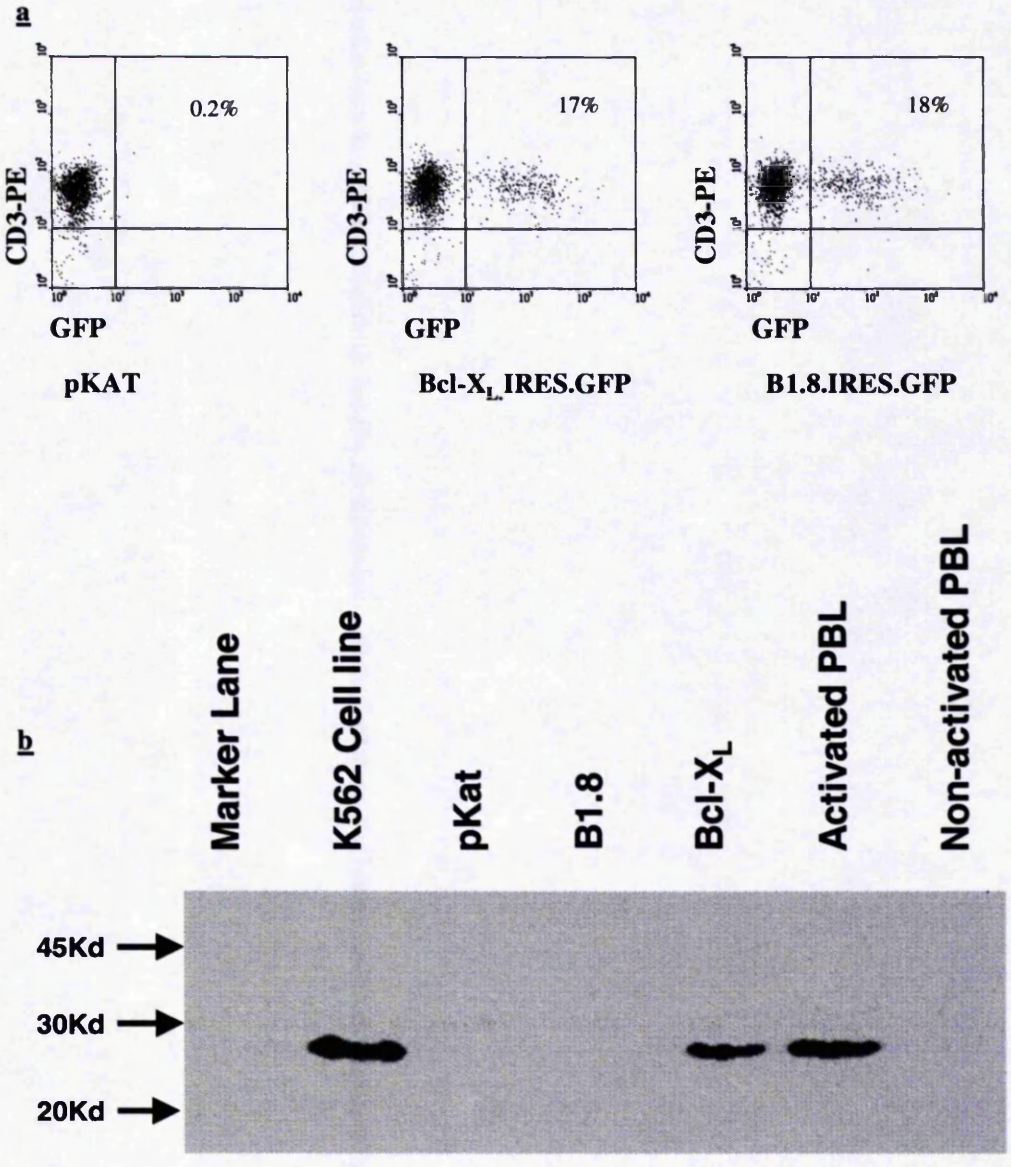


Figure 3.9 – Human lymphocyte transduction and Bcl-X_L expression - rKat.Bcl-X_L.IRES.EGFP retrovirus made by transient transfection of 293T cells using a calcium phosphate co-precipitation method (Packaging plasmid, pKat, alone and an rKat vector containing an irrelevant ScFv, rKat.B1.8.IRES.EGFP used as controls). Spin transduction method used to infect T cells (approx 17% GFP positive by FACS, *Figure 3.9a*). D+4 cells lysed. Proteins (200µg) subjected to 12%SDS/PAGE and Western blot analysis with a monoclonal anti-Bcl-X antibody (clone 2H12, Pharmingen). The Bcl-X_L -expressing K562 cell line is shown as a positive control along with protein lysates prepared from iCD3/iCD28 activated and resting lymphocytes (*Figure 3.9b*).

3.9 Western Blot for Bcl-X_L expression in transduced Lymphocytes

Following estimation of protein concentration (see materials and methods 2.2.5.2), equal amounts of protein lysate from the above experiment were separated by SDS/PAGE (12% gel, 200μg). Proteins were transferred to a nitrocellulose membrane by western blotting and comparative levels of Bcl-X_L expression were assessed by probing the membrane using a mouse monoclonal anti-Bcl-X antibody (Pharmingen) (materials and methods 2.2.5) (*Figure 3.9b*).

Figure 3.9b again shows antibody detected immuno-reactive bands correlating with the predicted molecular weight of Bcl-X_L. The K562 cell line was used as a positive control and Bcl-X_L expression was clearly detected. Only faint expression was seen in control pKat and B1.8 transduced lymphocytes with no expression detected in naïve non-stimulated cells. The degree of Bcl-X_L expression demonstrated in the Bcl-X_L transduced lymphocyte lysate (17% transduced according to GFP expression) appeared to approach that of freshly CD3/CD28 co-stimulated lymphocytes.

3.10 Expression of Bcl-X_L promotes *in vitro* survival of PBL in pro-apoptotic conditions

In these experiments the ability of Bcl-X_L over-expressing lymphocytes to resist apoptosis when compared with non-Bcl-X_L expressing gene-modified lymphocytes was tested in an *in vitro* assay.

3.10.1 Expression of Bcl-X_L promotes *in vitro* survival of PBL in pro-apoptotic conditions – short term assay

Peripheral blood lymphocytes were isolated from a healthy donor, activated on immobilised anti-CD3/anti-CD28 antibodies (iCD3/iCD28) for 48 hours and transduced (methods 2.2.6.6.2). Post transduction, cells were expanded on iCD3/iCD28 for 72hours. Transduction efficiencies of 8-20% were achieved as measured by flow cytometry analysis of GFP expression. Unsorted Bcl-X_L-transduced and control B1.8-transduced lymphocytes were each split into 4 groups of differing culture conditions ($1.5x10^6$ cells/group) and subsequently cells were cultured for 7 days. In two groups (T cell media alone with or without anti-CD3 antibody (OKT3 hybridoma – J Embleton) the culture conditions (ie in the absence of IL-2 and with unopposed CD3 stimulus)

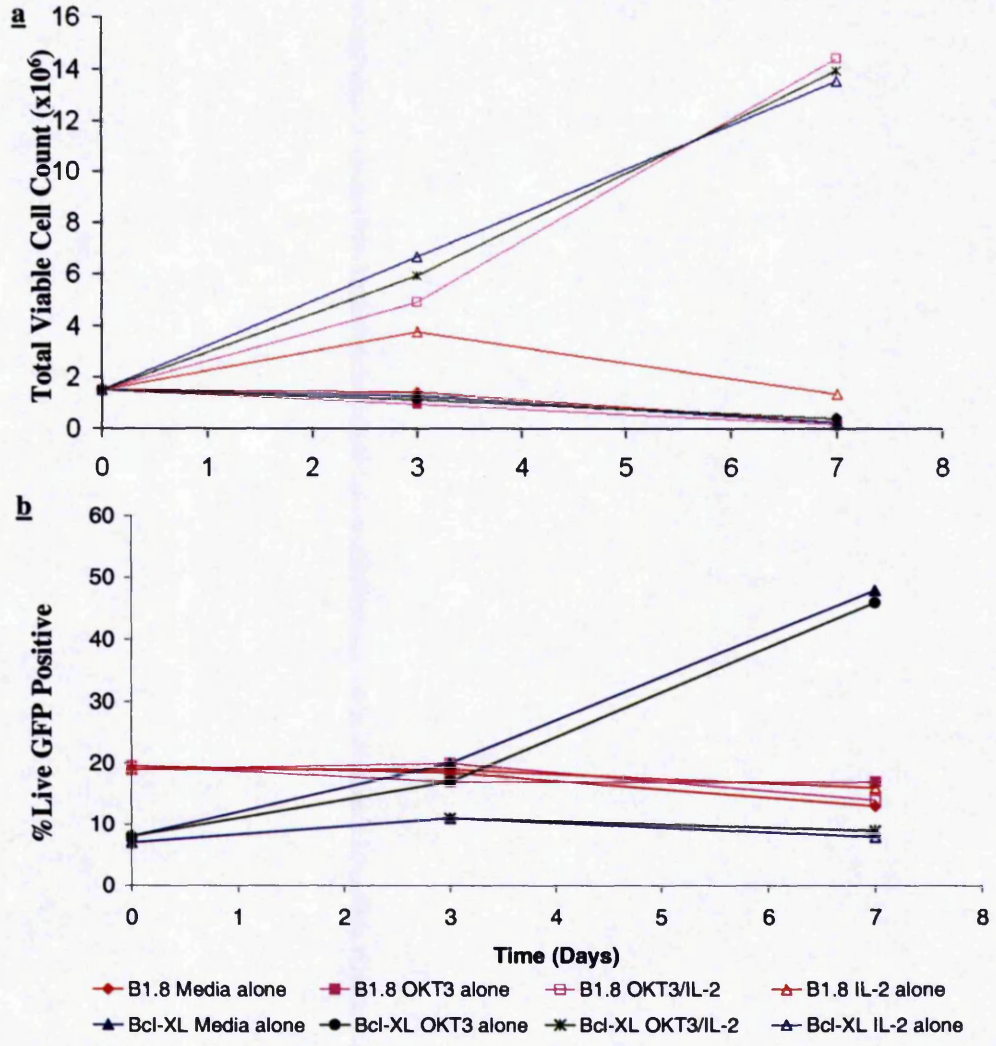


Figure 3.10 - Expression of Bcl-X_L promotes in vitro survival of PBL in pro-apoptotic conditions, but does not increase proliferation during expansion – short term assay - Unsorted Bcl-X_L-transduced and control B1.8-transduced lymphocytes (6-6.5x10^6 cells per group) were cultured in T cell media+/-OKT3, IL-2, or both for 7 days. Total viable cell number was measured by trypan blue exclusion (*Figure 3.10a*). After gating on the live lymphocyte population (Region R2 on representative flow cytometry FSC vs SSC dot plots, see *Figure 3.11c*) the %GFP positive live cells was measured by flow cytometry (*Figure 3.10b*). In culture conditions favouring lymphocyte apoptosis (T cell media alone with or without OKT3),although the overall number of viable cells decreases, the proportion of GFP positive cells in the live Bcl-X_L-transduced population increases approximately six-fold when compared with the control transduced population, indicating a survival advantage for Bcl-X_L-transduced cells. In conditions favouring expansion (IL-2 with or without OKT3), there is no change in the proportion of live transduced cells.

would be expected to induce lymphocyte apoptosis (Groux, Monte et al. 1993). In the other two groups (T cell media with IL-2, with or without OKT3) lymphocytes would be expected to expand.

On days 0, 3 and 7, the total viable cell number was estimated by trypan blue exclusion (*Figure 3.10a*) and the percentage of GFP positive cells (*Figure 3.10b*) within a live lymphocyte gating (similar to that shown in *Figure 3.11c*) as measured by flow cytometry analysis of aliquots of 10^5 cells from each group was quantified.

In culture conditions favouring lymphocyte apoptosis (T cell media alone with or without IL-2), although the overall number of viable cells decreased over the 7 days (*Figure 3.10a*), the proportion of GFP positive cells in the live Bcl-X_L-transduced population increased approximately six-fold when compared with the control transduced population (*Figure 3.10b*). This dramatic increase in the proportion of transduced cells (8% to 46-48% at Day 7) within the overall cell population indicated a survival advantage for the Bcl-X_L over-expressing cells that was not present in the control (B1.8) transduced population.

In conditions favouring expansion (IL-2 with or without OKT3), with the exception of the B1.8/IL-2 group (which failed to expand in this experiment), the total viable cell numbers expanded eight-fold in the seven day period. There was no increase, however, in the proliferation of either Bcl-X_L or B1.8 expressing lymphocytes compared to non-transduced cells (i.e. the relative proportion of GFP positive cells did not rise). These results are representative of four independent experiments (including one experiment where a commercial OKT3 antibody (Orthoclone) was used).

3.10.2 Expression of Bcl-X_L promotes *in vitro* survival of PBL in pro-apoptotic conditions – long term assay

In this larger scale assay unsorted Bcl-X_L-transduced and control B1.8-transduced lymphocytes (both approximately 15% GFP-positive, 6-6.5x10^6 cells/group) were cultured in pro-apoptotic culture conditions (T cell media with or without OKT3) between days 0-10 and 22-30. On day 10, dead cells in each group were removed by centrifugation on a Ficoll gradient and the remaining cells were resuspended in fresh media and expanded in IL-2 (100u/ml) for 12 days. As before, total viable cell number was estimated by trypan blue exclusion (*Figure 3.11a*) and the percentage of GFP positive cells (*Figure 3.11b*) within a live lymphocyte gating (*Figure 3.11c*) as

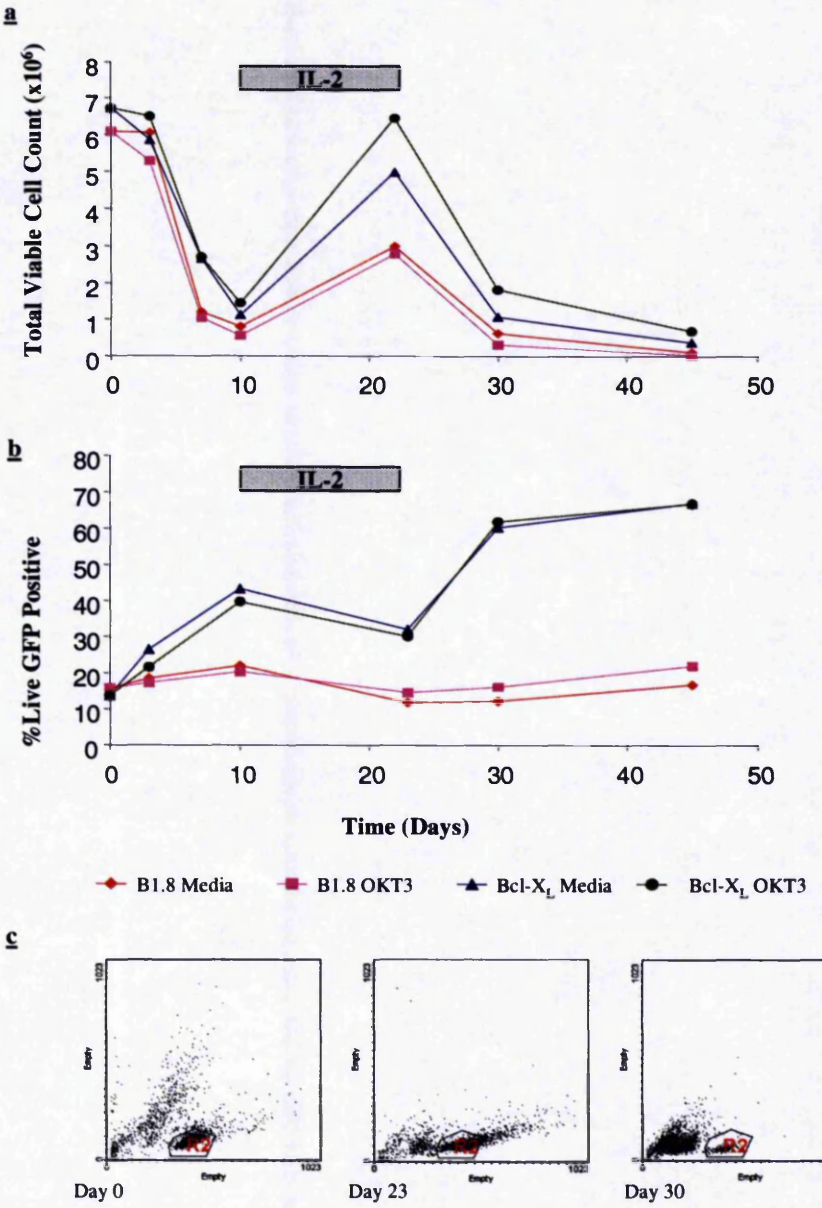


Figure 3.11 - Expression of Bcl-X_L promotes survival of peripheral blood lymphocytes - Unsorted Bcl-X_L-transduced and control B1.8-transduced lymphocytes (6-6.5x10^6 cells per group) were cultured in growth media+/-OKT3 antibody between days 0-10 and 22-30. On day 10, dead cells in each group were removed by centrifugation on a ficol gradient and remaining cells were resuspended in fresh media+IL-2 (100u/ml). Total viable cell number was measured by trypan blue exclusion (*Figure 3.11a*). After gating on the live lymphocyte population (Region R2 on representative flow cytometry FSC vs SSC dot plots, *Figure 3.11c*) the %GFP positive live cells was measured by flow cytometry (*Figure 3.11b*). The proportion of GFP positive cells in the live Bcl-X_L-transduced population increased compared with the control transduced population when cells were cultured in pro-apoptotic conditions, indicating a survival advantage for Bcl-X_L-transduced cells.

quantified by flow cytometry analysis of aliquots of 10^5 cells from each group was measured every 72-96 hours.

In *Figure 3.11a* it can be seen that, over the initial 10 days, in all 4 groups the total number of viable cells decreased in apoptotic conditions. However, by day 10 more viable cells were present in the Bcl-X_L transduced groups compared to control groups ($1.5x10^6$ Bcl-X_L-transduced cells in OKT3 compared with $0.55x10^6$ control cells in OKT3). Over the corresponding time period, the proportion of GFP positive cells in the live Bcl-X_L transduced populations increased 3-fold, compared with only a 1.3- fold increase in the percentage of live GFP positive cells in the control B1.8 transduced groups (*Figure 4b*). Again, this rise in the proportion of transduced cells within the overall cell population indicated a survival advantage for the Bcl-X_L over-expressing cells, confirming the results of the short-term experiments performed in section 3.10.1. Following the addition of IL-2 to these cultures, the populations containing Bcl-X_L transduced cells expanded at a faster rate than the controls (eg on Day 22, $5\text{-}6.5x10^6$ total Bcl-X_L transduced cells compared with $2.8\text{-}3.0x10^6$ control cells - *Figure 3.11a*) and maintained an enriched population of transduced cells (>30% GFP positive compared with 12% at start of experiment, *Figure 3.11b*). Importantly, again, no increase in proliferation of Bcl-X_L expressing lymphocytes was seen (i.e. the proportion of GFP positive cells did not rise during this period). By Day 45, following a further round of cytokine deprivation, there were very few remaining viable cells in the control population with the percentage of GFP positive cells essentially unchanged. In contrast, there was three times more viable cells in the Bcl-X_L transduced populations with a five-fold increase in the percentage of GFP positive cells (68% of remaining viable cells expressed GFP).

3.11 Culturing Bcl-X_L transduced cells at different cell densities in pro-apoptotic and normal growth conditions

This experiment was performed as a subsidiary experiment to 3.10 in order to examine whether cell concentration affected either gene-modified cell expansion in normal growth conditions or gene-modified lymphocyte apoptosis in pro-apoptotic conditions. T lymphocytes were isolated from a healthy donor, activated on immobilised anti-CD3/anti-CD28 antibodies (iCD3/iCD28) for 48 hrs and transduced with rKat.Bcl-X_L.IRES.EGFP retrovirus using a spin transduction method (2.2.6.6.2). Post transduction, cells were expanded on iCD3/iCD28 for 72hrs. Bcl-X_L transduced

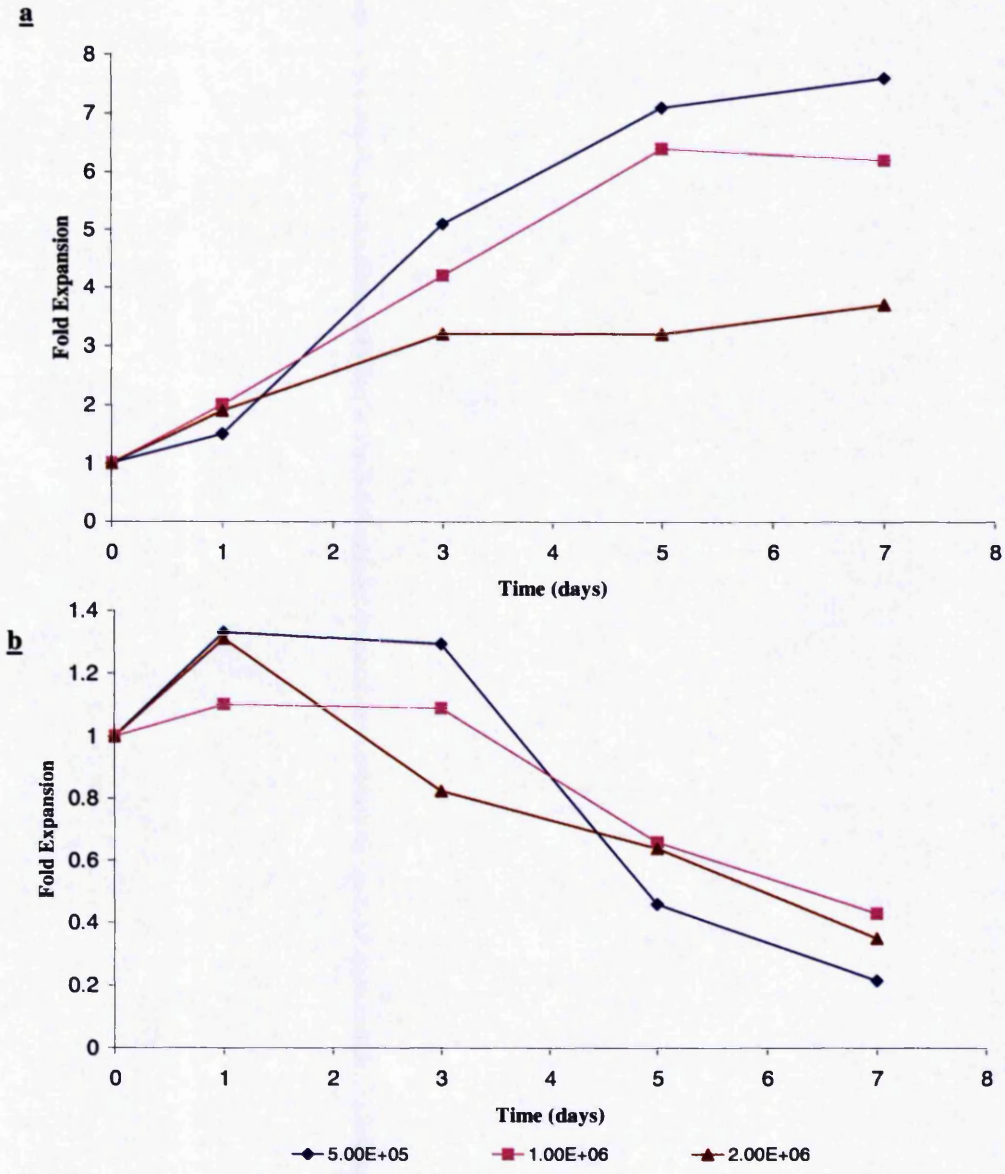


Figure 3.12 Culturing Bcl-X_L transduced cells at different cell densities in pro-apoptotic and normal growth conditions - Lymphocytes were isolated from a healthy donor, activated on immobilised anti-CD3/anti-CD28 antibodies (iCD3/iCD28) for 48 hrs and transduced with rKat.Bcl-xl.IRES.EGFP retrovirus using a spin transduction method. Post transduction, cells were expanded on iCD3/iCD28 for 72hrs. Bcl-X_L-transduced lymphocytes (6% GFP positive) were then cultured in growth media with either IL-2 (100u/ml) (*Figure 3.12a*) or OKT3 antibody (1µg/ml) (*Figure 3.12b*) at three different cell concentrations 0.5, 1 and $2x10^6$ cells/ml. The total number of viable cells in each group was counted by trypan blue exclusion every 48 hrs and cell concentration was adjusted accordingly.

lymphocytes were then cultured in T cell media with either IL-2 (100iu/ml) or OKT3 antibody (1μg/ml) at three different cell concentrations 0.5, 1 and $2x10^6$ cells/ml for 7 days. The total number of viable cells in each group was counted by trypan blue exclusion every 48 hrs and the cell concentration was adjusted accordingly.

In the presence of IL-2, T cell expansion over the seven-day period of the experiment was reduced when cells were cultured at the higher concentration of $2x10^6$ cells/ml (*Figure 3.12a*). At Day 7 there had been a 3.7-fold increase in the total number of viable cells in this group, compared with a 6.2-fold increase in the group of cells cultured at $1x10^6$/ml and a 7.6-fold increase in those cells cultured at $0.5x10^6$/ml. There was no clear difference in expansion between these two lower concentration groups.

In pro-apoptotic conditions there did not appear to be any clear difference or trend in terms of protection from apoptosis between the groups cultured at the three different cell concentrations during the 7-day period (*Figure 3.12b*). After seven days, 22% of the cells cultured at $0.5x10^6$/ml remained viable, 43% of those cultured at $1x10^6$/ml and 35% of those cultured at $2x10^6$/ml.

3.12 Bcl-X_L expressing peripheral blood lymphocytes are resistant to apoptosis induced by co-culture with tumour cells.

Peripheral blood lymphocytes were isolated from a healthy donor, activated on immobilised anti-CD3/anti-CD28 antibodies (iCD3/iCD28) for 48 hrs and transduced (methods 2.2.6.4 and 2.2.6.6.2). Post transduction, cells were expanded on iCD3/iCD28 for 72hrs and subsequently in IL-2 alone for 7-10 days in order to obtain an adequate cell number. Unsorted Bcl-X_L-transduced and control B1.8-transduced lymphocytes (both approximately 15% GFP positive) were then co-cultured with 293T embryonal kidney and HeLa cervical tumour cells over 13 days (see methods 2.2.6.8). On days 0, 2, 7 and 13, cells from each group were stained with annexin V and the cell viability probe 7-AAD, and analysed by triple-colour flow cytometry. *Figure 3.13a+b* shows the percentage of live GFP positive cells in each group over time, with the same data shown as a log plot to illustrate the rate of cell death in *Figure 3.13c+d*. These figures were calculated by gating on the GFP positive cells ($>10^2$, *Figure 3.13e*) in order to exclude tumour cells. Within this population, cells that stained with either Annexin V or 7-AAD were classed as apoptotic and the percentage of remaining live cells was calculated (*Figure 3.13f*).

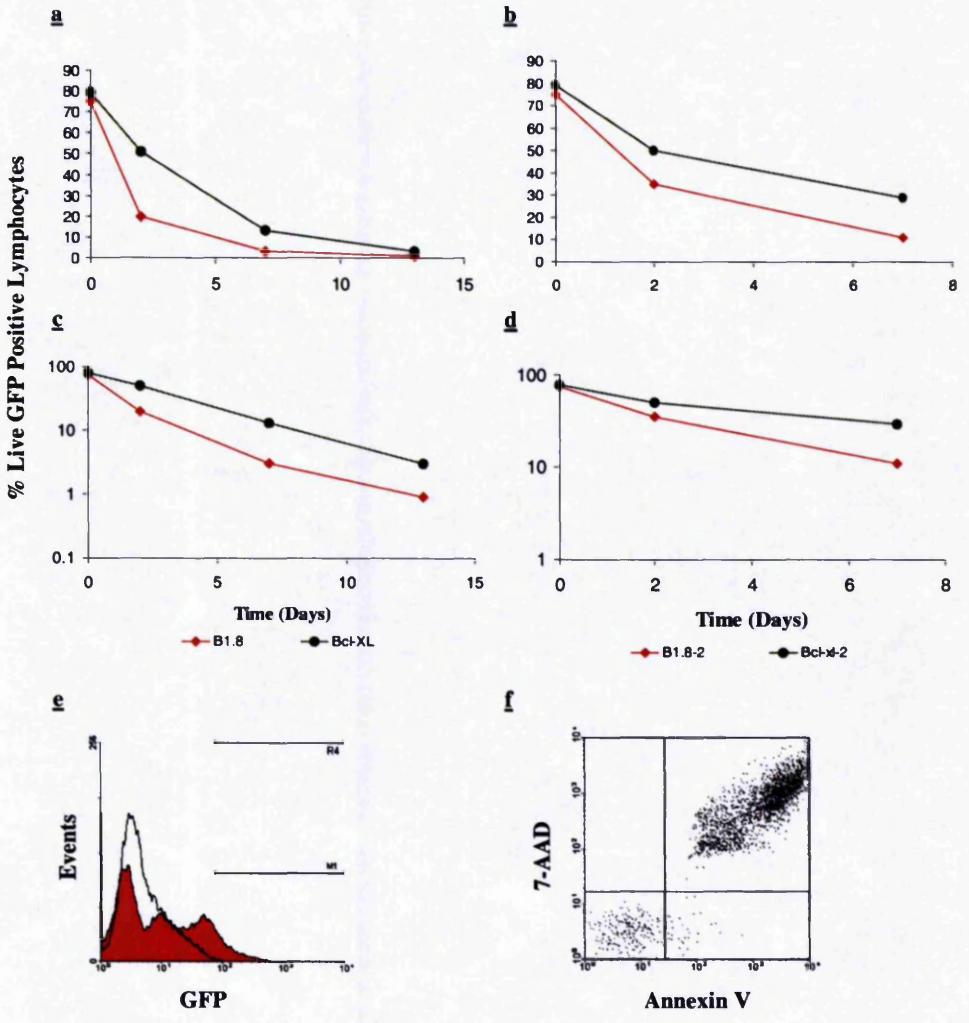


Figure 3.13 Bcl-X_L Expressing Peripheral Blood Lymphocytes are Resistant to Apoptosis Induced by Co-culture with Tumour Cells - Bcl-X_L, and control, B1.8 transduced lymphocytes ($2x10^6$ cells/ml) were co-cultured with 293T and HeLa tumour cell lines in the absence of IL-2. On days 0, 2, 7 and 13 aliquots of $2x10^5$ cells from each group were stained with PE-conjugated Annexin V and the viability probe 7-AAD, and analysed by triple-colour flow cytometry. *Figure 3.13a-d* shows the percentage of live GFP positive cells in each group over time. This figure was calculated by gating on the GFP positive cells (*Figure 3.13e*). Within this population, cells that stained with either Annexin V or 7-AAD were classed as apoptotic and the percentage of remaining live cells was calculated (*Figure 3.13f*). *Figure 3.13a+c* show results of co-culture on HeLa cells, with *Figure 3.13b+d* results for 293T cells. *Figure 3.13c+d* show log plots to assess rate of cell death. This experiment was repeated with similar results obtained.

Lymphocyte viability diminished in both groups over time. *Figure 3.13a-d* illustrates that, when cultured on both 293T and HeLa tumour cells, the rate of decrease in the percentage of viable GFP positive cells was clearly reduced in the Bcl-X_L over-expressing population when compared with the control B1.8 transduced population. For example, after 48hrs of co-culture on Hela cells (*Figure 3.13a*), 50% of Bcl-X_L over-expressing lymphocytes remain viable, compared with only 20% of control B1.8 transduced lymphocytes. With longer-term culture this difference is maintained. After 7 days 13% of Bcl-X_L over-expressing lymphocytes remained viable, compared with only 3% of control B1.8 transduced lymphocytes. Similar results were obtained with co-culture on 293T cells. After 7 days of co-culture (*Figure 3.13b*), 29% of Bcl-X_L over-expressing lymphocytes remained viable, compared with only 11% of control B1.8 transduced lymphocytes.

In addition the same assay was repeated (over a 48-hour time period) with both groups cultured in IL-2 (100u/ml) (*Figure 3.14*). Again, the Bcl-X_L transduced lymphocytes appear protected from apoptosis with the percentage of viable cells falling from 88% to 82%, compared with the control B1.8 transduced cells where the proportion fell from 72% to 33% viable after 48hours co-culture on HeLa cells (*Figure 3.14*).

In order to clarify a possible mechanism by which the HeLa and 293T cells could be causing lymphocyte apoptosis, the level of tumour cell Fas ligand expression was quantified by staining the tumour cells with a mouse anti-human Fas ligand antibody (NOK-1). Two alternative secondary antibody approaches were then used; a FITC-conjugated anti-mouse antibody and an anti-mouse biotin antibody followed by PE-Cy7-conjugated streptavidin (*Figure 3.15*). Cells were then analysed by flow cytometry. Using either approach there was no evidence of Fas ligand expression on the surface of HeLa cells. A small proportion (approximately 5%) of 293T cells expressed Fas ligand when staining with the biotin-streptavidin secondary antibodies (*Figure 3.15b*), although the alternative approach did not confirm this.

If true, these results would indicate that the lymphocyte apoptosis seen in these experiments is not likely to be mediated through the Fas/Fas ligand pathway. The interpretation of these results however, is limited by the lack of a positive control population. Another possible explanation was that rapid cleavage of Fas ligand from the cell surface of the tumour cells occured as has been described (Schneider, Thome et al. 1997). One way to investigate this further would be to repeat this experiment in the

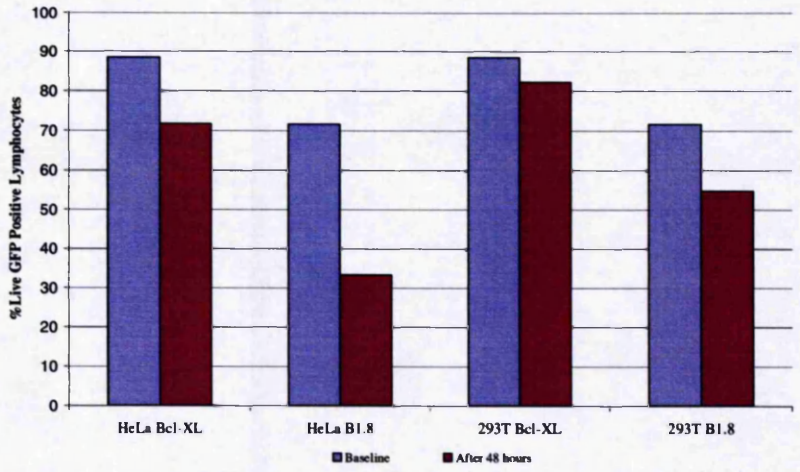


Figure 3.14 Bcl-X_L Expressing Peripheral Blood Lymphocytes are Resistant to Apoptosis Induced by Co-culture with Tumour Cells in the presence of IL-2 - Bcl-X_L, and control, B1.8 transduced lymphocytes ($2x10^6$ cells/ml) were co-cultured with 293T and HeLa tumour cell lines with IL-2 (100iu/ml). At baseline and after 48hrs, aliquots of $2x10^5$ cells from each group were stained with PE-conjugated Annexin V and the viability probe 7-AAD, and analysed by flow cytometry. *Figure 3.14* shows the percentage of live GFP positive cells in each group at baseline and after 48hrs. This figure was calculated in the same way as *Figure 3.13* by gating on the GFP positive cells (see *Figure 3.13e*). Within this population, cells that stained with either Annexin V or 7-AAD were classed as apoptotic and the percentage of remaining live cells was calculated (see *Figure 3.13f*).

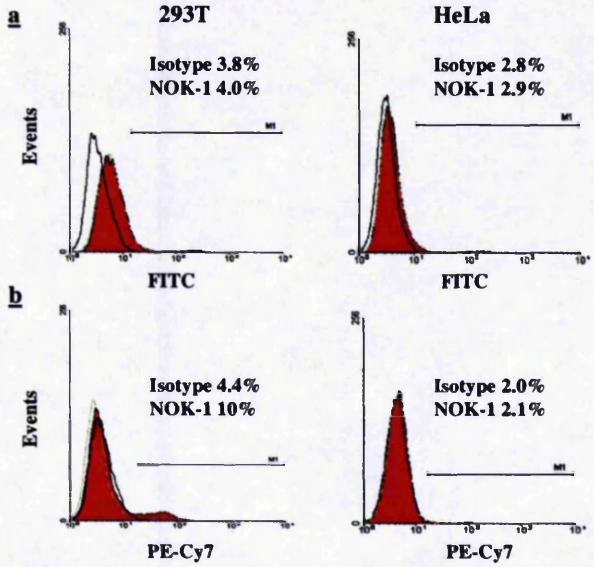


Figure 3.15 293T and HeLa cell lines – Fas ligand expression - Tumour cell Fas ligand expression was quantified by staining 293T and HeLa cells with a mouse anti-human Fas ligand antibody (NOK-1). Two alternative secondary antibody approaches were then used; a straight FITC-conjugated anti-mouse antibody (*Figure 3.15a*) and an anti-mouse biotin antibody followed by PE-Cy7-conjugated streptavidin (*Figure 3.15b*). Cells were then analysed by flow cytometry. Sample histogram is solid red with control cells black line and isotype antibody labelled cells green.

presence of a metalloproteinase inhibitor and examine whether this increases levels of Fas ligand expression. Another alternative would be to repeat the tumour cell co-culture experiment in the presence of the Nok-1 antibody to see if this resulted in a reduced level of T cell apoptosis.

3.13 Bcl-X_L expressing peripheral blood lymphocytes are not tumourigenic in an in vivo NOD/SCID model

One concern is that the over-expression of Bcl-X_L in lymphocytes may result in the promotion of malignancy or induction of autoimmunity. In order to study this, unsorted Bcl-X_L over-expressing human lymphocytes were injected intraperitoneally into NOD/SCID mice ($7.5x10^6$/mouse, n=5) daily for five days. Flow cytometry analysis was performed prior to injection in order to examine the phenotype of the injected cells (*Figure 3.16a*). This indicated that 34% of cells were GFP positive (ie expressing the rKat.Bcl-X_L.IRES.GFP vector). Transduced cells were 81% CD8+ve, 10% CD4+ve and 11% CD25+ve. In addition, western blot analysis of transduced cells was performed using a monoclonal anti-Bcl-X antibody. The same membrane was then stripped (methods 2.2.5.5) and restained with an anti-CD3ζ antibody in order to quantify amount of protein loaded. The relative band intensities were then analysed by densitometry; taking account of this the level of Bcl-X_L expression in the injected cell population was approximately five-fold that of control cells (*Figure 3.16b*).

Over a 90-day observation period all animals remained healthy. Mean body weight of the group increased by 16% during the course of the study and no clinical signs of tumourigenicity or auto-immunity were identified during this period. Upon post-mortem, no evidence of tumour formation or any other abnormalities were found. Splenocytes and bone marrow cells were also harvested from freshly killed animals (methods 2.2.6.5 and 2.2.7.1) and analysed by flow cytometry after staining with an anti-human CD3 antibody to check for any evidence of survival or proliferation of Bcl-X_L expressing human T cells. *Figure 3.16c+d* illustrates that no evidence of this was detected with anti-human CD3 staining levels similar to background isotype levels.

Foot Note - this animal model was performed at Covance Laboratories Ltd (Harrogate, UK). Lymphocyte transduction, pre-injection flow cytometry and analysis of necropsy samples, however, were carried out in the Department of Medical Oncology, PICR.

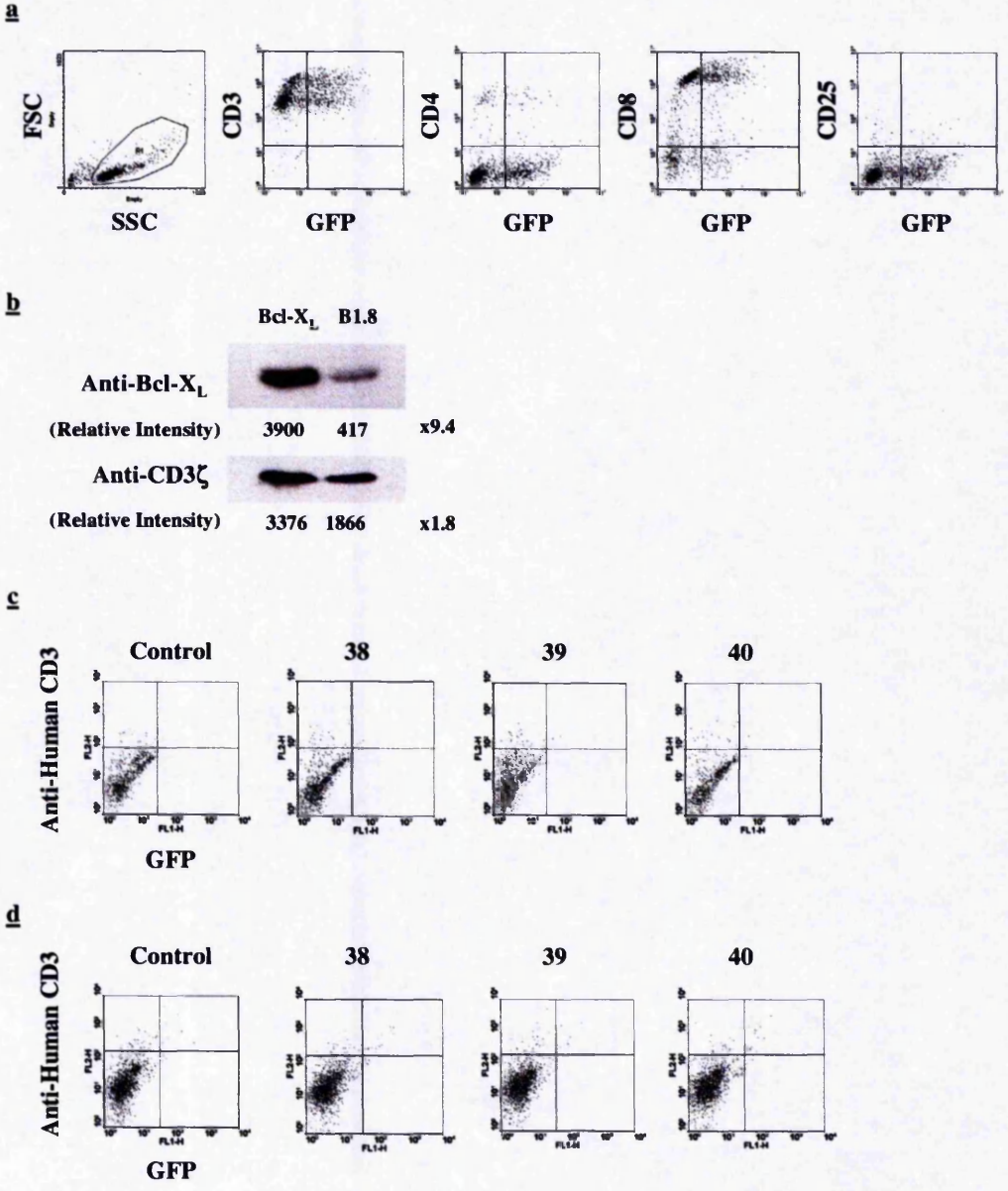


Figure 3.16 In vivo Bcl-X$_L$ safety model – (a) Cell surface markers of T cells on day of injection: cells were 86% viable by live FSC vs SSC gate, 99.8%CD3 +ve, 34.2% GFP +ve, 10% CD4+ve, 81% CD8+ve and 11% CD25+ve, (b) Western Blot analysis of transduced cells used in animal model. After staining with a monoclonal anti-Bcl-X antibody, the same membrane was stripped (methods 2.2.5.5) and restained with an anti-CD3ζ antibody in order to quantify amount of protein loaded. The relative band intensities were analysed using Labworks software; taking account of this the level of Bcl-X$_L$ expression was approx five-fold that of control cells. Splenocytes (c) and bone marrow cells (d) were also harvested from freshly killed animals and analysed by flow cytometry after staining with an anti-human CD3 antibody to check for any evidence of surviving human T cells. Representative samples from a control and three injected mice (38, 39 and 40) are shown.

3.14 Discussion

In this chapter, an attempt has been made to modulate the resistance of cells to apoptosis and improve survival by transducing Jurkat cells, and subsequently human peripheral blood lymphocytes using a retroviral vector which expresses Bcl-X_L linked via an internal ribosome entry site to the marker green fluorescent protein. It was shown that Bcl-X_L expression in unselected Jurkat cells can give a degree of protection against Fas antibody induced apoptosis (*Figure 3.8*). Subsequent *in vitro* assays using transduced primary human lymphocytes clearly demonstrated that over-expression of Bcl-X_L promotes the survival, but not proliferation of transduced lymphocytes deprived of cytokines such as interleukin-2, with or without the AICD inducing anti-$CD3_\varepsilon$ antibody, OKT3 (*Figures 3.10-11*). Furthermore, Bcl-X_L over-expression in human lymphocytes also decreased, but did not prevent apoptosis induced by long-term co-culture with the HeLa and 293T tumour cell lines (*Figures 3.13-14*). In a clinical setting this simple approach could potentially improve gene-modified cell survival and reduce the requirement for additional systemic IL-2 and the severe toxicity with which this may be associated (Rosenberg, Lotze et al. 1989).

The control of lymphocyte death/apoptosis is clearly vital to the regulation of the immune system. Too much apoptosis may result in immunodeficiency and a risk from infection, too little in autoimmune disease. Lymphocyte apoptosis commonly occurs by one of two major mechanisms. As alluded to above, a lack of antigen-specific receptor stimulation can result in lymphocytes not producing vital cytokines that are required for survival and dying by 'neglect'. Alternatively, a more active mechanism involves stimulation of 'death receptor' pathways such as the CD95 (Fas) receptor with its natural ligand CD95L culminating in the death of the cell by apoptosis (Krammer 2000).

Both of these mechanisms may play a part in the poor *in vivo* survival of gene-modified lymphocytes in adoptive cancer therapy. Systemic exogenous administration of IL-2 has been shown to potentiate the anti-tumour effect of TILs (Rosenberg, Spiess et al. 1986) in murine models and the mechanism by which this occurs is thought to be the maintainenance of growth and viability of the re-infused cells. Indeed, in recent work, Liu and Rosenberg have demonstrated that human melanoma-reactive lymphocytes transduced with the IL-2 gene can grow in the absence of exogenous IL-2 whilst maintaining specific antitumour activity (Liu and Rosenberg 2001). Natural killer-like T

lymphocytes transfected with IL-2 have also been shown to have significantly higher cytotoxic activity *in vitro* and are under investigation in phase I human trials (Schmidt-Wolf, Finke et al. 1999). However, in contrast, in natural immune responses, IL-2 serves a dual function: although mandatory for initial clonal expansion, the presence of IL-2 in the later phases of the immune response results in T cells becoming progressively more sensitive to apoptosis (Van Parijs, Refaeli et al. 1999) (see section 5.1 and *Figure 5.2*). Therefore reducing the requirement for exogenous IL-2 during adoptive cellular therapy (by over-expression of Bcl-X_L) could not only reduce potential side-effects, but also in itself render T cells more resistant to apoptosis.

Expression of Fas ligand (CD95L) on tumour cells leading to stimulation of lymphocyte death receptor pathways has been proposed as a mechanism of 'tumour escape' from immune control (Hahne, Rimoldi et al. 1996). More recent work however suggests that rather than lymphocytes undergoing apoptosis following contact with tumour Fas ligand, it is more likely that the death receptor signalling results from what is known as activation-induced cell death (AICD) (see section 5.1). Lymphocyte TCR activation (in the absence of sufficient co-stimulation) leads to expression of CD95L on the T cell surface and results in the cell eliminating nearby CD95 positive T cells (Zaks, Chappell et al. 1999) (Krammer 2000).

A role for Bcl-X_L in the prevention of death receptor induced apoptosis is controversial with some groups suggesting that Bcl-X_L over-expression has no impact on cell death induced by Fas antibodies even though it efficiently inhibits apoptosis induced in other ways (eg cytokine withdrawal, or irradiation). Other groups (Huang, Hahne et al. 1999) have found that only membrane-bound CD95L reliably induces apoptosis, independently of the Bcl-2 genes, whereas results using CD95 antibodies are variable. Activation of death receptors results in the formation of a death-inducing signalling complex (DISC) on the cell membrane. This links with the fas-associated death domain (FADD) and results in proteolytic activation of caspase 8. Caspase 8 can rapidly activate caspase 3 and trigger a cascade of protease activation that results in the apoptosis of the cell. However, the amount of caspase 8 activated is often insufficient to cause the caspase cascade, and an amplification loop is required via the mitochondria. Caspase 8 activates the bcl-2 family member Bid. This causes mitochondria to release pro-apoptotic molecules such as cytochrome C and Apaf 1, which in turn trigger the caspase cascade and the apoptosis of the cell (see *Fig 5.2*). Bcl-X_L, is thought to prevent apoptosis by stabilising the mitchondrial membrane thus preventing release of

molecules such as cytochrome C. In this model (Krammer 2000), lymphocytes not requiring mitochondrial amplification for death receptor apoptosis (Type I cells) are unlikely to benefit from Bcl-X_L over-expression as a way of preventing it. In more resistant T cells (Type II) however, blocking mitochondrial amplification by Bcl-X_L over-expression can prevent death receptor-induced apoptosis. It is likely that during the immune response the shift that is seen from apoptosis resistance to sensitivity corresponds with a shift from a Type II to a Type I phenotype. In view of this in Chapter 5, the co-delivery of an alternative anti-apoptotic gene, cFLIP, to transduced lymphocytes as a potentially more efficient way of protecting cells from death receptor induced apoptosis was studied.

Pirtskhalaishvili *et al* (Pirtskhalaishvili, Shurin et al. 2000) recently used an adenoviral vector to transduce Bcl-X_L into dendritic cells. Bcl-X_L over-expressing dendritic cells were shown to be more resistant to apoptosis induced by a prostatic cancer cell line. Subsequently, in a murine prostate cancer model, intra-tumoural administration of dendritic cells transduced with the Bcl-X_L gene resulted in a significant inhibition of tumour growth compared with the administration of non-transduced dendritic cells.

Unlike Bcl-2, Bcl-X_L expression has not been reported to produce malignancy. Our *in vitro* data shows no evidence of increased proliferation or clone formation following Bcl-X_L gene transfer and in an *in vivo* model NOD/SCID mice injected with large numbers of Bcl-X_L over-expressing lymphocytes on consecutive days showed no signs of ill health over a 90 day observation period. However, Bcl-X_L expression is seen in a number of malignancies (Puthier, Derenne et al. 1999; Nicot, Mahieux et al. 2000) and clearly a potential concern that would have to be fully addressed before this approach could be tested in humans is the risk of either tumour induction or, indeed, autoimmunity. Further, long-term *in vivo* models would be required to address such concerns and study the survival of Bcl-X_L transduced lymphocytes.

In summary, the results in this chapter indicate that expression of Bcl-X_L in therapeutic lymphocyte infusions could enhance the long-term survival and persistence of transduced cells in vivo, either by reducing apoptosis caused by cytokine deprivation, or by reducing AICD. This novel physiological approach has the potential to enhance the clinical outcome of gene-modified (eg chimeric T cell receptor (Gross, Waks et al. 1989)) T cell therapy, the adoptive transfer of specific T cells and, in the allogeneic setting, donor lymphocyte infusions.

The next stage of this work was to study the functional effects of Bcl-X_L expression when combined in retroviral vectors with other therapeutic genes. This work is described in Chapter 4.

4 Results – Co-expressing pro-survival genes with other genes

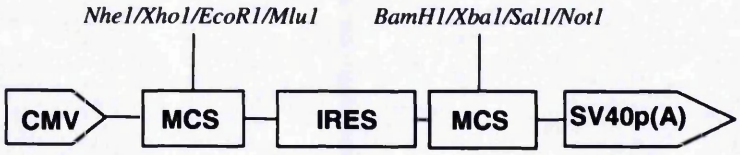
4.1 Introduction

The experiments performed in Chapter 3 demonstrate that, at least in *in vitro* systems, transducing lymphocytes with an anti-apoptotic gene such as Bcl-X_L has the potential to improve the survival of the transduced cell. Transducing cells to express an anti-apoptotic protein alone could potentially improve clinical outcome of adoptive cellular therapy in certain situations, such as the use of donor lymphocyte infusions to treat relapsed leukaemias, or by improving the survival of antigen-specific T cell clones (see Chapter 7) or tumour-infiltrating lymphocytes. However, the process of genetic modification itself could potentially impair the function of such cells (Sauce, Bodinier et al. 2002) thereby negating any benefits seen from survival gene expression.

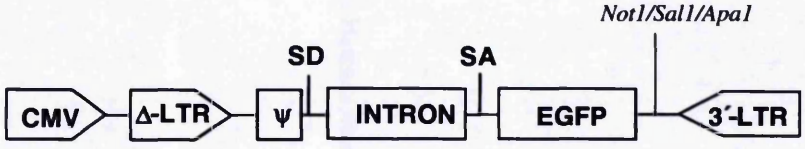
An alternative application, and the major focus of this work, would be to attempt to improve the survival of lymphocytes already undergoing retroviral genetic modification. This could potentially be done by constructing a retroviral vector that co-expressed a survival gene such as Bcl-X_L in addition to the main gene being used to modify the cell; for example, in conjunction with a chimeric T cell receptor gene (Eshhar, Waks et al. 1993) used to target lymphocytes against cancer (see section 1.2.5 and Chapter 7).

Currently there are two main methods through which heterologous gene products can be expressed in a single vector. Separate independent promoters can be used, but this can result in promoter interference (ie transcription from one promoter suppressing the transcription from another) (Emerman and Temin 1984). An alternative is the use of internal ribosome entry site (IRES) sequences. IRES sequences allow the initiation of translation in a cap-independent manner: ribosomes bind internally at the initiating AUG without scanning the 5' nontranslated region of the transcript (Mountford and Smith 1995). This allows the efficient expression of two genes from the same promoter, eliminating problems of promoter interference. IRES sequences derived from the 5' nontranslated regions of the encephalomyocarditis (EMCV) genome (such as that found in the pIRES vector, Clontech, California, USA) have been shown to have higher translation efficiency than other IRES sequences and are functional in most cell types.

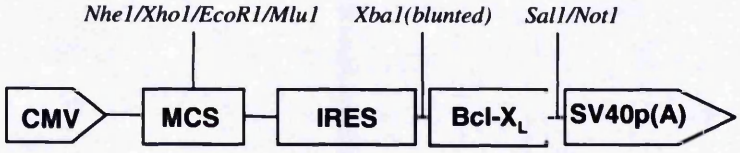
4.1a. pIRES



4.1b. rKat.GFP



4.1c. pIRES.Bcl-X_L



4.1d. rKat.GFP.IRES. Bcl-X_L

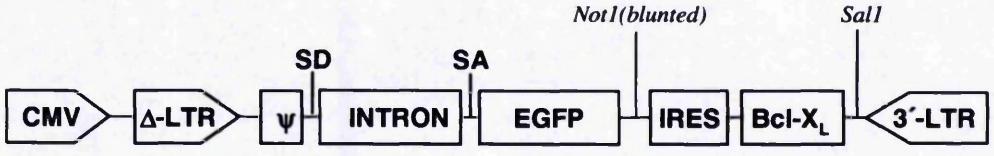


Figure 4.1 – Construction of rKat.GFP.IRES.Bcl-X_L – a) Basic pIRES vector containing multiple cloning site either side of IRES, b) rKat.EGFP vector, c) pIRES.Bcl-X_L vector, d) rKat vector expressing GFP and Bcl-X_L. ψ = packaging signal, SD, SA are splice donor and splice acceptor sites respectively. MCS, multiple cloning site.

As a consequence they are widely used in gene transfer research (Mountford and Smith 1995).

Studies of IRES-dependent second gene expression have generally shown it to be significantly lower than cap-dependent first gene expression. One recent publication studying different gene/target cell combinations, both *in vitro* and *in vivo,* demonstrated variable second gene expression ranging from 6% to 100% that of the first gene (although in most cases it ranged between 20 and 50%) (Mizuguchi, Xu et al. 2000).

Clearly this was an important consideration when designing a bicistronic retroviral vector to co-express Bcl-X_L with a chimeric T cell receptor. The functional effects of chimeric T cell receptor expression had so far only been studied (by other members of the Dept of Medical Oncology) using vectors expressing the chimeric receptor as the first gene in a bicistronic construct, but similarly this was also the case with studying the effects of Bcl-X_L expression (see Chapter 3). In terms of modifying T cell function overall it was considered likely that maximal chimeric T cell receptor expression would be important in achieving an anti-tumour effect. Consequently, in this chapter a vector was constructed that expressed Bcl-X_L as the second gene in a bicistronic vector (rKat.GFP.IRES.Bcl-X_L). The relative expression of Bcl-X_L in cells transduced using this vector when compared with cells transduced using rKat.Bcl-X_L.IRES.GFP was then studied, and similar functional assays to those done in Chapter 3 were performed.

4.2 Construction of retroviral vector rKat.EGFP.IRES.Bcl-X_L

The original reverse pcr primer (BCL-XL-REVR, section 2.1.3) used to amplify Bcl-X_L from PBL cDNA contained an error. The intention had been to include restriction sites *Mlu1* and *Sal1* 3' to Bcl-X_L in order to facilitate future sub-cloning. In fact, both sites were inserted in reverse orientation. Fortunately, the reverse of *Mlu1* is *Fsp1* and since this restriction site was not present in the Bcl-X_L cDNA, the cloning of rKat.Bcl-X_L.IRES.EGFP could proceed as described (section 3.2-3.3). However, in order to insert Bcl-X_L into the rKat vector beyond the IRES, a repeat reverse primer was designed (BCL-XL-REV2, section 2.1.3) containing *Mlu1* and *Sal1* as originally planned. Using the new reverse primer Bcl-X_L was amplified from the rKat.Bcl-X_L.IRES.EGFP vector plasmid and again cloned into the TOPO TA^{TM} vector (Invitrogen). After a diagnostic digest using *EcoR1* (the TA vector contains *EcoR1* sites either side of the pcr insert), five miniprep clones were sequenced to confirm the presence of the restriction sites and exclude any replication errors and an appropriate clone was selected.

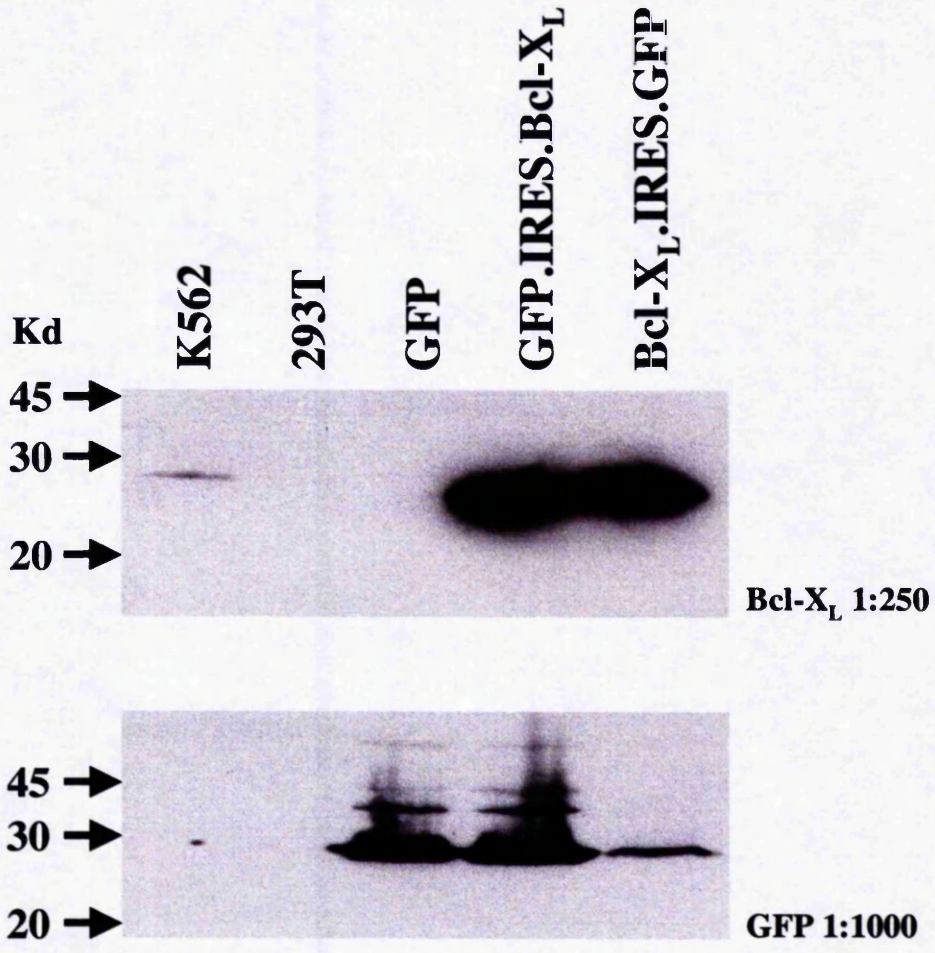


Figure 4.2 Western Blots for Bcl-X_L and GFP Expression in Transfected 293T cells - 293T cells were transiently transfected by calcium phosphate co-precipitation (methods 2.2.6.2.1) using the retroviral vectors rKat.GFP, rKat.GFP.IRES.Bcl-X_L and rKat.Bcl-X_L.IRES.GFP. 72hrs later cells were lysed, separated by SDS/PAGE (12% gel, 100µg/lane) and expression of Bcl-X_L was assessed by western blot using a mouse monoclonal anti-Bcl-X_L antibody at dilution of 1:250 (Pharmingen) with an anti-mouse HRP secondary antibody (1:2000 dilution) (upper blot). The gel was run in duplicate and the second membrane was developed using an anti-GFP HRP antibody (1:1000 dilution)(lower blot). The expected molecular weights of Bcl-X_L and GFP protein are 28 and 27 kD, respectively. For comparison lysates of the K562 and untransfected 293T cell lines are also shown.

pIRES (*Figure 4.1a*) is a mammalian expression vector containing multiple cloning sites (MCS) either side of an internal ribosome entry site (IRES) (derived from the ECMV genome) allowing high level expression of two genes of interest (see section 4.1). As a first step the vector pIRES.Bcl-X_L was cloned (*Figure 4.1c*). Bcl-X_L cDNA cut out of the TA vector *EcoR1(blunted)/Sal1* was inserted into pIRES cut *Xba1(blunted)/Sal1*. An IRES.Bcl-X_L fragment was then cut out of pIRES.Bcl-X_L *Xho1(blunt)/Sal1* and cloned into rKat.GFP (*Figure 4.1b*) that had been cut *Not1(blunt)/Sal1*, making the vector rKat.GFP.IRES.Bcl-X_L (*Figure 4.1d*).

4.3 Western blot for Bcl-X_L expression in Bcl-X_L-transfected 293T cells

293T cells were transfected using the rKat.GFP.IRES.Bcl-X_L vector, along with rKat.GFP and rKat.Bcl-X_L.IRES.GFP vectors for comparison (methods 2.2.6.2). Forty-eight hours post transfection 293T cells were lysed and separated by SDS/PAGE (12% gel, 100µg/lane) in duplicate. The expression of both Bcl-X_L and GFP was assessed by separate western blots using a mouse monoclonal anti-Bcl-X antibody (Pharmingen) and an anti-GFP HRP antibody, respectively (methods 3.2.5) (*Figure 4.2*).

The results of these blots (*Figure 4.2*) confirmed that the rKat.GFP.IRES.Bcl-X_L vector was functional following transfection of the vector into 293T cells. Comparable intensity anti-Bcl-X_L antibody detected immunoreactive bands are seen at the expected molecular weight of wild-type Bcl-X_L (28kD) in both Bcl-X_L.IRES.GFP-transfected and GFP.IRES.Bcl-X_L-transfected 293T cells with no evidence of expression in the GFP-transfected or non-transfected 293T cells. The anti-GFP stained membrane shows bands at the expected molecular weight of GFP (27kD) in all three vector-transfected lysates. In this case expression of GFP appeared reduced in cells transfected by the rKat.Bcl-X_L.IRES.GFP vector in which GFP expression was IRES-dependent rather than cap-dependent (in contrast to BCl-X_L expression which appeared similar).

4.4 Human lymphocyte transduction and Bcl-X_L expression with IRES.Bcl-X_L vector

Human peripheral blood lymphocytes (PBL) were obtained from a healthy donor and pre-activated on immobilised anti-CD3 and anti-CD28 monoclonal antibodies with IL-2 (30u/ml) for 48hours as previously described (materials and methods 2.2.6.4).

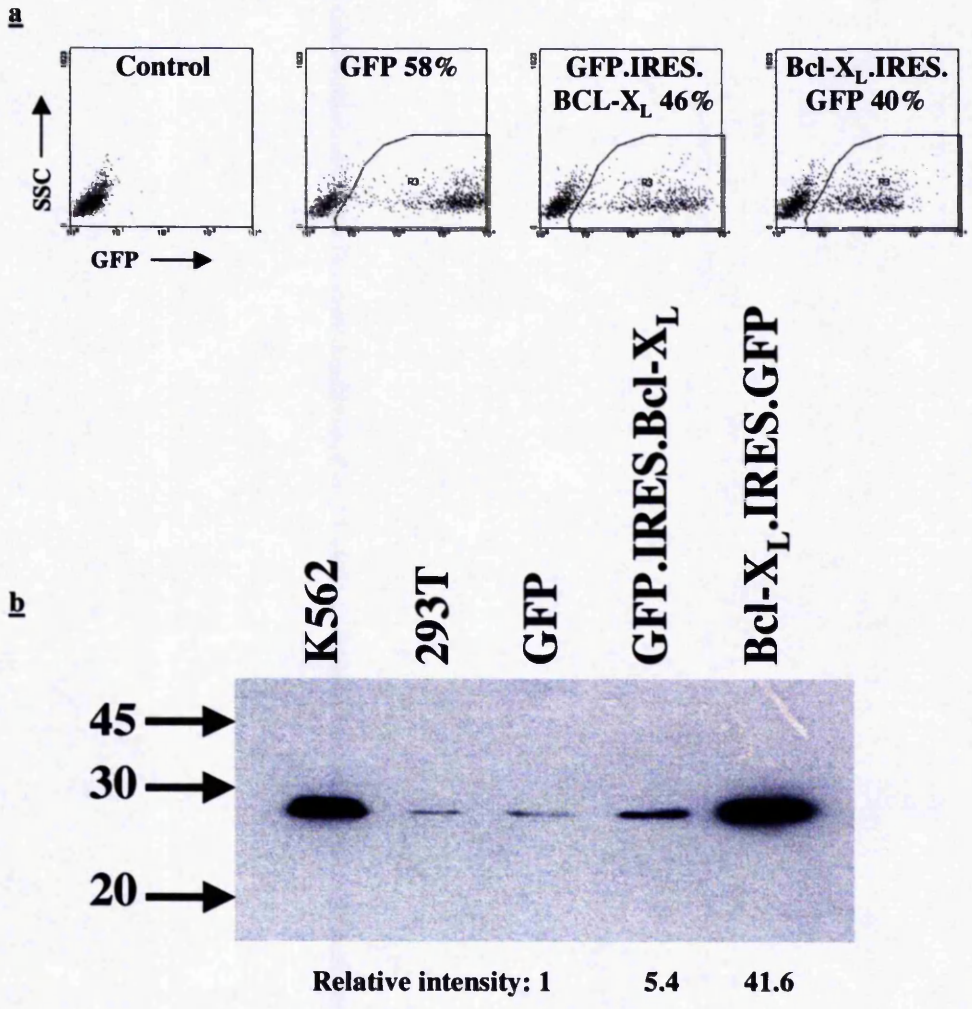


Figure 4.3 – Human lymphocyte transduction and Bcl-X_L expression using IRES.Bcl-X_L vector - rKat.GFP, rKat.GFP.IRES.Bcl-X_L and rKat.Bcl-X_L.IRES.GFP retrovirus was used to transduce T cells and GFP expression was analysed by flow cytometry (*Figure 4.3a*). D+4 cells lysed. Proteins (10^6 cells/lane) subjected to SDS/PAGE and Western blot analysis with a monoclonal anti-Bcl-X antibody (Pharmingen) (*Figure 4.3b*). The Bcl-X_L -expressing K562 cell line (100µg) is shown as a positive control. Quantified relative image intensity was calculated using Labworks™ software.

rKat.GFP, rKat.GFP.IRES.Bcl-X_L and rKat.Bcl-X_L.IRES.GFP retrovirus was again made by transient 293T transfection, and a spin transduction method (methods 2.2.6.6.2) was then used to transduce lymphocytes. 72 hours later, transduction efficiency as measured by GFP expression was analysed by flow cytometry (*Figure 4.3a*). Efficiencies of 58%, 46% and 40% respectively were achieved in the rKat.GFP, rKat.GFP.IRES.Bcl-X_L and rKat.Bcl-X_L.IRES.GFP groups. As anticipated, there was a noticable difference in mean fluorescence intensity (MFI) of GFP expression between the three groups of transduced cells. Measured MFI was 1854, 534 and 124 in the respective rKat.GFP, rKat.GFP.IRES.Bcl-X_L and rKat.Bcl-X_L.IRES.GFP groups indicating lower levels of expression when the GFP gene was combined in a bicistronic construct as the cap-dependent gene and lower still when expressed as the IRES-dependent second gene.

The remaining cells were lysed in RIPA buffer. Protein lysates (10^6 cells/lane) were subjected to SDS/PAGE and Western blot analysis with a monoclonal anti-Bcl-X antibody (Pharmingen) (*Figure 4.3b*).

Of immediate note is the increased intensity of the immunoreactive band seen in the lane loaded with the positive control K562 lysate when compared with *Figure 4.2* despite equal protein loading. This is likely to be a reflection of the different levels of protein expression between 293T cells and lymphocytes and represents differing exposure periods between the two blots.

The relative image intensity of the immunoreactive bands was quantified using Labworks™ software. In contrast to the Bcl-X_L protein expression seen in transfected 293T cells, in transduced human lymphocytes IRES-dependent Bcl-X_L expression (after transduction with rKat.GFP.IRES.Bcl-X_L), although fivefold higher than in control rKat.GFP transduced cells, was only 13% that of cap-dependent Bcl-X_L expression (following transduction with rKat.Bcl-X_L.IRES.GFP). This was despite relatively equivalent transduction efficiencies. This reduced IRES-dependent gene expression compared with cap-dependent expression of Bcl-X_L was very similar (but the reverse of) the changes in MFI of GFP expression demonstrated by flow cytometry of the three groups of cells.

In sections 4.5-4.6 we examined whether this significantly reduced degree of Bcl-X_L expression was sufficient to reproduce the functional effects demonstrated in Chapter 3.

4.5 *In Vitro* Survival Assay

In this experiment (and the tumour co-culture experiment described in section 4.6) the ability of lymphocytes to resist apoptosis when over-expressing Bcl-X_L as the second gene in a bicistronic construct (rKat.GFP.IRES.Bcl-X_L) was tested in an *in vitro* assay and compared with lymphocytes modified to express Bcl-X_L as the first gene (rKat.Bcl-X_L.IRES.GFP) and non-Bcl-X_L expressing gene-modified lymphocytes (rKat.GFP).

Peripheral blood lymphocytes isolated from a healthy donor were transduced with retroviral vectors rKat.GFP, rKat.Bcl-X_L.IRES.GFP and rKat.GFP.IRES.Bcl-X_L (methods 2.2.6.4 and 2.2.6.6). Post transduction, cells were expanded in IL-2 (100u/ml) for 72hrs. Transduction efficiencies of 24-44% were achieved as measured by flow cytometry analysis of GFP expression (*Figure 4.4a*). As before transduced lymphocytes were cultured for 17 days ($1x10^6$ cells/group), either in T cell media alone (*Figure 4.4d)* or with the anti-CD3 antibody (OKT3, Orthoclone) (ie pro-apoptotic conditions) (*Figure 4.4c*). On days 0, 10 and 17 aliquots of cells were stained with a PE-conjugated Annexin V antibody and the viability marker 7-AAD and analysed by triple-colour flow cytometry. The percentage of GFP positive cells viable by Annexin V/7-AAD exclusion from each group was quantified at each time point (*Figure 4.4b* shows density plots for days 0 and 10 illustrating how this was calculated).

As in the similar experiments performed in Chapter 3, the overall number of viable GFP positive cells decreased in all 3 groups over the 17 days. At day 0 (ie 72 hours post transduction) there was a higher percentage of viable cells in the rKat.Bcl-X_L.IRES.GFP and rKat.GFP.IRES.Bcl-X_L transduced populations (92% and 86% respectively), compared with the control rKat.GFP transduced cells (72%). When cells were cultured in T cell media alone (*Figure 4.4d*) again the viability of the rKat.Bcl-X_L.IRES.GFP transduced cells decreased at a lower rate than the control rKat.GFP transduced cells. For example after 10 days, 43% remained viable by Annexin V/7-AAD exclusion compared with only 13% of control rKat.GFP transduced cells. Unfortunately, the viability of the rKat.GFP.IRES.Bcl-X_L transduced cells was not similarly sustained. By day 10 only 16% remained viable, suggesting that the amount of protein produced by IRES-dependent Bcl-X_L expression may not be sufficient to achieve a functional effect in preventing apoptosis.

When cells were cultured in T cell media and OKT3, the difference in rate of decline in viability of transduced cells appeared less obvious. It was noticable however, that at

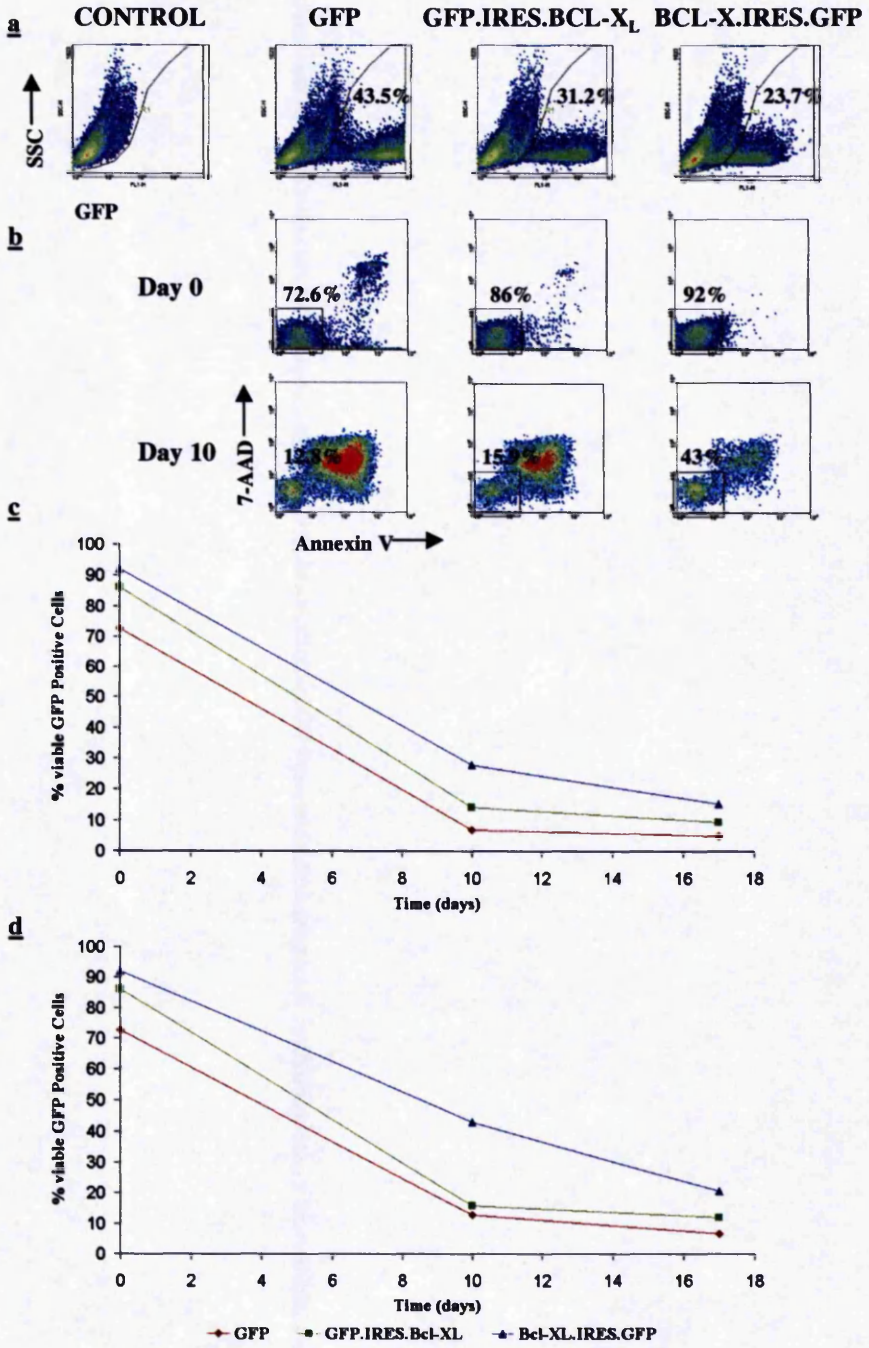


Figure 4.4 – In Vitro Survival assay - Transduced lymphocytes (*Figure 4.*4a) were cultured for 17 days (1x10^6 cells/group), either in T cell media alone or with the anti-CD3 antibody (OKT3, Orthoclone) (ie pro-apoptotic conditions). On days 0, 10 and 17 aliquots of cells were stained with a PE-conjugated Annexin V antibody and the viability marker 7-AAD and analysed by triple-colour flow cytometry. The percentage of GFP positive cells viable by Annexin V/7-AAD exclusion (*Figure 4.4b*) from each group was quantified at each time point (*Figure 4.4c* shows cells cultured in T cell media and OKT3, with *Figure 4.4d* media alone).

each time point (as with *Figure 4.4d*) there was an observed 'level of expression' effect, with the highest proportion of cells viable in the rKat.Bcl-X_L.IRES.GFP group, followed by the rKat.GFP.IRES.Bcl-X_L, followed by the control rKat.GFP group with the lowest percentage of cells viable.

4.6 *In Vitro* Tumour Co-culture Assay

Peripheral blood lymphocytes isolated from a healthy donor were transduced with retroviral vectors rKat.GFP, rKat.Bcl-X_L.IRES.GFP and rKat.GFP.IRES.Bcl-X_L (methods 2.2.6.4 and 2.2.6.6). Post transduction, cells were expanded in IL-2 (100u/ml) for 72hrs. Transduction efficiencies of 22%, 18% and 25% respectively, were achieved as measured by flow cytometry analysis of GFP expression. Unsorted transduced lymphocytes were then co-cultured with HeLa tumour cells over a period of 14 days (see methods 2.2.6.8). Transduced cells were cultured either with or without IL-2 (100u/ml) in triplicate wells. On days 0, 1, 2 and 14 aliquots of cells from each group were stained with Annexin V and the cell viability probe 7-AAD, and analysed by triple-colour flow cytometry. *Figure 4.5b+c* shows the percentage of viable GFP positive cells in each group over time (*Figure 4.5b* without IL-2 and *Figure 4.5c* with IL-2). These figures were calculated by gating out HeLa cells (*Figure 4.5a, far left*) and gating on the GFP positive cells (*Figure 4.5a, middle two plots*). Within this population, cells that stained with either Annexin V or 7-AAD were classed as apoptotic (*Figure 4.5a, far right*) and the percentage of remaining live cells was calculated (*Figure 4.5b+c*).

In the absence of IL-2 (*Figure 4.5b*), rKat.Bcl-X_L.IRES.GFP transduced lymphocytes were again clearly protected from apoptosis during co-culture. This was most noticeable after 14 days when 63% of GFP positive cells remain viable compared with only 6% of rKat.GFP transduced cells. In the short term rKat.GFP.IRES.Bcl-X_L cells were also protected to a certain extent when compared with the control population. For example, after 48 hours 69% remained viable compared with 54% rKat.GFP transduced cells. However, by 14 days, the survival of rKat.GFP.IRES.Bcl-X_L cells was similar to that of the control rKat.GFP population.

Interestingly (and in contrast to the results obtained in *Figure 3.14*), when transduced lymphocytes were co-cultured with tumour cells in the presence of IL-2 (*Figure 4.5c*), the differences in viability between the three groups were much less noticeable. The survival of rKat.Bcl-X_L.IRES.GFP transduced cells was essentially unchanged, however

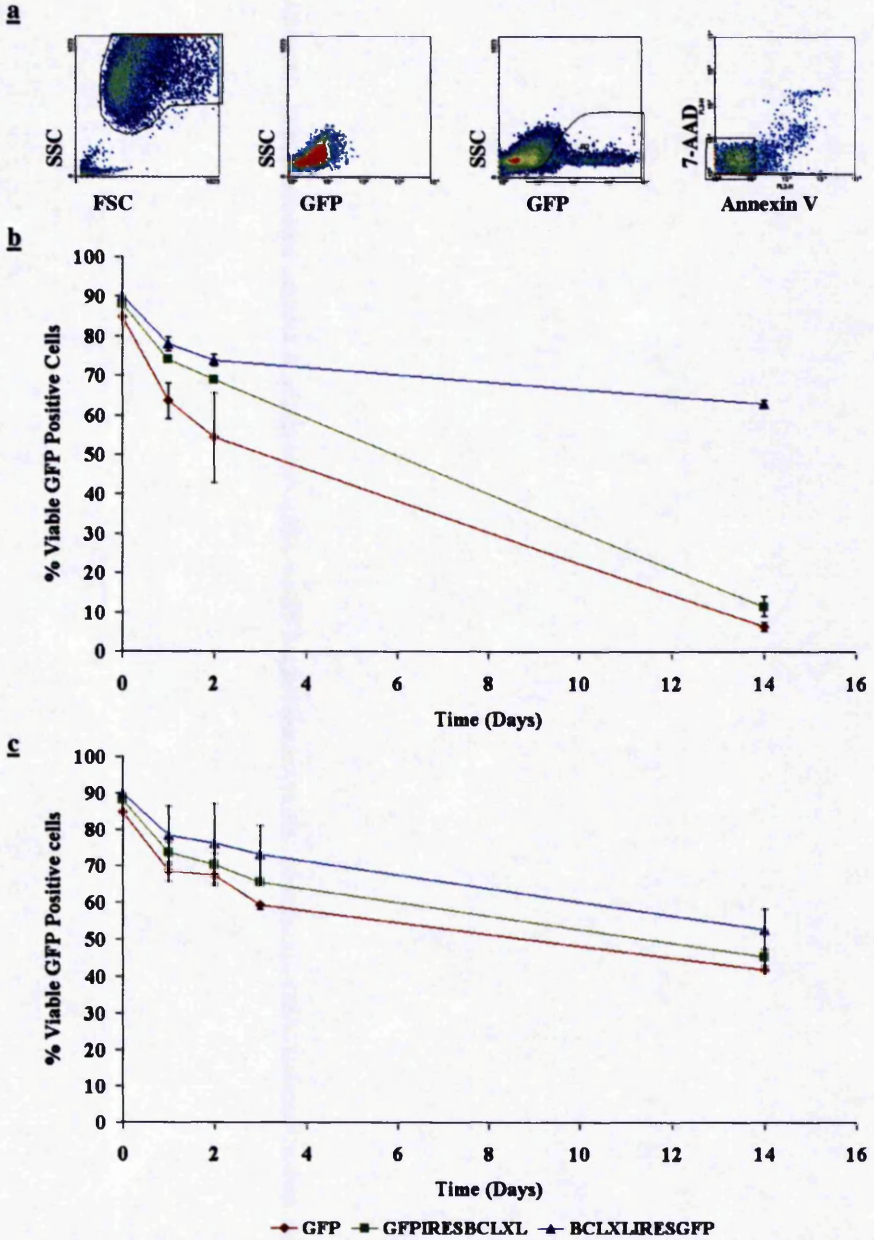


Figure 4.5 Bcl-X_L Expressing Peripheral Blood Lymphocytes are Resistant to Apoptosis Induced by Co-culture with Tumour Cells -Unsorted transduced lymphocytes were co-cultured with HeLa cells over a period of 14 days either with or without IL-2 (100u/ml) in triplicate wells. On days 0, 1, 2 and 14 aliquots of cells from each group were stained with Annexin V and the cell viability probe 7-AAD, and analysed by triple-colour flow cytometry. *Figure 4.5b+c* shows the percentage of viable GFP positive cells in each group over time (*Figure 4.5b* without IL-2 and *Figure 4.5c* with IL-2). These figures were calculated by gating out HeLa cells (*Figure 4.5a, far left*) and gating on the GFP positive cells (*Figure 4.5a, middle two plots*). Within this population, cells that stained with either Annexin V or 7-AAD were classed as apoptotic (*Figure 4.5a, far* right) and the percentage of remaining live cells was calculated (*Figure 4.5b+c*). Results shown are means (+/- standard deviation error bars) of triplicate wells.

the viability of the control rKat.GFP and the rKat.GFP.IRES.Bcl-X_L transduced populations was much improved. This would support the hypothesis that in these assays Bcl-X_L over-expression was predominantly protecting the lymphocytes from the effects of growth factor withdrawal, rather than any pro-apoptotic stimulus either secreted by or on the cell surface of the tumour cells.

Although differences in viability between the three groups of transduced cells were small (and some error bars overlapped), it remained the case that at each time point there were more viable rKat.Bcl-X_L.IRES.GFP expressing lymphocytes (eg 53%+/-5% at Day 14) compared with rKat.GFP.IRES.Bcl-X_L expressing lymphocytes (45%+/-2% at Day 14), and more rKat.GFP.IRES.Bcl-X_L expressing cells than rKat.GFP expressing cells (42%+/-0.5% at Day 14).

4.7 Discussion

In this chapter, a retroviral vector was constructed that expressed Bcl-X_L as the second gene in a bicistronic construct (rKat.GFP.IRES.Bcl-X_L) along with the marker GFP. The expression of this vector was confirmed by western blot of transfected 293T cell lysates and, subsequently, the relative expression of Bcl-X_L in human lymphocytes transduced using this vector was examined. Although rKat.GFP.IRES.Bcl-X_L transduced cell populations expressed approximately fivefold higher levels of Bcl-X_L when compared with control transduced cell populations, this level of expression was much lower than in populations of cells transduced using the rKat.Bcl-X_L.IRES.EGFP vector (where the expression levels were approximately forty-fold higher than in control transduced cell populations).

In vitro assays were then performed to assess the functional effect of IRES-dependent Bcl-X_L expression. In these assays again it was clearly demonstrated that Bcl-X_L expression can protect transduced lymphocytes from apoptosis caused by withdrawal of growth factors (such as IL-2) and (to a lesser extent) the activation induced cell death (AICD) effect of culture with the anti-CD3 antibody (OKT3). In the tumour co-culture assays Bcl-X_L expression was again protective, although experiments performed in the presence and absence of IL-2 suggested that this protection was most noticable in the absence of IL-2 with much less of an effect when cells were cultured with IL-2.

As previously discussed in section 3.14, it is not entirely clear to what extent Bcl-X_L expression can protect against death receptor-induced apoptosis such as might occur

either following CD3 stimulation (which can lead to upregulation of Fas ligand on the lymphocyte cell surface and ensuing Fas/Fas ligand interactions with neighbouring cells (Krammer 2000)), or during tumour cell co-culture (where tumour cell surface expression of death receptor ligands (Hahne, Rimoldi et al. 1996) or secretion of immunosuppressive cytokines can result in activation of death receptor pathways). The evidence presented in this chapter is consistent with this uncertainty and suggests that Bcl-X_L over-expression is most effective at preventing apoptosis due to a lack of growth factor stimulation.

The major aim of the work presented in this chapter was to examine whether the (reduced) expression of Bcl-X_L as the second gene in a bicistronic construct using an internal ribosome entry site to drive expression was sufficient to protect the lymphocyte from apoptosis. The functional *in vitro* assays performed indicate that this level of Bcl-X_L expression does result in a certain amount of protection, although this protection is far less than that achieved in cells transduced with a vector expressing Bcl-X_L as the first gene in the construct. Given that the Bcl-X_L expression achieved was only approximately 13% that of cap-dependent Bcl-X_L expression this result was not entirely unexpected. It would appear that a certain level of protein expression is needed in order to significantly inhibit apoptosis.

A future assay to further investigate this relationship between expression and functional effect could involve using pharmacologically controlled promotors to vary the level of gene expression from a given vector.

In the tumour co-culture experiments performed in this chapter cells were analysed every 24 hours for the first 72 hours in order to more closely study the early time period. In the experiment performed in the absence of IL-2, there is a suggestion that for the first 48 hours IRES-dependent Bcl-X_L expression may be almost as effective as cap-dependent expression, however, this effect was certainly lost by the day 14 analysis.

The experiments described in Chapter 3 confirmed the theory that survival gene over-expression could help to reduce gene-modified lymphocyte apoptosis in an *in vitro* system. The results from this chapter indicated that to achieve a significant effect, a relatively high level of expression was required and this was not achieved by co-expressing Bcl-X_L as the second gene in a bicistronic construct.

In conclusion, on balance it was felt unlikely that the functional effects of IRES-dependent Bcl-X_L expression would result in a significant reduction in apoptosis following therapeutic cellular adoptive transfer. In view of this, the cloning of the retroviral construct rKat.OM1.C23.CD3TGDA.IRES.Bcl-X_L (where C23.CD3TDGA was a chimeric T cell receptor specific to the tumour antigen, CEA – see Chapters 1 and 7) was not undertaken.

One future approach that could potentially increase IRES-dependent gene expression might be the incorporation of scaffold attachment region (or SAR) elements into the retroviral vector (Agarwal, Austin et al. 1998). DNA sequences encoding SAR elements derived from the human β-interferon gene have been shown to enhance retroviral transgene expression in primary T cells (Agarwal, Austin et al. 1998).

Ultimately the purpose of the work was to improve the survival of and enhance the clinical effect of lymphocytes following adoptive transfer. Although the *in vitro* results were interesting, this sort of approach would only be likely to be clinically useful if an effect could be demonstrated in an *in vivo* setting. With a view to setting up murine models to investigate this, in Chapter 6 the retroviral transduction of murine lymphocytes was studied. Given the problems encountered in this chapter in achieving sufficient co-expression of two genes, the genetic modification of an antigen-specific murine T cell line with survival genes was also attempted and modified cells were tested in an established *in vivo* model.

In terms of co-expression of Bcl-X_L with chimeric T cell receptors two other approaches were examined. Since it was not clear what level of chimeric T cell receptor expression was required to achieve targeted cytotoxicity, but it was clear that high level Bcl-X_L expression was needed, the retroviral vector rKat.Bcl-X_L.IRES.C23.CD3TDGA was constructed by a four step cloning procedure. Using this vector should have allowed the investigation of combined high level Bcl-X_L expression with (probably) lower level T cell chimeric receptor expression. Unfortunately, although diagnostic digests performed after each cloning step produced band patterns of the expected size, when the final vector was transfected into 293T cells (on two occasions) no expression of either Bcl-X_L or the chimeric receptor were detected by western blotting of transfected cell lysates. Part of the plasmid was sequenced and found to contain both the chimeric receptor sequence and the Bcl-X_L sequence intact and the cause of this lack of expression

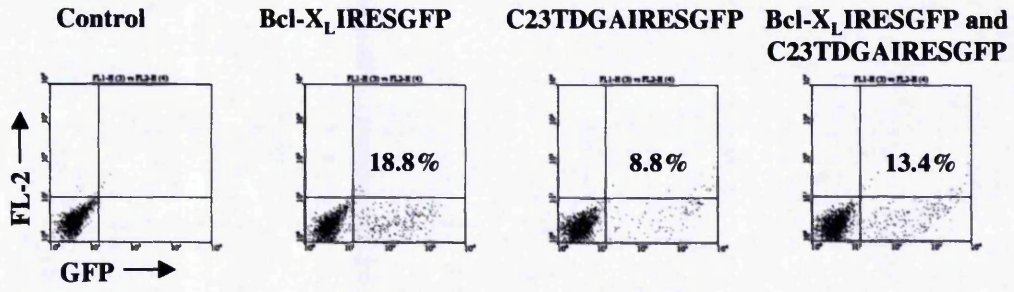


Figure 4.6 Transducing peripheral blood lymphocytes using combined retroviral supernatants - rKat.Bcl-X_L.IRES.EGFPand rKat.C23.CD3TDGA.IRES.GFP retrovirus was made by transient transfection of 293T cells using a calcium phosphate co-precipitation method. A spin transduction method was used to infect T cells. In combined group, an equal volume of each retroviral supernatant was mixed together immediately prior to transduction. Cells were analysed by flow cytometry 72 hours post transduction.

remains unclear. Unfortunately, in the time available it was not possible to repeat the construction of this vector.

The other alternative approach considered was to attempt double transductions. In other words, transducing a population of cells with two different retroviral vectors at the same time (in this case rKat.Bcl-X_L.IRES.GFP viral supernatant was mixed with rKat.C23.CD3TDGA.IRES.GFP viral supernatant immediately prior to transduction) (*Figure 4.6*). Although this sort of approach will result in a population of cells transduced by both vectors (ie expressing both transgenes), unless transduction efficiencies are very high this is likely to be a small percentage of cells when compared with the overall population. In addition, in order to ascertain which cells have been doubly transduced it is necessary to have different marker genes in each vector in order that cells expressing both transgenes can be identified. Given these difficulties this technique was not pursued. It would, however, from a scientific point of view, be a reasonable future approach in order to attempt to examine combined high-level expression of two genes if this could not be achieved using a single vector.

5 Results - Improving lymphocyte survival by over-expression of cFLIP$_S$

5.1 Introduction

Optimal lymphocyte activation, proliferation and survival generally occurs when a TCR-antigen signal is received in combination with co-stimulatory signals such as the interaction between the CD28 receptor and CD80/CD86 ligands expressed on antigen-presenting cells (Sperling and Bluestone 1996). The anti-apoptotic gene Bcl-X_L had been shown to be up-regulated following CD28 co-stimulation (Boise, Minn et al. 1995; Radvanyi, Shi et al. 1996; Sperling, Auger et al. 1996) and, on this basis, in Chapters 3 and 4 the effect of over-expressing Bcl-X_L in gene-modified lymphocytes was studied. Bcl-X_L prevents apoptosis caused by intracellular stress signals that result in the release of cytochrome c from damaged mitochondria, thereby preventing the stimulation of intracellular receptor proteins such as apoptotic protease-activating factor 1 (Apaf-1) (*Figure 5.1*) (Cory and Adams 2002). Whilst in certain circumstances Bcl-X_L expression can help to prevent apoptosis induced by death receptors (see Discussion, Chapter 3) it is likely that in this situation the predominant effect was to reduce the level of apoptosis caused by growth factor withdrawal.

More recently, however, CD28 co-stimulation has been shown to result in two other important downstream effects in addition to upregulation of Bcl-X_L (see *Figure 5.1* and below). During an immune response, T lymphocytes go through several phases (see *Figure 5.2*): following antigenic activation there is initially an IL-2 dependent clonal expansion and differentiation into effector cells; next there is an effector phase in which T lymphocytes either provide help, or kill target cells; and finally a down phase during which most of the antigen-specific T cells undergo apoptosis (Krammer 2000; Thome and Tschopp 2001). Eventually most cells are eliminated, but certain cells survive and become 'memory' T cells. This process of elimination of activated T cells is called activation-induced cell death (AICD) and serves to maintain lymphocyte homeostasis, thereby preventing autoimmunity, or uncontrolled proliferation leading to malignancy.

One of the main ways by which cytotoxic T lymphocytes can kill target cells is through

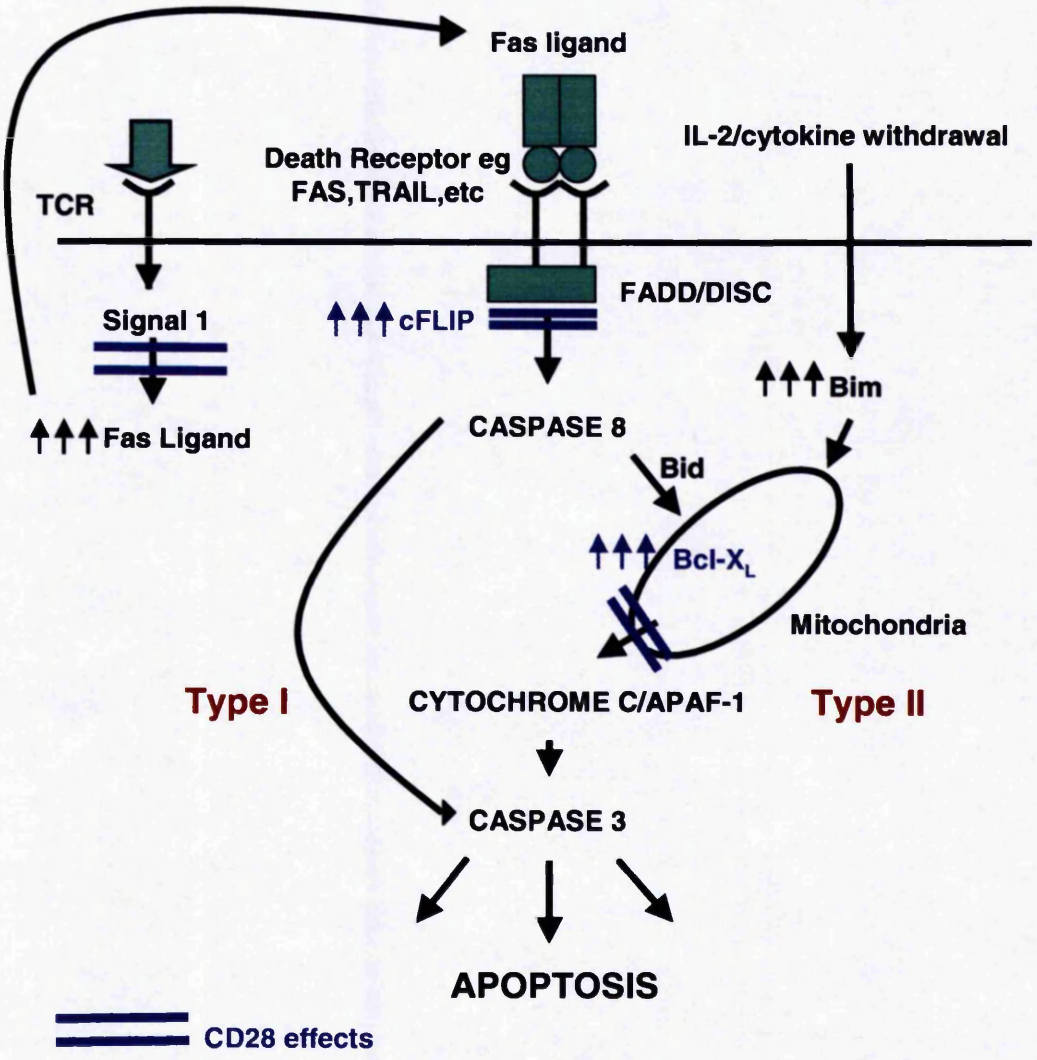


Figure 5.1 Lymphocyte apoptosis (modified from Krammer, 2000 and Kirchoff, Muller et al. 2000) - Figure illustrates simplified death receptor signalling pathways in lymphocytes (see text). It has been proposed that CD28 co-stimulation acts in three ways to inhibit apoptosis. TCR activation induces Fas ligand promotor activity leading to AICD. CD28 reduces Fas ligand promotor activity. CD28 co-stimulation also upregulates $cFLIP_S$ which reduces DISC activity and Caspase 8 activation. Finally, CD28 co-stimulation strongly up-regulates Bcl-X_L expression, preventing release of Cytochrome C from mitochondria and subsequent stimulation of apoptotic protease-activating factor , APAF-1. For an explanation of Type I and Type II death receptor pathways see text – page 96. DISC, death inducing signalling complex; FADD, fas associated death domain; TRAIL, TNF related apoptosis inducing ligand.

the Fas ligand-dependent triggering of so-called death receptors on the surface of the target cell. Clustered FasL/Fas receptors recruit the Fas-associated death domain (FADD) adapter protein to form the death-inducing signalling complex (DISC) resulting in the activation of caspase 8 by enzymatic cleaveage and apoptosis via the downstream caspase cascade. Interestingly, in a type of negative feedback loop, this is also the major mechanism by which lymphocytes undergo apoptosis during AICD. Following TCR activation, activated lymphocytes are induced to express Fas ligand on the cell surface and this results in the cell triggering CD95 receptors on nearby T cells (Krammer 2000) (see *Figure 5.1*).

Aside from increased Bcl-X_L expression, CD28 co-stimulation has recently been shown to reduce this AICD process by two mechanisms that reduce death receptor-induced apoptosis. Firstly, Fas ligand mRNA and protein has been shown to be (temporarily) downregulated resulting in reduced death receptor activation in neighbouring cells (Kirchhoff, Muller et al. 2000). The other mechanism involves the strong upregulation of another anti-apoptotic protein, $cFLIP_S$ (Van Parijs, Refaeli et al. 1999; Kirchhoff, Muller et al. 2000).

FLIPs (or FLICE (an old name for caspase 8)-inhibitory proteins) were first identified in herpes viruses. Viruses have evolved distinct mechanisms of evading the hosts' immune response and delaying the apoptotic death of infected cells. Following transfection, viral FLIP proteins were shown to inhibit the apoptosis caused by various death receptor pathways (Thome, Schneider et al. 1997). Two cellular homologues of vFLIP have since been identified: short FLIP ($cFLIP_S$) and long FLIP ($cFLIP_L$) (Irmler, Thome et al. 1997). When expressed both $cFLIP_S$ and $cFLIP_L$ have shown a protective role against apoptosis induced by death receptors (Irmler, Thome et al. 1997; Schneider, Thome et al. 1997).

Both cFLIP proteins bear structural homology with caspase-8 (Thome and Tschopp 2001). They are composed of two upstream death effector domains (DEDs) which are able to act as dominant negative inhibitors by binding to the FADD adaptor protein and preventing the processing and release of active caspase 8, thereby inhibiting death receptor induced apoptosis (*Figure 5.1*). $cFLIP_L$ also has a caspase 8 homologous carboxy terminal region which lacks catalytic activity due to the substitution of several amino acids. Embryonic fibroblasts from cFLIP-knockout mice are highly sensitive to

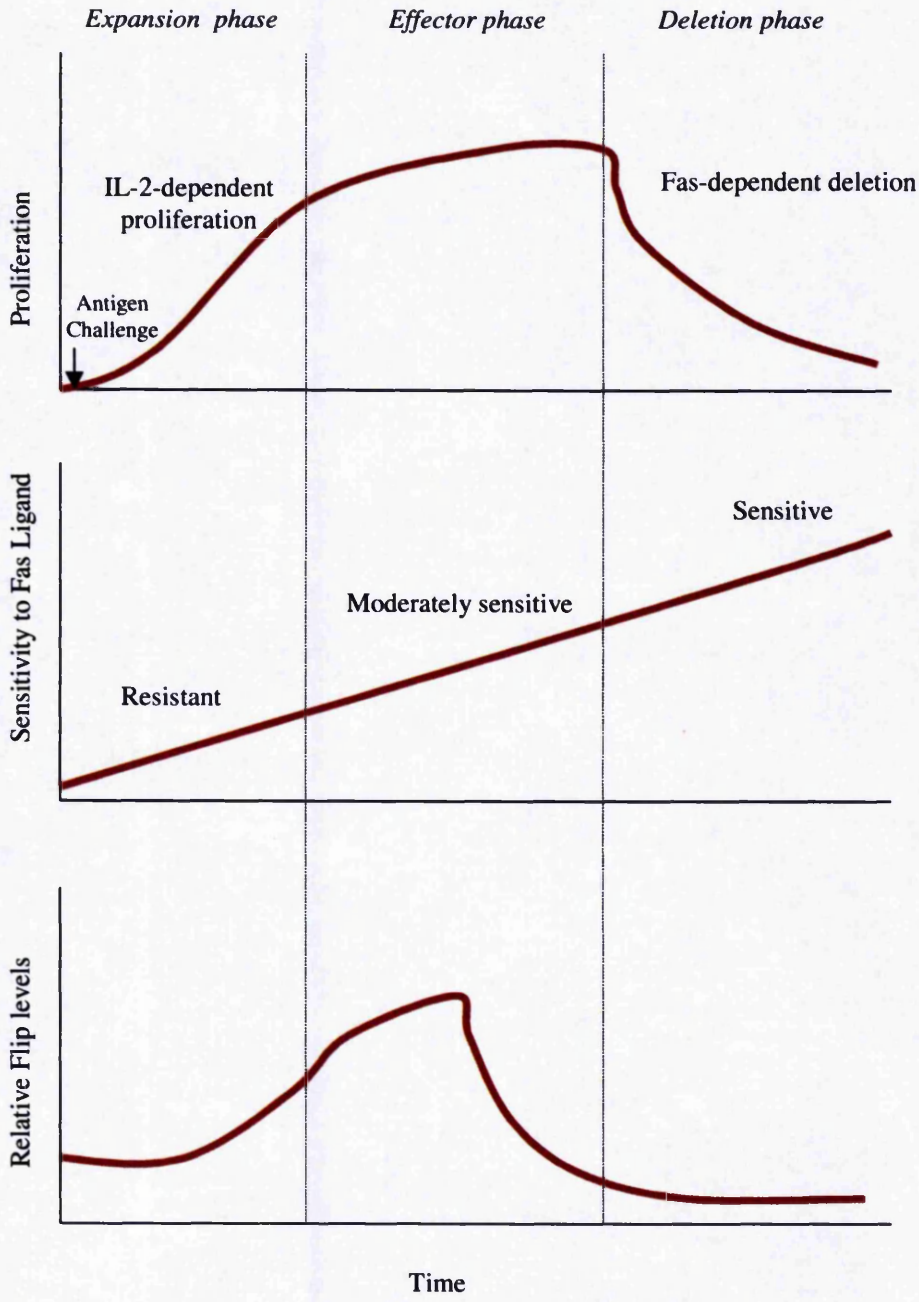


Figure 5.2 Activation induced cell death of T lymphocytes (adapted from Thome and Tschopp 2001) – Following an immune challenge, T cells undergo an IL-2 dependent proliferation and differentiation into effector cells. Subsequently most effector cells are eliminated by AICD, but certain cells survive and become memory cells. During the expansion phase lymphocytes are resistant to apoptosis, but become increasingly sensitive to cell death during the effector phase. cFLIP expression levels correlate with changes in the sensitivity of T cells to apoptosis. Memory cells are thought to be resistant to apoptosis due to high levels of cFLIP expression.

Fas ligand- or TNF-induced apoptosis, clearly showing an anti-apoptotic role of cFLIP (Yeh, Itie et al. 2000). These knockout mice also suggest a role for cFLIP in embryonic development, with cFLIP-deficient animals not surviving beyond day 10 of embryogenesis (with signs of cardiac failure and impaired heart development).

During the expansion phase of an immune response, lymphocytes are resistant to Fas ligand, but become increasingly sensitive to this form of cell death during the effector phase. cFLIP expression levels have been shown to correlate with these changes in sensitivity of T cells to Fas ligand (*Figure 5.2*) (Irmler, Thome et al. 1997; Kirchhoff, Muller et al. 2000). Although it is currently unclear how antigen-activated T cells become memory cells one theory is that this could be caused by an apoptosis resistant phenotype caused by high levels of cFLIP expression (Thome and Tschopp 2001).

In a similar way to the work presented in Chapter 3 using Bcl-X_L, the aim of the work in this chapter was to obtain cDNAs encoding the cFLIP proteins (long and short) and incorporate them into vectors used for cancer gene therapy, with a view to protecting the gene-modified lymphocytes from the effects of activation induced cell death and potentially improving *in vivo* lymphocyte survival.

5.2 cFLIP$_S$ PCR

Peripheral blood lymphocytes were isolated from a healthy donor and activated on iCD3 and iCD28 antibodies. RNA was extracted from $5x10^6$ cells using the RNeasy protocol (Qiagen) and cDNA was made by reverse transcription (methods 2.2.3.1). The human cFlip$_S$ gene was cloned from this cDNA by the PCR technique (methods 2.2.3.2). Primers CFLIPSH-FOR and CFLIPSH-REV (materials 2.1.3) were designed according to the published sequence (GenBank accession no. U97075). Additional restriction enzyme site sequences were added to enable subsequent sub-cloning (*EcoR1* and *Xba1* in the forward primer and *Mlu1* and *Sal1* in the reverse primer). Gel electropheresis of the PCR reaction revealed a band at the expected DNA size of cFLIP$_S$ (approximately 670 base pairs). This cFLIP$_S$ cDNA PCR product was cloned into the TOPO TA™ vector (Invitrogen, Paisley, UK) according to the manufacturers instructions and a diagnostic restriction enzyme digest was performed to confirm the presence of an insert in the minipreps. Prior to further vector construction, the cFlip$_S$ gene was sequenced.

Primers CFLIPLONG-FOR and CFLIPLONG-REV (materials 2.1.3) were also designed according to the published sequence of the cFLIP$_L$ gene. Unfortunately,

attempts to clone this gene from the same cDNA used in the above procedure were not successful.

5.3 cFLIP$_S$ Sequencing

Five separate plasmid clones of TA-cFLIP$_S$ were used to sequence the cFLIP$_S$ gene (methods 2.2.2.5) using sequencing primers T7 and M13R (materials 2.1.3). Miniprep clone 7 was identical to the the published sequence (Genbank Accession No. U97075, *Table 5.1*) with appropriate restriction sites either end of the gene and a Maxiprep of this clone was prepared (methods 2.2.1.5).

5.4 Construction of retroviral vector rKat.cFLIP$_S$.IRES.GFP

The vector rKat.MCS.IRES.GFP (*Figure 5.3a*) had previously been cloned as described in section 3.3. The human cFLIP$_S$ cDNA was cut out from TA-cFlip$_S$ as an *EcoR1/Sal1* fragment and inserted *EcoR1/Sal1* in front of the IRES, generating the vector rKat.cFLIP$_S$.IRES.GFP (*Figure 5.3b*). Using this vector, a bicistronic mRNA encompassing the cFLIP$_S$ and GFP cDNA was generated under the control of the LTR promoter.

Insertion of the cFLIP$_S$ cDNA into the rKat.MCS.IRES.GFP vector was confirmed both by diagnostic restriction enzyme digest and PCR (using the CFLIPSH-FOR and CFLIPSH-REV primers).

5.5 Western Blot for cFLIP$_S$ expression in cFLIP$_S$-transfected 293T cells

This experiment was performed in order to confirm that the cFLIP$_S$ cDNA within the rKat.cFLIP$_S$.IRES.GFP vector was functional and resulted in cFLIP$_S$ protein expression following cell transfection. Retroviral supernatant used in subsequent human lymphocyte and Jurkat cell line transduction experiments was produced by transient calcium phosphate transfection of the 293T tumour cell line (methods 2.2.6.2.1). In a similar way to the experiments performed in Chapters 3 and 4, 293T cells were transfected using the retroviral vectors rKat.GFP and rKat.cFLIP$_S$.IRES.GFP. Forty-eight hours post transfection 293T cells were lysed, separated by SDS/PAGE (12% gel, 100µg/lane) and the expression of cFLIP$_S$ was assessed by western blot (*Figure 5.4*) using a mouse monoclonal IgG$_1$ anti-FLIP$_{S/L}$ antibody (Santa Cruz) (methods 3.2.5).

Table 5.1 – cFLIP$_S$ Gene Sequence

```
C - ATGTCTGCTGAAGTCATCCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGCTCTTTTTGTGCCGGG 79
P - ATGTCTGCTGAAGTCATCCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGCTCTTTTTGTGCCGGG 79
7 - ATGTCTGCTGAAGTCATCCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGCTCTTTTTGTGCCGGG 79

C - ATGTTGCTATAGATGTGGTTCCACCTAATGTCAGGGACCTTCTGGATATTTTACGGGAAAGAGGTAAGCTGTCTGTCG 157
P - ATGTTGCTATAGATGTGGTTCCACCTAATGTCAGGGACCTTCTGGATATTTTACGGGAAAGAGGTAAGCTGTCTGTCG 157
7 - ATGTTGCTATAGATGTGGTTCCACCTAATGTCAGGGACCTTCTGGATATTTTACGGGAAAGAGGTAAGCTGTCTGTCG 157

C - GGGACTTGGCTGAACTGCTCTACAGAGTGAGGCGATTTGACCTGCTCAAACGTATCTTGAAGATGGACAGAAAAGCTG 235
P - GGGACTTGGCTGAACTGCTCTACAGAGTGAGGCGATTTGACCTGCTCAAACGTATCTTGAAGATGGACAGAAAAGCTG 235
7 - GGGACTTGGCTGAACTGCTCTACAGAGTGAGGCGATTTGACCTGCTCAAACGTATCTTGAAGATGGACAGAAAAGCTG 235

C - TGGAGACCCACCTGCTCAGGAACCCTCACCTTGTTTCGGACTATAGAGTGCTGATGGCAGAGATTGGTGAGGATTTGG 313
P - TGGAGACCCACCTGCTCAGGAACCCTCACCTTGTTTCGGACTATAGAGTGCTGATGGCAGAGATTGGTGAGGATTTGG 313
7 - TGGAGACCCACCTGCTCAGGAACCCTCACCTTGTTTCGGACTATAGAGTGCTGATGGCAGAGATTGGTGAGGATTTGG 313

C - ATAAATCTGATGTGTCCTCATTAATTTTCCTCATGAAGGATTACATGGGCCGAGGCAAGATAAGCAAGGAGAAGAGTT 391
P - ATAAATCTGATGTGTCCTCATTAATTTTCCTCATGAAGGATTACATGGGCCGAGGCAAGATAAGCAAGGAGAAGAGTT 391
7 - ATAAATCTGATGTGTCCTCATTAATTTTCCTCATGAAGGATTACATGGGCCGAGGCAAGATAAGCAAGGAGAAGAGTT 391

C - TCTTGGACCTTGTGGTTGAGTTGGAGAAACTAAATCTGGTTGCCCCAGATCAACTGGATTTATTAGAAAAATGCCTAA 469
P - TCTTGGACCTTGTGGTTGAGTTGGAGAAACTAAATCTGGTTGCCCCAGATCAACTGGATTTATTAGAAAAATGCCTAA 469
7 - TCTTGGACCTTGTGGTTGAGTTGGAGAAACTAAATCTGGTTGCCCCAGATCAACTGGATTTATTAGAAAAATGCCTAA 469

C - AGAACATCCACAGAATAGACCTGAAGACAAAAATCCAGAAGTACAAGCAGTCTGTTCAAGGAGCAGGGACAAGTTACA 547
P - AGAACATCCACAGAATAGACCTGAAGACAAAAATCCAGAAGTACAAGCAGTCTGTTCAAGGAGCAGGGACAAGTTACA 547
7 - AGAACATCCACAGAATAGACCTGAAGACAAAAATCCAGAAGTACAAGCAGTCTGTTCAAGGAGCAGGGACAAGTTACA 547

C - GGAATGTTCTCCAAGCAGCAATCCAAAAGAGTCTCAAGGATCCTTCAAATAACTTCAGGATGATAACACCCTATGCCC 625
P - GGAATGTTCTCCAAGCAGCAATCCAAAAGAGTCTCAAGGATCCTTCAAATAACTTCAGGATGATAACACCCTATGCCC 625
7 - GGAATGTTCTCCAAGCAGCAATCCAAAAGAGTCTCAAGGATCCTTCAAATAACTTCAGGATGATAACACCCTATGCCC 625

C - ATTGTCCTGATCTGAAAATTCTTGGAAATTGTTCCATGTG                                       665
P - ATTGTCCTGATCTGAAAATTCTTGGAAATTGTTCCATGTG                                       665
7 - ATTGTCCTGATCTGAAAATTCTTGGAAATTGTTCCATGTG                                       665
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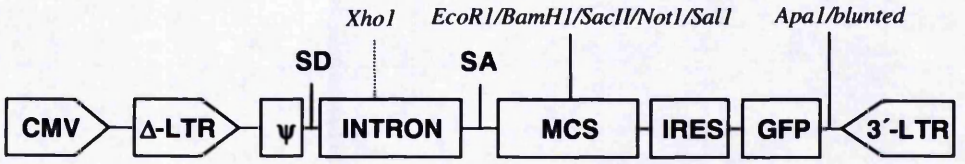
C-Consensus, P-Published sequence, 7-Sequence in miniprep clone 7 of TA-cFLIP$_S$

5.3a rKat.MCS.IRES.GFP



5.3b rKat.cFLIP$_S$.IRES.GFP

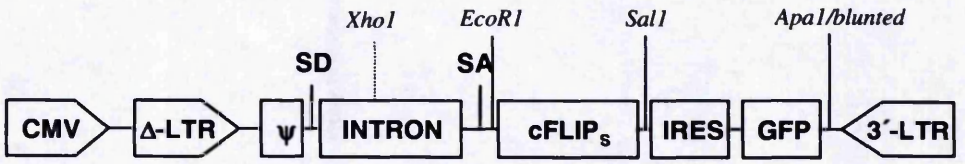


Figure 5.3 Construction of retroviral vector rKat.cFLIP$_S$.IRES.GFP - The vector rKat.MCS.IRES.EGFP (*Figure 5.3a*) had been previously constructed (see section 3.3). Human cFLIP$_S$ cDNA was cut out from TA-cFlip$_S$ as an *EcoR1/Sal1* fragment and inserted *EcoR1/Sal1* in front of the IRES, generating the vector rKat.cFLIP$_S$.IRES.EGFP (*Figure 5.3b*). Using this vector, a bicistronic mRNA encompassing the cFLIP$_S$ and GFP cDNA was generated under the control of the LTR promoter.

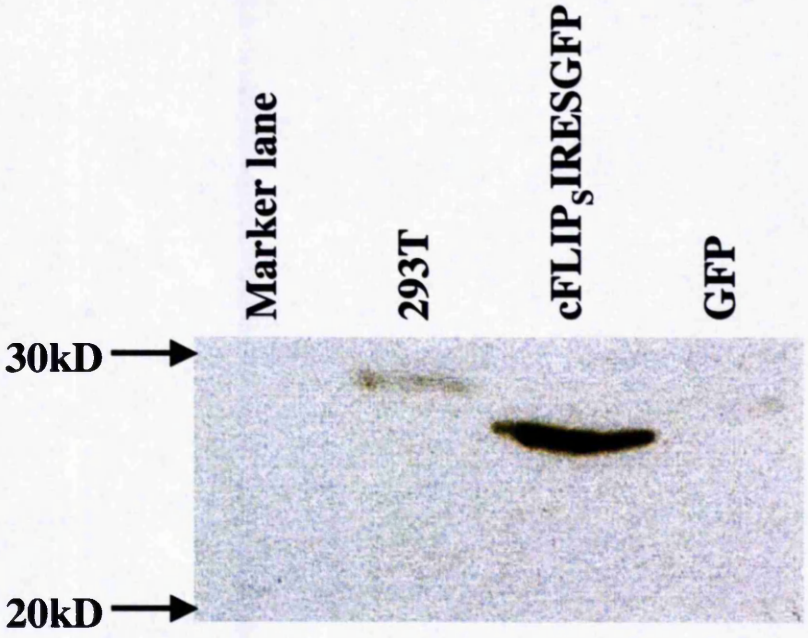


Figure 5.4 Western Blot for cFLIP expression in cFLIP$_S$-transfected 293T cells - 293T cells were transiently transfected by calcium phosphate co-precipitation (methods 2.2.6.2.1) using the retroviral vectors rKat.GFP and rKat.cFLIP$_S$.IRES.GFP. 48hrs later cells were lysed, separated by SDS/PAGE (12% gel, 100µg/lane) and expression of cFLIP$_S$ was assessed by western blot using a mouse monoclonal anti-cFLIP antibody at a dilution of 1:100 (Santa Cruz) with an anti-mouse HRP secondary antibody (1:1000 dilution). Over-expression of cFLIP$_S$ can clearly be seen in cFLIP$_S$-transfected 293T cells with no evidence of expression in GFP-transfected or non-transfected 293T cells.

Figure 5.4 clearly shows an anti-cFLIP$_S$ antibody detected immunoreactive band at the expected molecular weight of wild-type cFLIP$_S$ (26kD) in the lysate of rKat.cFLIP$_S$.IRES.GFP transfected cells with no evidence of expression in the GFP-transfected or non-transfected 293T cells.

5.6 cFLIP$_S$ transduced Jurkat cells are resistant to anti-Fas antibody induced apoptosis

Jurkat cells (10^5) were transduced with either rKat.GFP, rKat.Bcl-X$_L$.IRES.GFP, rKat.cFLIP$_S$.IRES.GFP, or an equal mixture of both rKat.Bcl-X$_L$.IRES.GFP and rKat.cFLIP$_S$.IRES.GFP retroviruses using a spin transduction method (methods 2.2.6.6.1). Transduction efficiencies ranging from 15% (rKat.cFLIP$_S$.IRES.GFP) to 63% (rKat.GFP) were achieved as assessed by flow cytometry analysis of GFP expression (*Figure 5.5*). In order to make the respective populations more comparable in terms of transduction efficiency the GFP positive cells within each group were then turbo-sorted by flow cytometry (methods 2.2.6.11). Sorted cells were expanded in T cell media. Further analyses of GFP expression by flow cytometry confirmed that post sorting the majority of cells in each group were GFP positive (*Figure 5.5*).

Transduced cells from each group were then suspended in fresh medium ($0.5x10^6$/ml) in triplicate wells of a 24-well tissue culture plate (2ml/well). Twenty-four hours later, the soluble anti-human CD95 (FAS) monoclonal antibody (R&D Systems) (1μg/ml) and Protein G (Sigma) (1μg/ml) was added to the cells. In a previous titration experiment (see section 3.7.1) at this concentration of Fas antibody, after 48 hours culture nearly 70% of Jurkat cells stained positive for Annexin V (ie were undergoing apoptosis). Control cells were cultured in the presence of an IgG$_1$ isotype control antibody and Protein G. At 24 and 48hrs, aliquots of cells were stained with PE-conjugated annexin V (Pharmingen) and the viability probe, 7-AAD and the percentage of apoptotic cells within the GFP positive cell population was assessed by triple-colour flow cytometry (in an identical way to the FACS density plots shown in *Figure 4.4a+b*, page 87).

Figure 5.6a illustrates the percentage of viable GFP positive cells (as assessed by this method) at each timepoint (results shown are means of triplicate wells+/-standard error of mean). *Figure 5.6b* shows the results after 48hrs culture. In the control rKat.GFP transduced population after 48 hours exposure to anti-Fas antibody 54% of cells were viable compared to 88% of cells not exposed to the anti-Fas antibody. In a result similar to that shown in *Figure 3.8*, Bcl-X$_L$ transduced cells were partially protected from this

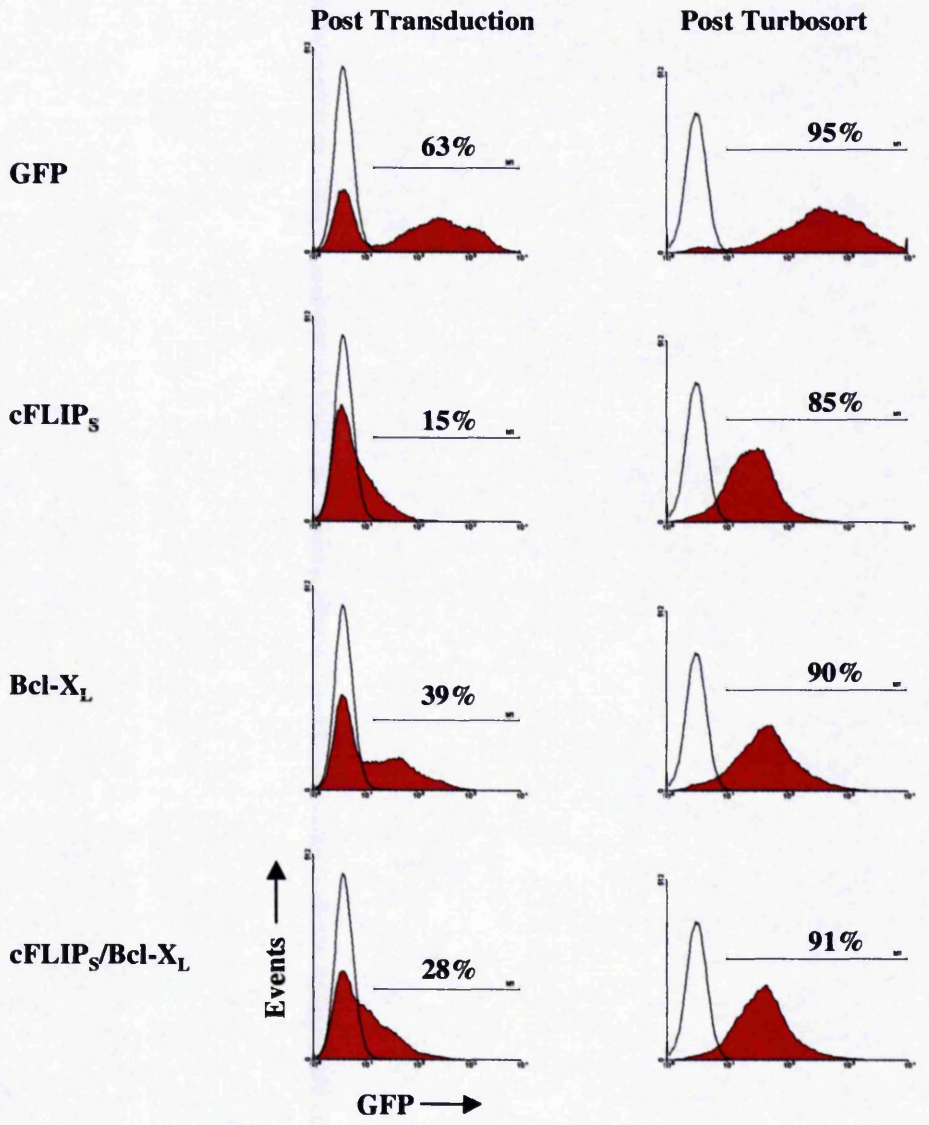


Figure 5.5 Transduction of Jurkat T cell line using Bcl-X_L and cFLIP$_S$ expression vectors - Jurkat cells (10^5) were transduced with either rKat.GFP, rKat.Bcl-X_L.IRES.GFP, rKat.cFLIP$_S$.IRES.GFP, or an equal mixture of both rKat.Bcl-X_L.IRES.GFP and rKat.cFLIP$_S$.IRES.GFP retroviruses using a spin transduction method (methods 2.2.6.6.1). Transduction efficiencies as assessed by flow cytometry analysis of GFP expression are illustrated (histograms on left). GFP positive cells within each group were then turbo-sorted by flow cytometry (methods 2.2.6.11). Further analyses of GFP expression by flow cytometry post cell sorting are shown as histograms on right.

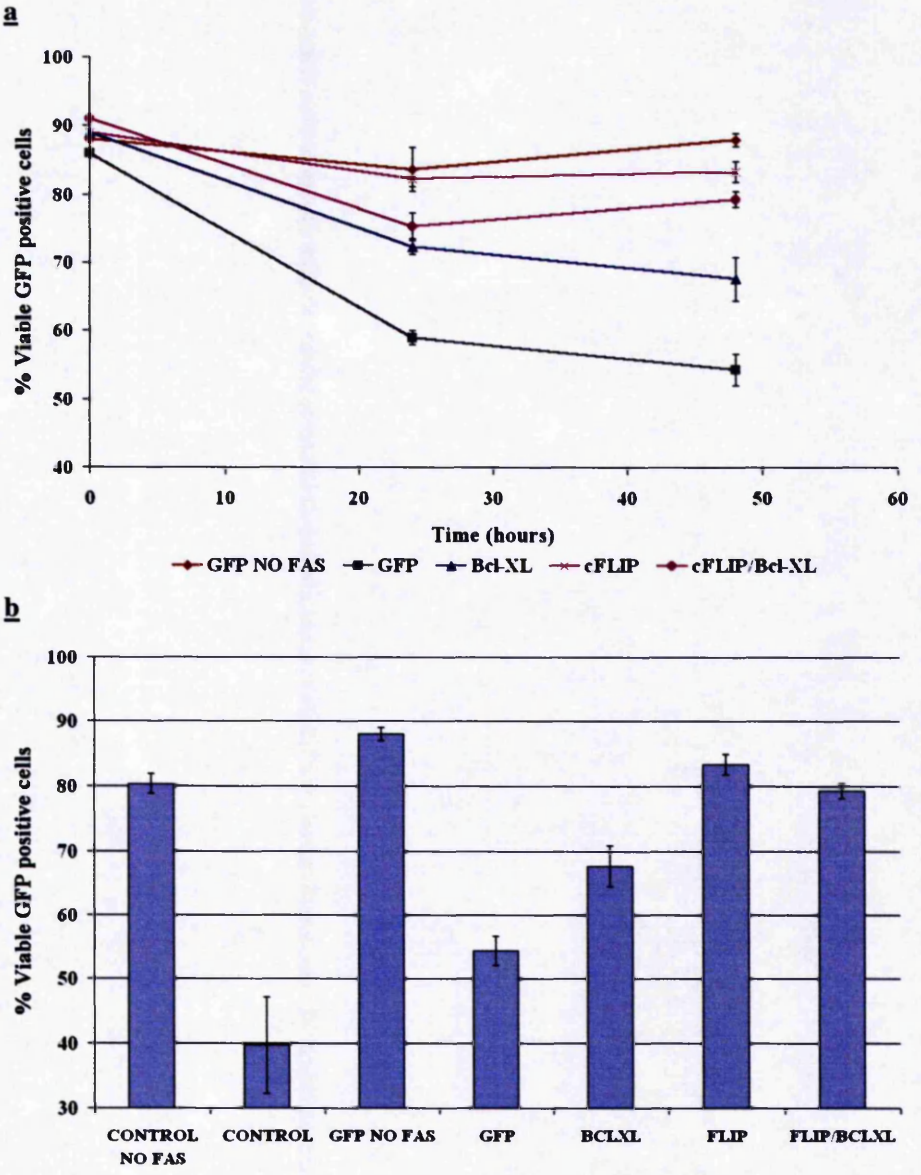


Figure 5.6 - cFLIP$_S$ transduced Jurkat cells are resistant to anti-Fas antibody induced apoptosis –Transduced Jurkat cells (*Figure 5.5*) and untransduced control cells were cultured with the soluble anti-human CD95 (FAS) monoclonal antibody (1µg/ml) and Protein G (1µg/ml). Control cells were cultured in the presence of an IgG_1 isotype control antibody and Protein G. At 24 and 48 hours, cells were stained with PE-conjugated annexin V and 7-AAD and the percentage of apoptotic cells within the GFP positive cell population was assessed by flow cytometry. *Figure 5.6a* illustrates the percentage of viable GFP positive cells at each time-point. *Figure 5.6b* shows the results at 48 hours. Results shown are means of triplicate wells (+/- standard error of mean). This experiment was repeated once and a similar result was obtained.

effect with 68% of cells remaining viable at the same time point. Of most interest, however, was the fact that in the $cFLIP_S$ transduced cell population there was almost complete protection from the effects of the anti-Fas antibody with only a slight fall in the percentage of viable cells after 48hours culture (83% viability after 48hours compared with 89% at baseline). The viability of $cFLIP_S$ transduced cells cultured in the presence of the anti-Fas antibody was similar to that of control cell populations that had not been cultured with anti-Fas antibody.

The cell population modified to express both cFLIP and Bcl-X_L also demonstrated increased resistance to apoptosis when compared with either control or Bcl-X_L transduced cells (79% viability at 48 hours). This experiment was repeated once and a similar result was obtained.

5.7 Human lymphocyte transduction with $cFLIP_S$ expression vector

Human peripheral blood lymphocytes (PBL) were obtained from a healthy donor and pre-activated on immobilised anti-CD3 and anti-CD28 monoclonal antibodies with IL-2 (30iu/ml) for 48hours as previously described (materials and methods 2.2.6.4). rKat.GFP, rKat.MCS.IRES.GFP and rKat.cFLIP.IRES.GFP retrovirus was again made by transient 293T transfection, and a spin transduction method (methods 2.2.6.6.2) was then used to transduce the T lymphocytes. Seventy-two hours later, transduction efficiency as measured by GFP expression was analysed by flow cytometry (*Figure 5.7a*). Efficiencies of 61%, 44% and 10% respectively were achieved in the rKat.GFP, rKat.MCS.IRES.GFP and rKat.$cFLIP_S$.IRES.GFP groups. The remaining cells were lysed in RIPA buffer. Protein lysates (10^6 cells/lane) were subjected to SDS/PAGE, western blotting and analysed by probing with a monoclonal anti-cFLIP antibody (Santa Cruz) (*Figure 5.7b*).

Figure 5.7b illustrates that although anti-cFLIP antibody detected immunoreactive bands at the expected molecular weight of wild-type $cFLIP_S$ (26kD), there did not appear to be any increase in band intensity in the rKat.cFLIP.IRES.GFP transduced cell lysate when compared with lysates prepared from lymphocytes transduced with the control vectors.

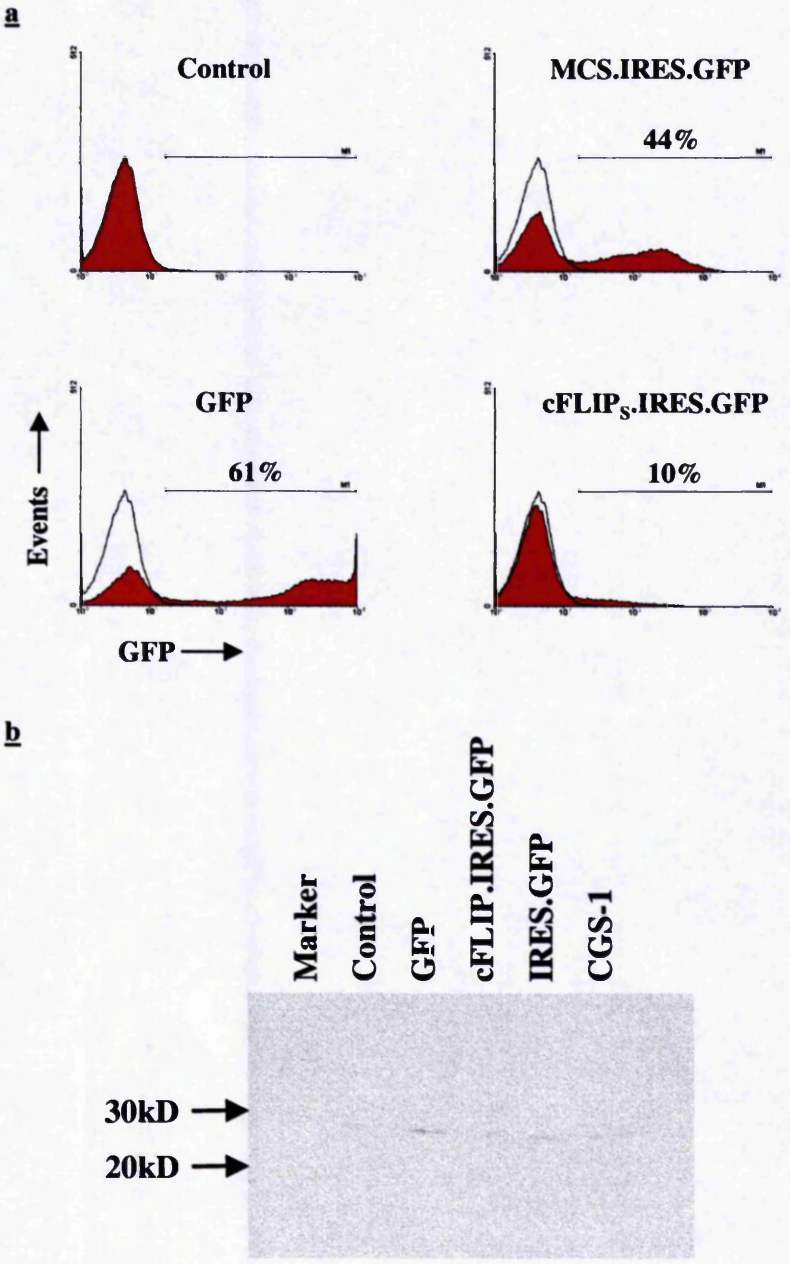


Figure 5.7 Human lymphocyte transduction with cFLIP expression vector - Human peripheral blood lymphocytes (PBL) were transduced with rKat.GFP, rKat.MCS.IRES.GFP and rKat.cFLIP$_S$.IRES.GFP retrovirus. 72 hours later, transduction efficiency as measured by GFP expression was analysed by flow cytometry (*Figure 5.7a*). The remaining cells were lysed in RIPA buffer. Protein lysates (10^6 cells/lane) were subjected to SDS/PAGE and Western blot analysis with a monoclonal anti-cFLIP antibody (*Figure 5.7b*).

5.8 Discussion

In this chapter, the cDNA of the anti-apototic protein $cFLIP_S$ was incorporated into a retroviral expression vector, rKat, with $cFLIP_S$ expression linked via an internal ribosome entry site to the transduction marker protein GFP.

An original intention had also been to clone the cDNA encoding $cFLIP_L$, however several attempts to amplify this cDNA from PBL cDNA were unsuccessful. Although $cFLIP_L$ may be a more potent inhibitor than $cFLIP_S$ at comparable expression levels (Irmler, Thome et al. 1997), over-expression in several studies has paradoxically shown a cytocidal effect (Inohara, Koseki et al. 1997; Irmler, Thome et al. 1997; Shu, Halpin et al. 1997). The reason for this is currently unclear, but may result from the non-physiological aggregation of its pro-apoptotic interaction partners FADD and caspase-8 under conditions of over-expression (Thome and Tschopp 2001). In view of this and given the problems with cloning, it was decided to concentrate on $cFLIP_S$.

Western blot of rKat.$cFLIP_S$.IRES.GFP vector-transfected 293T cell lysate confirmed the presence of an immunoreactive protein at the expected molecular weight of $cFLIP_S$, and the vector was subsequently used to transduce the Jurkat T cell line. Transduction efficiency of Jurkat cells using the rKat.$cFLIP_S$.IRES.GFP vector was poor (approximately 15%, *Figure 5.5*) and consequently, in order to increase the population of $cFLIP_S$ over-expressing cells within the population, and also to compare populations of equal transduction efficiency, cells were then bulk-sorted by flow cytometry.

$CFLIP_S$-expressing Jurkat cells were then compared to Bcl-X_L-expressing Jurkat cells (and cells expressing both transgenes) in an *in vitro* assay testing the resistance of the cells to anti-Fas antibody induced apoptosis. Over the 48hour time period, as anticipated, $cFLIP_S$-expressing Jurkat cells were almost completely resistant to the pro-apoptotic effects of an anti-Fas antibody in two independent experiments. This result demonstrates the functionality of the cFLIP cDNA cloned into the vector and illustrates the potential for $cFLIP_S$ to prevent Fas death receptor-induced apoptosis. In a similar result to Chapter 3 Bcl-X_L expression conferred partial protection from apoptosis. The population of cells transduced with both $cFLIP_S$ and Bcl-X_L expression vectors were also almost completely protected from apoptosis, however, it is difficult to know how this result should be interpreted since it is not possible to tell what proportion of this population were expressing either transgene.

rKat.cFLIP$_S$.IRES.GFP retroviral supernatant for these experiments was made by transient transfection of 293T cells. Although the titre of the supernatant was not formally tested, both the relatively low expression seen on western blot of the 293T cells themselves and the poor transduction efficiency of the (usually highly transducible) Jurkat cell line indicate that the construct may have resulted in a relatively low titre retrovirus. Subjectively, following transfection with rKat.cFLIP$_S$.IRES.GFP, for a reason that is not clear, the viability of the transfected 293T cells appeared poor.

Certainly, when this retrovirus was used to transduce human lymphocytes, transduction efficiencies of only 2-10% were achieved, and in subsequent western blots of transduced cell lysates it was not possible to discern significant cFLIP$_S$ over-expression when compared to control transduced lymphocytes.

Unfortunately, in the time available for this thesis it was not possible to carry out further experiments to improve upon human lymphocyte transduction efficiency. A logical next step, however, would be to create a high-titre stable rKat.cFLIP$_S$.IRES.GFP PG13 packaging cell line after cloning by limiting dilution. (high efficiency PG13 transductions using amphotropic 293T virus were performed but there was insufficient time to perform the cloning). If transduction efficiency could be improved further, *in vitro* and *in vivo* studies investigating this approach could then be performed.

Whilst co-expression of cFLIP$_S$ in a bicistronic gene therapy construct could have advantages over Bcl-X$_L$ in terms of the prevention of death receptor induced apoptosis, it is important to note that it would have a minimal effect on cell death caused by cytokine withdrawal (Van Parijs, Refaeli et al. 1999). Future *in vivo* studies could compare the survival of cFLIP$_S$-expressing lymphocytes with Bcl-X$_L$-expressing lymphocytes in order to ascertain the relative importance of each pathway in preventing apoptosis.

Although potentially improving the clinical efficacy and survival of gene-modified lymphocytes, it is important to note that, as with Bcl-X$_L$, cFLIP over-expression may pose several safety issues that would need to be closely studied prior to clinical development.

Fas- or Fas ligand-deficient (*lpr* and *gld*) mice and humans with autoimmune lymphoproliferative syndrome (ALPS) (associated with mutations in Fas, FasL, or other Fas pathway genes) both develop systemic autoimmunity characterised by an

accumulation of lymphocytes and the production of multiple autoantibodies (Nagata 1997; Siegel, Chan et al. 2000). In addition, in murine studies where cFLIP was constitutively expressed following retroviral transduction of bone marrow, self-tolerance was broken and the mice developed an accumulation of activated B cells and signs of autoimmunity (Van Parijs, Refaeli et al. 1999).

Tumour cell FLIP expression has also been implicated as a tumour progression factor. Over-expression is seen in several tumours and tumour cell lines (Tepper and Seldin 1999; Yeh, Itie et al. 2000) and has been correlated with tumour escape from T-cell immunity and enhanced tumour progression *in vivo* (French and Tschopp 1999).

Concern regarding the oncogenic potential of cFLIP and its role in breaking immune tolerance would mean that the expression of such a gene in a clinical gene therapy vector to improve survival would have to be carefully controlled. However, if future experiments demonstrate the principle that cFLIP expression can improve the anti-tumour efficacy of gene-modified lymphocytes, there are several approaches that might allow controlled protein expression and thereby minimising potential side-effects. Pharmacologically-controllable promotors (Alvarez-Vallina, Agha-Mohammadi et al. 1997) could be incorporated and additional safety features such as the inclusion of suicide genes in the vector could be employed. In order to reduce the risk of autoimmunity further, cFLIP-expressing lymphocytes could be targeted only to antigens that were entirely tumour-specific and not expressed at low levels on normal tissues.

6 Results – Retroviral transduction of murine T cells

6.1 Introduction

In the work presented so far (and also in Chapter 7), the genetic modification of human peripheral blood lymphocytes has been investigated and the functional effects of this gene expression demonstrated in *in vitro* assays. Ultimately, however, the purpose of these approaches was to either improve lymphocyte survival (Chapters 3-5) or lymphocyte targeting (Chapter 7) following *in vivo* adoptive transfer. Whilst human cells could be tested in immunodeficient models (see 7.11-12), it was clearly important to also assess effects in an immunocompetent setting. Bearing this in mind, and with a view to developing syngeneic murine models of adoptive cellular transfer, in this chapter the genetic modification of murine lymphocytes was attempted.

There were two key aims to this section of work. Firstly, given the difficulty encountered in Chapter 4 in achieving sufficient co-expression of a survival gene along with a therapeutic gene, the genetic modification of an antigen-specific primary murine T cell line to over-express Bcl-X_L was investigated. This cell line, with specificity against a B cell lymphoma idiotype, had previously been shown to have activity following adoptive transfer in an established *in vivo* lymphoma model (Armstrong and Dermime 2004). The aim of the work therefore was to investigate whether genetic modification of these cells to express Bcl-X_L could improve this *in vivo* activity. Clearly, this had the advantage of only requiring the expression of a single functional transgene and appeared to be the simplest method of studying this hypothesis *in vivo*.

Secondly, the genetic modification of fresh murine lymphocytes was also studied. This was with a view to developing the *in vitro* work described in Chapter 7 investigating the targeting of lymphocytes through the expression of tumour vasculature-specific chimeric T cell receptors (see section 1.2.5 for explanation of chimeric T cell receptors). Again, the efficacy of these tumour-targeted murine T cells could then be tested *in vivo*. Also, in the event that the constructs co-expressing chimeric receptors with survival genes could be improved, this would provide a means of also testing these constructs *in vivo*.

Factors required for the efficient genetic modification of primary human T-lymphocytes using replication-defective murine retroviruses (generally based on the moloney

leukaemia virus) are now well established (Finer, Dull et al. 1994; Bunnell, Muul et al. 1995; Movassagh, Boyer et al. 2000). The most effective, high viral titre producing, packaging cell lines have been identified and this has been combined with other improvements in the transduction protocol such as centrifugation (Movassagh, Boyer et al. 2000) or co-localisation of virus and target cell on recombinant fibronectin fragments (Pollok, Hanenberg et al. 1998) to produce high levels of transduction (see Chapter 1, section 1.2.3).

In contrast, retrovirus-mediated gene transfer in to murine T lymphocytes has proven more difficult with reduced transduction efficiencies when compared with human cells. Recently, however, several papers have reported improved retroviral gene transfer through a variety of alternative transduction protocols and packaging cell lines (Hagani, Riviere et al. 1999; Haynes, Snook et al. 2001; Annenkov, Daly et al. 2002; Niederman, Ghogawala et al. 2002).

As with human cells, high efficiency retroviral transduction of murine lymphocytes is thought to be dependent upon several factors. Retroviral integration requires target cells to be undergoing mitosis (Miller, Adam et al. 1990) and optimal lymphocyte activation conditions are therefore vital (Hagani, Riviere et al. 1999). The other major determinants are the concentration of retroviral stock used and the susceptibility of the lymphocyte to infection with the particular retroviral vector under investigation. This final factor is largely, but not exclusively, determined by cellular expression of the appropriate cell surface receptor capable of binding the particular retroviral envelope glycoprotein (Miller 1996). In other haematopoietic cells activation conditions can affect retroviral receptor expression levels (Orlic, Girard et al. 1996) and it may well be therefore that these factors are not independent, but inter-related.

Murine lymphocytes express both the cationic amino acid transporter (CAAT) that acts as the receptor for ecotropic retroviruses and, also, phosphate transporters that are the receptors for amphotropic retroviruses. Both of these types of retrovirus are commonly used for murine cell transduction. The use of high viral titre producing clones of the ecotropic Gp+e86 packaging cell line in a co-culture protocol has recently been demonstrated to give reproducible high level expression of a scFv chimeric T cell receptor in murine T lymphocytes (Darcy, Haynes et al. 2000; Haynes, Snook et al. 2001) and this protocol was tested in this chapter. The use of another ecotropic packaging cell line, EcoPack^{TM}2-293 (Clontech, CA, USA) was also examined. These

cells are easy to transfect and can produce high viral titres of greater than 10^6 virus/ml 24 to 72 hours post transfection. This removes the requirement to clone high titre producers and is useful when testing several different constructs although has the disadvantage that, unlike stable packaging cell lines, a repeat transfection needs to be performed each time virus is prepared.

The PT67 packaging cell line expresses a dualtropic envelope based on the 10A1 serotype of murine leukaemia virus. This envelope recognises both the gibbon ape leukaemia virus receptor (Glvr-1 or Pit-1) and also the amphotropic retrovirus receptor (Ram-1 or Pit-2) that are both expressed on murine lymphocytes (Miller and Chen 1996). In another recent publication, transduced PT67 cells produced high titres of retrovirus that gave high efficiency gene transfer in to antigen-specific primary mouse lymphocytes (when combined with centrifugation), without the need for cloning high titre producing packaging cell lines (Annenkov, Daly et al. 2002). These polyclonal packaging cell populations retained their viral titre for greater than one year. Potentially using these cells would have the advantages of a stable packaging cell line without the need for long periods of cloning.

Another alternative to ecotropic or amphotropic packaging cell lines is to pseudotype retrovirus with the G-glycoprotein of vesicular stomatitis virus (VSV-G). VSV-G binds to ill-defined phospholipids expressed in all mammalian species (Schlegel, Tralka et al. 1983) and one advantage of this approach is that VSV-G pseudotyped retrovirus is sufficiently robust to be concentrated by ultra-centrifugation enabling very high titre viral stock to be produced (Burns, Friedmann et al. 1993). In a recent publication this approach resulted in high efficiency gene transfer into murine T lymphocytes (Niederman, Ghogawala et al. 2002), however others have reported this to be less efficient than retrovirus vectors expressing ecotropic envelope proteins (Hagani, Riviere et al. 1999).

6.2 Transduction of an idiotype-specific primary murine T cell line to over-express Bcl-X_L

As discussed in the introduction to this chapter, in order to further investigate the hypothesis that the over-expression of the survival gene Bcl-X_L could improve the *in vivo* survival and, hence, therapeutic efficacy of adoptive T cell transfer, the retroviral genetic modification of an idiotype specific primary murine T cell line using the rKat.Bcl-X_L.IRES.GFP vector (see Chapter 3) was investigated.

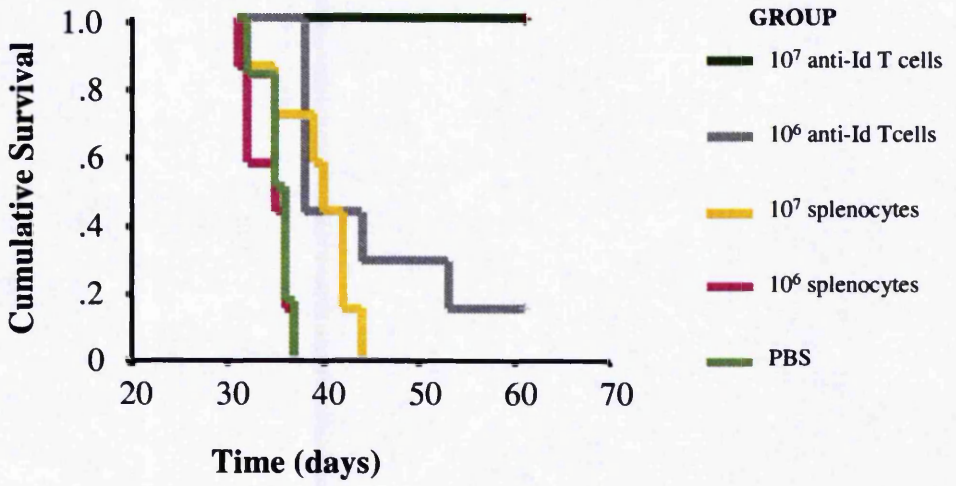


Figure 6.1 Protection from A20 tumour challenge by adoptive transfer of antigen-specific T cells in a BALB/c syngeneic A20 lymphoma model (reproduced with kind permission of Dr AC Armstrong) – 10^6 A20 murine lymphoma cells were injected intravenously. 72hrs later, BALB/c mice (n=10/group) received either 10^6 or 10^7 idiotype-specific T cells, non-targeted splenocytes or PBS injections intravenously. Results of this study are shown as a Kaplan-Meier survival curve. Control mice injected with either PBS or non-targeted all succumbed to the tumour within 45 days. In contrast mice treated with 10^7 idiotype-specific T cells were entirely protected from the A20 tumour challenge with 100% survival past day 60.

The Ig idiotypic determinants of B cell lymphomas, formed by rearrangement of V_H and V_L genes are unique tumour antigens. In work done by Dr Anne Armstrong (Department of Medical Oncology) (Armstrong, Dermime et al. 2002), a recombinant adenoviral vaccine encoding the lymphoma idiotype for the murine A20 lymphoma cell line was used to vaccinate BALB/c mice. Splenocytes isolated from the vaccinated mice were stimulated weekly using irradiated XS52 cells (a dendritic cell line) transduced to express the A20 scFv (XS52.A1.A20) and in this way A20-specific cytotoxic primary murine T cell lines were generated that were able to lyse A20 lymphoma cells in ^{51}chromium-release cytotoxicity assays.

A syngeneic BALB/c A20 murine lymphoma model was then used to examine the ability of adoptively transferred idiotype-specific T cells (injected intravenously) to prevent the development of A20 lymphomas in mice previously injected (also intravenously) with 10^6 A20 lymphoma cells. The results of this study are illustrated in *Figure 6.1* (with permission from Dr Armstrong) as a Kaplan-Meier survival curve. Control mice injected with either PBS or non-targeted splenocytes all succumbed to the tumour within 45 days. In contrast mice treated with 10^7 idiotype-specific T cells were entirely protected from the A20 tumour challenge with 100% survival past day 60. One of 10 of mice treated with 10^6 idiotype-specific T cells was protected from the effects of the tumour with another mouse having an extended survival before succumbing. The aim of this work was therefore to repeat this model comparing idiotype-specific lymphocytes transduced with rKat.Bcl-X_L.IRES.GFP with non-transduced lymphocytes giving a dose of 10^6 cells, the hypothesis being that Bcl-X_L expression should improve *in vivo* survival of lymphocytes and consequently increase the efficacy of these cells in this model.

6.2.1 Creation of a Gp+e86 rKat.Bcl-X_L.IRES.GFP stable packaging line

Amphotropic rKat.Bcl-X_L.IRES.GFP retrovirus and control rKat.B1.8.IRES.GFP retrovirus) were made by transient transfection of 293T cells (methods 2.2.6.2.1 and 2.2.6.3.1). Gp+e86 packaging cells were transduced using these retroviruses and the transduced cells cloned by limiting dilution as described in methods 2.2.6.6.1.

Cell colonies that fluoresced green under ultraviolet light were picked and expanded. Several attempts were made to estimate the viral titre of these clones by using supernatant to infect the murine 3T3 cell line (methods 2.2.6.3.3). Unfortunately, the

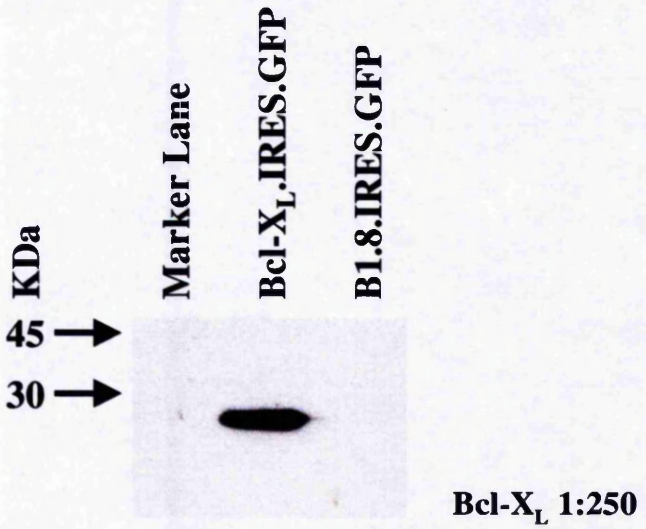


Figure 6.2 Western blot for Bcl-X_L expression in rKat.Bcl-X_L.IRES.GFP Gp+e86 packaging cell line, clone 18 - To confirm that the Gp+e86 rKat.Bcl-X_L.IRES.GFP packaging cell line selected (clone 18) was expressing Bcl-X_L the cells were lysed, separated by SDS/PAGE (12% gel, 300µg/lane) and expression of Bcl-X_L was assessed by western blot using a mouse monoclonal anti-Bcl-X antibody (Clone 2H12, Pharmingen).

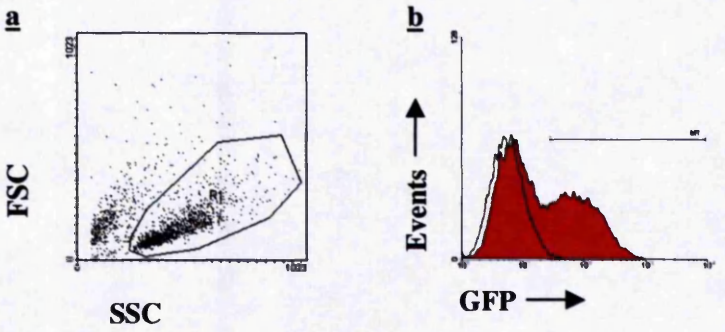


Figure 6.3 Assessment of viability and transduction efficiency – Lymphocytes transduced in the experiments described in 6.2.2 were analysed by flow cytometry 72 hours post transduction. Percentage viability of the cell population was measured by a live lymphocyte gate on FSC vs SSC analysis (*Figure 6.3a*) and the percentage of transduced cells determined by analysis of GFP expression (*Figure 6.3b*).

viral stock tested failed to transduce the 3T3 cells at all even when used neat. Alongside these titration experiments however, virus from several of the clones was used to transduce fresh murine splenocytes (see 6.3.1 and *Figure 6.7a*). Retrovirus harvested from rKat.Bcl-X_L.IRES.GFP clone 18 resulted in a transduction efficiency of greater than 40% of fresh splenocytes when used in a retronectin-mediated protocol (*Figure 6.7a*). It was evident therefore that these cells were producing a reasonable viral titre and on the basis of these results clone 18 was selected to produce retrovirus in the following series of experiments, along with a similar rKat.B1.8.IRES.GFP producer clone. It subsequently became clear that the '3T3' cells used at the time of these particular experiments had become resistant to any form of retroviral infection and consequently were discarded.

To confirm that the Gp+e86 rKat.Bcl-X_L.IRES.GFP packaging cell line selected (clone 18) was indeed expressing Bcl-X_L the cells were lysed, separated by SDS/PAGE (12% gel, 300µg/lane) and expression of Bcl-X_L was assessed by western blot (*Figure 6.2*).

6.2.2 Optimising retroviral transduction

A series of experiments were performed testing different methods of activation and transduction of the A20-specific murine T lymphocytes. The results of these experiments are summarised in *Table 6.1*. During these experiments lymphocytes were cultured as described in 2.2.6.1.2. Supernatant harvested from the Gp+e86 packaging cell lines (or the cells themselves in the co-culture experiments) cloned in 6.2.1 was used for all experiments. Details of activation and transduction methods are described in 2.2.6.5 and 2.2.6.6.3.

In experiment 1, cells were antigenically-stimulated (with irradiated XS52.A1.A20 dendritic cells – see 2.2.6.1.2) 48 hours before supernatant transduction on fibronectin-coated plates. This resulted in good cell viability but minimal cell transduction efficiency. Pre-activating the cells on anti-CD3 and anti-CD28 antibody coated plates gave a slight increase in transduction (experiment 2) but reduced the viability of the lymphocytes. An attempt was made to sort the rKat.Bcl-X_L.IRES.GFP-transduced population by flow cytometry following this experiment. Unfortunately, these cells failed to expand and eventually died.

A co-culture protocol similar to that described by Darcy (Darcy, Haynes et al. 2000) was tested in experiments 3-5. During experiment 3 however, it became evident that the

Table 6.1 Optimising retroviral transduction of A20-specific murine T cells

No	Activation Conditions	Transduction Method[+]	% Viability of Cell Population[*]		Transduction Efficiency[*] (%GFP positive)	
			Bcl-X_L	B1.8	Bcl-X_L	B1.8
1	DC 48hrs prior to transduction	RN-supernatant	78	73	0.7	0.2
2	CD3/CD28	RN-supernatant	24	27	8[^]	1
3a	CD3/CD28	Co-culture	48	-	4	-
3b	PHA	Co-culture	56	50	3	2
4[++]	PHA	Co-culture	39	29	8	13
5	PHA	Multiple Co-culture	5	1	96	87
6	DC 6hrs prior to transduction	RN-supernatant	34	36	**41**[**]	**20**[**]

(***Key***: DC – antigenic dendritic cell stimulation (see 2.2.6.1.2); CD3/CD28 – activation on immobilised anti-CD3 (clone 2C11) and anti-CD28 (clone 37.51) antibodies (2.2.6.5); PHA – phytohaemagglutinin; RN – Retronectin™; [+]see 2.2.6.6.3 for description of methods; [*]measured by flow cytometry 72hrs post transduction (see *Fig 6.3*); [^]GFP-positive population sorted by flow cytometry but failed to expand; [++]Splenocytes isolated from freshly sacrificed vaccinated mice were used as opposed to established T cell line; [**]these cells were used in the experiments described in 6.2.3-6.2.4.)

lymphocytes were actually targeting the packaging cells, to the extent that after 72hrs co-culture very few packaging cells remained alive. The cause of this is not entirely clear, but may have been due to an allogeneic response from the BALB/c lymphocytes against the Swiss embryo-based Gp+e86 (3T3) packaging cells. In experiment 4, fresh splenocytes from vaccinated mice (see 6.2) were transduced with a view to subsequently generating A20-specific T cells that were also over-expressing Bcl-X_L. The transduction efficiency using this method was a little better, but it was not possible to generate A20-specific cells post-transduction due to an inability to expand the cells (despite regular re-stimulation with irradiated XS52.A1.A20 dendritic cells).

In experiment 5, lymphocytes were re-plated onto fresh packaging cells every twenty-four hours for three days. Whilst this significantly increased transduction rates, it also killed the majority of the cells.

It appeared that when the cells were aggressively activated transduction rates increased, but viability of the population decreased. Considering this, in experiment 6, cells were antigenically-activated in a similar way to experiment 1, except on this occasion the 48hr transduction process was commenced 6hrs after DC stimulation, rather than 48. One week later, the transduction process was repeated using the same cells. This experiment gave a significantly improved transduction efficiency of 41% in the rKat.Bcl-X_L.IRES.GFP transduced population with moderate viability and these cells were then further tested in 6.2.3-6.2.4.

6.2.3 Bcl-X_L transduced A20-specific T cells retain cytolytic activity against A20 tumour cells

Transduced lymphocytes from experiment 6 (see 6.2.2) were expanded in IL-2 (50u/ml) and restimulated weekly with irradiated XS52.A1.A20 cells (as described in 2.2.6.1.2). Over the next 3 weeks, cell numbers expanded approximately eight-fold, but then reached a plateau in both groups with no further proliferation over the next 4 weeks of culture (*Figure 6.4a*).

Since it was evident that the lymphocytes were no longer expanding (despite varying the concentration of IL-2 and interval between stimulations) it was decided to use the cells available in further *in vitro* and *in vivo* assays. Unfortunately, the control rKat.B1.8.IRES.GFP transduced cells became infected at this point and were discarded.

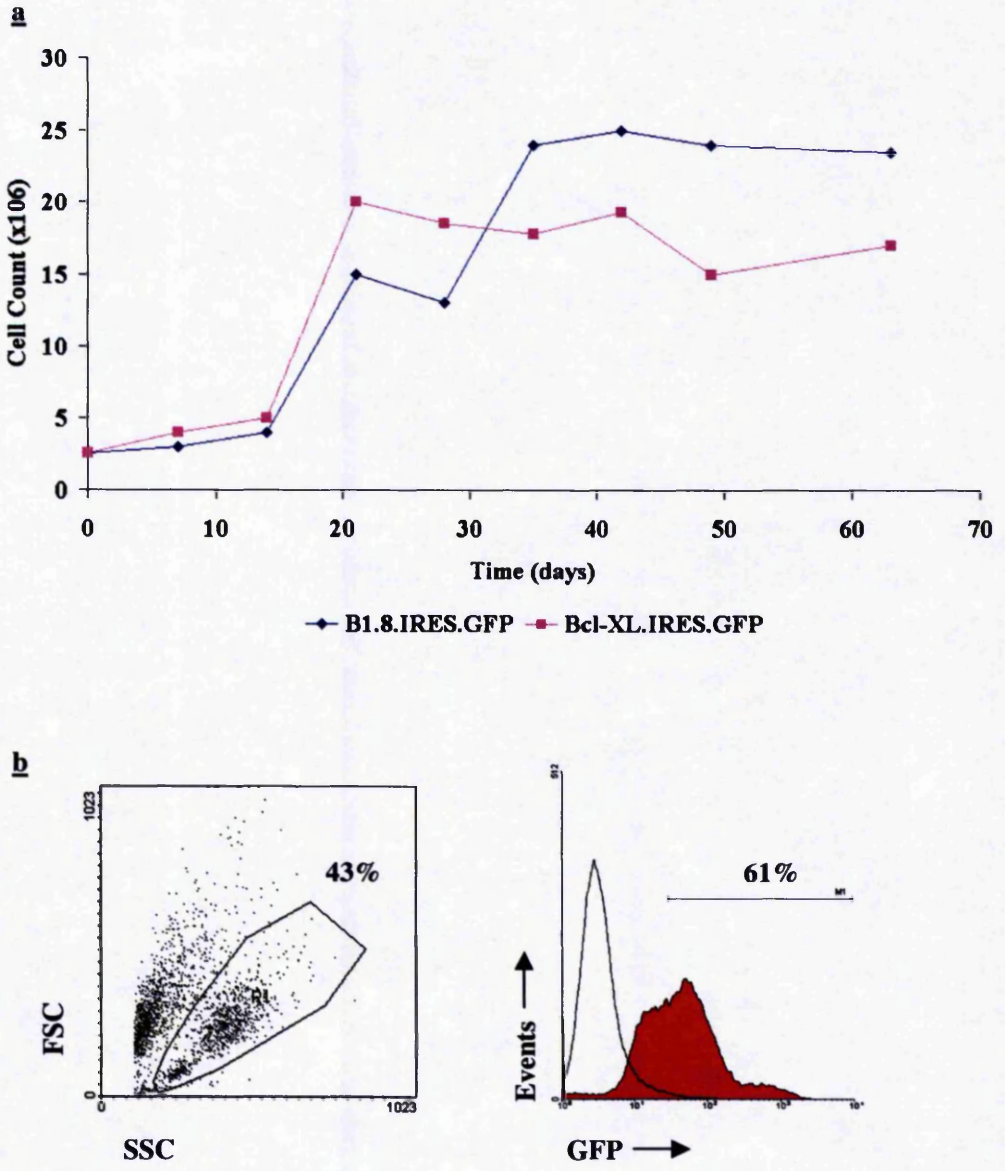


Figure 6.4 Expansion of rKat.Bcl-X_L.IRES.GFP transduced A20-specific T cells – Transduced lymphocytes from experiment 6 (see 6.2.2) were expanded in IL-2 (50u/ml) and restimulated weekly with irradiated XS52.A1.A20 cells (as described in 2.2.6.1.2) (*Figure 6.4a*). Flow cytometry analysis of GFP expression was performed on day 63 following transduction (*Figure 6.4b*).

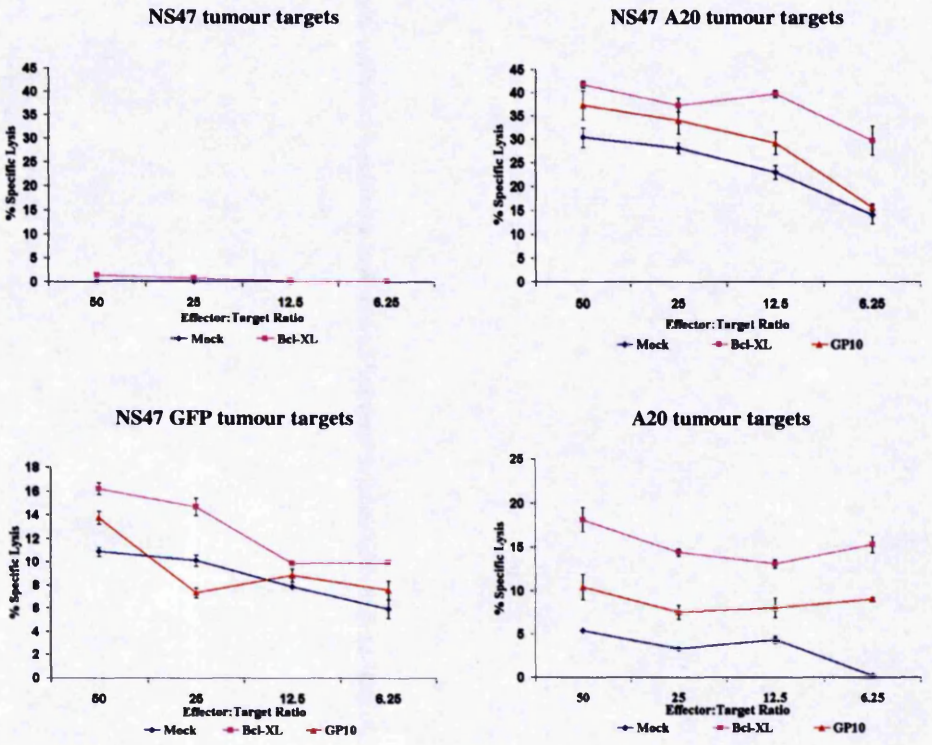


Figure 6.5 rKat.Bcl-X_L.IRES.GFP transduced A20-specific murine T cells retain cytolytic activity against A20 tumour cells – Cytolytic activity of the Bcl-X_L-transduced lymphocytes was tested in a ^{51}Chromium release assay along with mock non-transduced A20-specific T cells that had been cultured for the same period of time as the transduced cells (mock) and another group of A20-specific T cells that had been recently defrosted (gp10). Target cells were A20 lymphoma cells, the syngeneic NS47 fibroblast line, NS47 cells transduced to express A20 (NS47 A20) and NS47 cells transduced with GFP (NS47 GFP) (obtained from Dr Anne Armstrong, Department of Medical Oncology).

Flow cytometry analysis performed on day 63 following transduction (*Figure 6.4b*) indicated that the proportion of Bcl-X_L-transduced cells had expanded to 61% of the total population from 40% immediately post transduction.

Cytolytic activity of the Bcl-X_L-transduced lymphocytes was tested in a ^{51}Cr release assay along with mock non-transduced A20-specific T cells that had been cultured for the same period of time as the transduced cells (mock) and another group of A20-specific T cells that had been recently revived (gp10) (methods 2.2.6.9). Target cells were A20 lymphoma cells, the syngeneic NS47 fibroblast line, NS47 cells transduced to express A20 idiotype (NS47 A20) and NS47 cells transduced with GFP (NS47 GFP) (obtained from Dr Anne Armstrong, Department of Medical Oncology).

Figure 6.5 illustrates that the Bcl-X_L-transduced T cells had maintained a high specific lytic activity against the NS47-A20 and A20 tumour cell lines with no detectable lysis of NS47 cells. Against both NS47-A20 and A20 tumour cells, lysis was higher with the Bcl-X_L-transduced T cells than with the non-transduced cells.

6.2.4 Bcl-X_L transduced A20-specific T cells do not show improved efficacy in a BALB/c syngeneic A20 lymphoma model

Having confirmed that the Bcl-X_L-transduced A20-specific lymphocytes retained specific cytolytic activity against A20-expressing target cells, their activity was tested *in vivo*.

BALB/c mice (n=21) were injected intravenously, via a tail vein, with 10^6 A20 tumour cells in 200µl PBS. Seventy-two hours later mice were divided into three groups (n=7/group) and treated with one intravenous dose of 10^6 A20-specific T cells (gp10 ie the recently defrosted cells), 10^6 Bcl-X_L-transduced A20-specific T cells or PBS control. Mice were observed as described in methods (2.2.7.2) and sacrificed when first noted to be showing any signs of distress.

The results of this experiment are shown in *Figure 6.6* as a kaplan-meier survival curve. Median survival was 41 days for the group treated with Bcl-X_L-transduced A20-specific T cells, 34 days for the non-transduced A20-specific T cell group and 30 days in the control PBS treated group. Unfortunately, none of these differences were significant in terms of survival between the three treatment groups. The final animals in each group were sacrificed between 40 and 45 days post tumour cell injection (apart from one

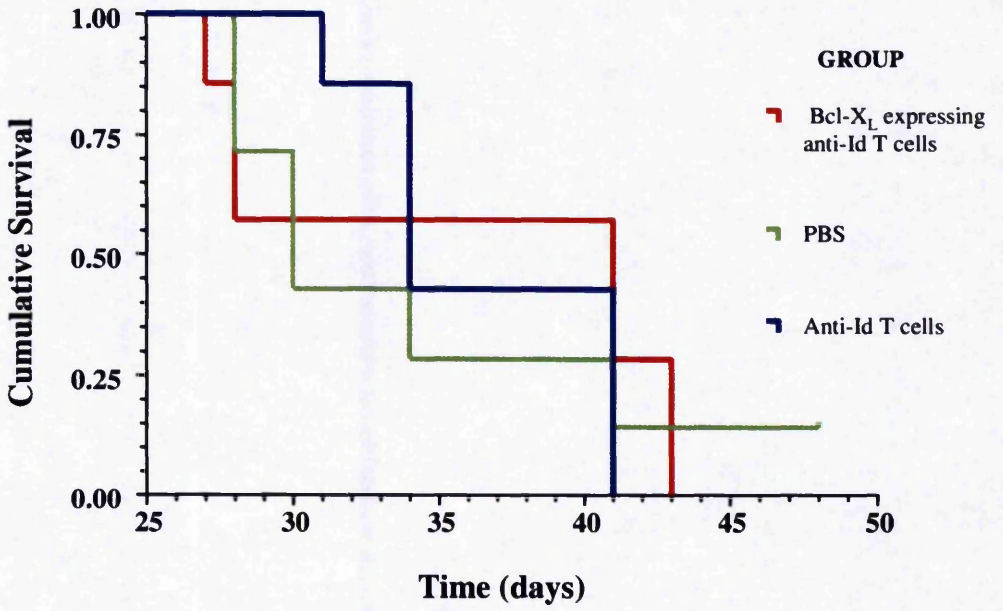


Figure 6.6 Bcl-X_L transduced A20-specific T cells do not show improved efficacy in a BALB/c syngeneic A20 lymphoma model – BALB/c mice (n=21) were injected with 10^6 A20 tumour cells. Seventy-two hours later mice were divided into three groups (n=7/group) and treated with one intravenous dose of 10^6 A20-specific T cells, 10^6 Bcl-X_L-transduced A20-specific T cells or PBS control. Mice were observed and sacrificed when first noted to be showing any signs of distress. Results are shown as a kaplan-meier survival curve.

mouse in the control PBS group who failed to develop any signs of lymphoma); a similar survival to the control groups in the previous experiment shown in *Figure 6.1.*

6.3 Optimising fresh murine splenocyte transduction

Primarily with a view to developing a syngeneic *in vivo* model to examine the use of gene-modified lymphocytes expressing pro-survival proteins or tumour-specific chimeric T cell receptors (see Chapter 7), the genetic modification of fresh murine lymphocytes was investigated.

6.3.1 Comparing methods of non-enriched splenocyte transduction

In parallel with the work described in 6.2.2, an initial series of experiments was performed studying splenocyte isolation, activation and transduction protocols and comparing several different packaging cell lines for the production of retrovirus expressing alternative envelope proteins. The results of these experiments are summarised in *Table 6.2.* In all experiments fresh spleens were obtained from recently sacrificed C57BL/6 mice. Further details of activation and transduction methods are described in 2.2.6.5 and 2.2.6.6.3.

In the initial experiment, red blood cells were removed by separation on a Lympho-Prep gradient. This resulted in a significant persisting red cell contamination and thereafter protocols employing a red cell lysis step were used.

In experiment 1, when activated on immobilised anti-CD3/anti-CD28 antibodies and used in a supernatant centrifugation protocol, both amphotropic *rKat* retrovirus derived from 293T cells transiently transfected with the *pKat* packaging plasmid and PG13 (GALV)-derived retrovirus gave minimal transduction rates. *RKat* retrovirus pseudotyped with VSV-G (prepared by transient transfection of 293T cells, but substituting *pKat* packaging plasmid with plasmids pRV67 and pHIT60, obtained from Dr Jonathan Rholl, Oxford Biomedica, Oxford, UK) was no better in experiment 2.

In the third experiment antibody activation was compared to concanavalin A (Con A) and fibronectin-mediated transduction was compared with a centrifugation protocol for viral supernatant expressing a number of different envelope proteins. There was no evidence of transduction in any of the Con A activated cell populations and these cells did not expand post transduction (*Figure 6.7b*). In the antibody-activated groups

Table 6.2 Comparing methods of non-enriched splenocyte transduction

No	Splenocyte isolation	Activation Conditions	Packaging cell line^	Transduction Method+	% Viability of Cell Populations*	Transduction Efficiency* (%GFP positive)
1	LP	CD3/CD28	Ampho /GALV	spin-supernatant	57-64	A 0.4-3.0 G 0.4-0.7
2	RCL	CD3/CD28	Ampho /VSV-G	spin-supernatant	A 46-57 V 50-51	A 0.5-1.0 V 0
3	RCL	CD3/CD28, Con A	Ampho /VSV-G /Gp+e86	RN, spin-supernatant	C spin 15 C RN 45 Ab spin 16 Ab RN 25	C 0 Ab 3-42 (see *Fig 6.7a*)
4	RCL	PHA	Gp+e86	Co-culture	18-31	**7-52** (see *Fig 6.8*)
5	RCL	PHA	Eco-Pak	Co-culture	12-16	20-32**

(***Key***: LP – centrifugation on a Lympho-Prep gradient; RCL – red cell lysis (see 2.2.6.5); CD3/CD28 (Ab) – activation on immobilised anti-CD3 and anti-CD28 antibodies (2.2.6.5); PHA (P) – phyto-haemagglutinin; Con A (C) – concanavalin A; RN – Retronectin™; +see 2.2.6.6.3 for description of methods; *measured by flow cytometry 72hrs post transduction; ^Ampho (A) and VSV-G (V) refer to 293T cells transfected with pKat and *** packaging plasmids respectively, GALV (G) and Gp+e86 (GP) refers to retrovirus harvested from PG13 and Gp+e86 packaging cells respectively; **used in cytotoxicity assay *Fig 6.9*)

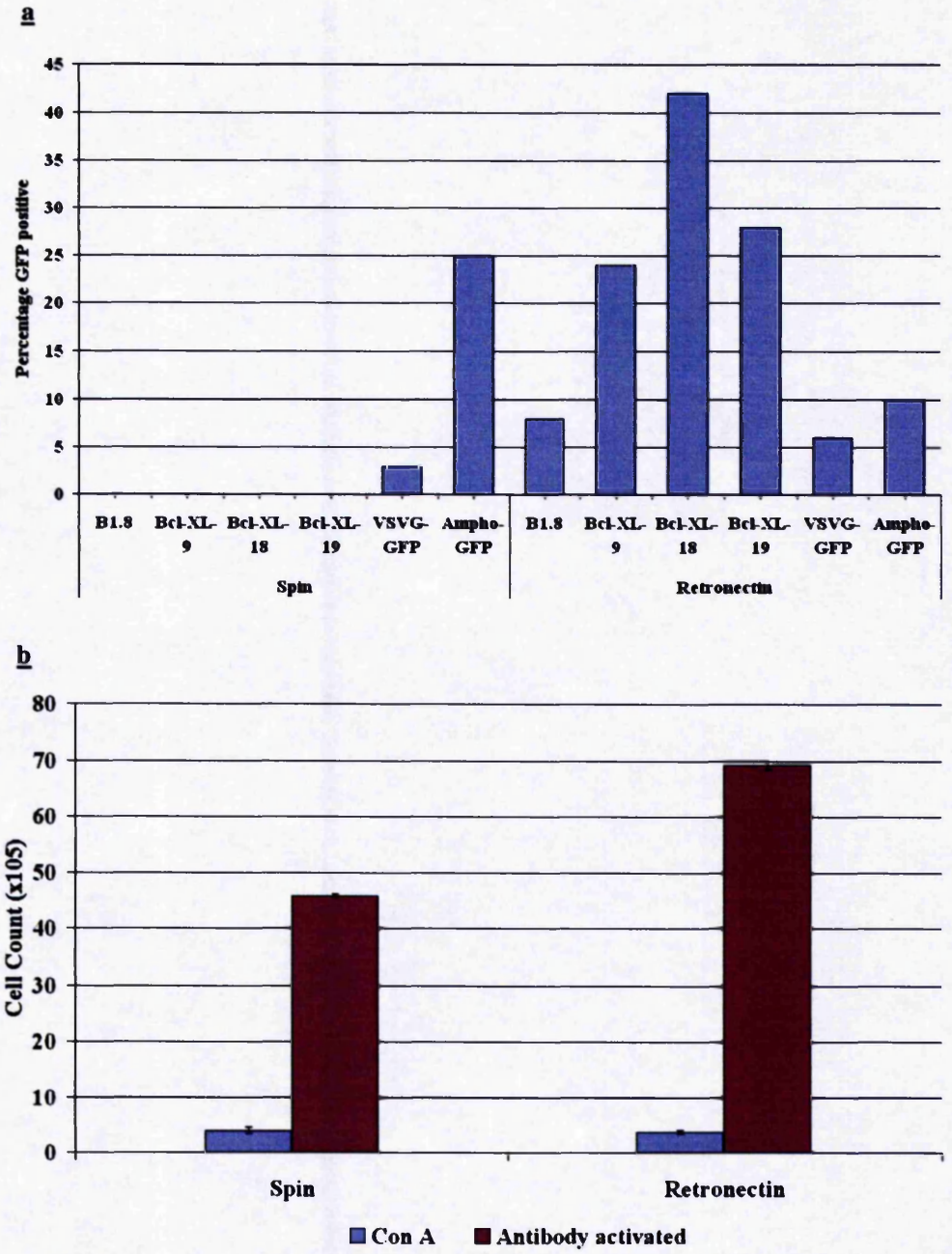


Figure 6.7 Comparing methods of non-enriched splenocyte transduction 1 – Results from experiment 3 (*Table 6.2*) are shown. *Figure 6.7a* shows transduction rates of antibody activated lymphocytes using viral supernatant expressing a number of different envelope proteins in fibronectin-mediated and centrifugation-based transduction protocols. There was no evidence of transduction in any of the Con A activated cell populations (data not shown) and these cells did not expand post transduction (*Figure 6.7b*). *Figure 6.7b* illustrates total cell counts 14 days post transduction ($1.5x10^6$/group initially).

transduced on fibronectin, transduction efficiency ranged from 6-42% (*Figure 6.7a*) with the highest levels of transduction seen using retrovirus made from several of the Gp+e86 rKat.Bcl-X_L.IRES.GFP clones that were tested (see 6.2.1). Strangely the ecotropic Gp+e86 retrovirus did not result in transduction when used as part of a centrifugation protocol. Amphotropic and VSV-G-pseudotyped retrovirus led to transduction rates of 6-25% with both protocols.

Figure 6.7b illustrates that 14 days post transduction on average the spin-transduced, antibody-activated groups had undergone a two-fold expansion, with the fibronectin-transduced, antibody-activated groups expanding three-fold. When analysed by flow cytometry however the proportion of live cells in these populations remained less than 20%.

A co-culture protocol similar to that described by Darcy (Darcy, Haynes et al. 2000) was tested in experiment 4. Splenocytes were co-cultured for 72hrs on Gpe+86 packaging cells with PHA and IL-2 (100iu/ml) activation. The Gpe86 Bcl-X_L.IRES. GFP (clone 18) and B1.8.IRES.GFP (clone 3) clones generated to transduce antigen-specific murine cell lines in section 6.2 were used. *Figure 6.8* shows the transduction rates obtained by culturing increasing numbers of packaging cells ($2.5x10^5$, $5x10^5$ and 10^6) with 10^7 splenocytes in a T25 flask. With the higher number of packaging cells (approx 80-90% confluent when splenocytes added) in both groups approximately 50% of cells expressed GFP after 72hrs. Clearly this result demonstrated that high titre clones of the ecotropic Gp+e86 cell line when used to infect splenocyte populations in a co-culture protocol can give efficient transduction rates.

In order to develop this further, the Darcy co-culture protocol was modified with the substitution of transiently transfected Eco-pack™ cells (see section 6.1) instead of the stable Gp+e86 cloned producers. Using the Eco-pack cell line had the advantage that several constructs could be tested rapidly without the need for prolonged periods of cloning. In addition, at that point in time no further GP+e86 producer cells were available.

Cells were trypsinised 24 hours post transfection and then plated into T25 flasks in the same way as the Gp+e86's. Reasonable transduction rates were obtained using the Eco-pack cell line transfected with the chimeric T cell receptor constructs rKat.CGS-

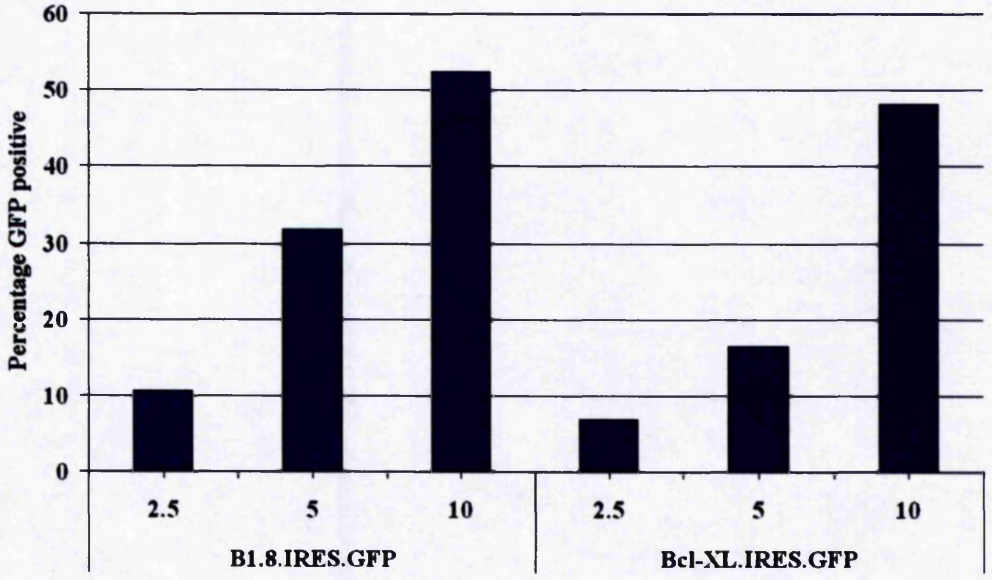


Figure 6.8 Comparing methods of non-enriched splenocyte transduction 2 - Splenocytes were co-cultured for 72hrs on the Gpe86 Bcl-X_L.IRES.GFP (clone 18) and B1.8.IRES. GFP (clone 3) packaging cell lines. The transduction rates obtained (% T cells GFP positive by flow cytometry analysis) by culturing increasing numbers of packaging cells ($2.5x10^5$, $5x10^5$ and 10^6) with 10^7 splenocytes in a T25 flask are illustrated.

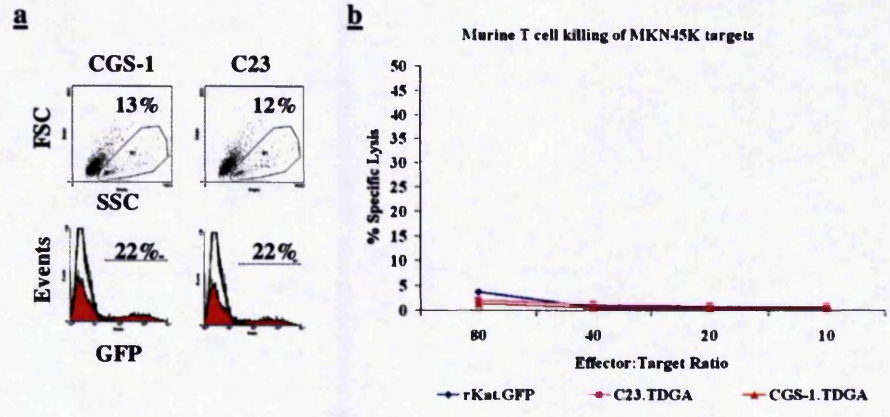


Figure 6.9 – Murine T lymphocytes co-cultured with Eco-pack producers are transduced but not functional in a ^{51}chromium-release cytotoxicity assay – Transduction rates and viabilities from experiment 5 (*Table 6.2*) are shown in *Figure 6.9a.* Post-transduction the splenocytes were centrifuged on a Lympho-prep gradient to remove the dead cells/debris and the function of the remaining chimeric-receptor-expressing cells was tested in a ^{51}Cr release assay against the CEA-expressing MKN45K gastric tumour cell line (*Figure 6.9b*).

1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP (22% GFP positive – *Figure 6.9a*). T cell expressing the C23.CD3ζTDGA chimeric receptor should target cells expressing CEA, and CGS-1.CD3ζTDGA receptor expressing T cells should target a protein found in tumour neovasculature (see Chapters 1 and 7 for fuller explanation).

Following transduction, the splenocytes were centrifuged on a Lympho-prep gradient to remove dead cells and debris and the function of the remaining chimeric-receptor-expressing cells was tested in a ^{51}Cr release assay (methods 2.2.6.9) against the CEA-expressing MKN-45-K gastric tumour cell line. *Figure 6.9b* illustrates that the C23.CD3ζ-expressing splenocytes (targeted to CEA) showed no evidence of cytotoxicity in this assay.

Figure 6.9a indicates the probable cause of this lack of function. As with many of the approaches tested so far, the proportion of viable cells following the transduction process was very low and the cells were clearly in a poor condition when the assay was performed.

Genetic modification of whole splenocyte populations has been successfully described (Hagani, Riviere et al. 1999). However, T lymphocytes constitute only 30% of the murine splenic cell population (Coligan 2002). The rest consists of a variety of different haematopoietic cells the majority of which will survive for only a short period of *in vitro* culture and one possibility is that this was impairing the activation and expansion of the T cells. An alternative explanation was that the T cells were suboptimally activated. The anti-CD3 and anti-CD28 antibodies used in the experiments to date (37.51 and 145-2C11) had previously been prepared 'in house' from hybridoma supernatant.

In view of these considerations, in 6.3.2 a method of T cell enrichment was studied and commercial anti-CD3 (145-2C11) and anti-CD28 (37.51) antibodies (Pharmingen) were purchased and used in the following experiments 6.3.3-6.4.

6.3.2 T cell enrichment using Nylon wool columns

The use of nylon wool columns to enrich for T cell populations was first described in 1973 (Julius, Simpson et al. 1973). It is a relatively simple procedure and has the advantage of not requiring antibodies or beads for separation, whilst still yielding populations of T cells sufficiently enriched for most purposes.

Table 6.3 Enriched T cell yield following nylon wool column separation

Experiment	No of splenocytes loaded on to column	No of cells obtained post enrichment	% Yield
1	$4.5x10^7$	$1x10^7$	22
2	$16x10^7$	$2.3x10^7$	14
3	$15x10^7$	$3x10^7$	20

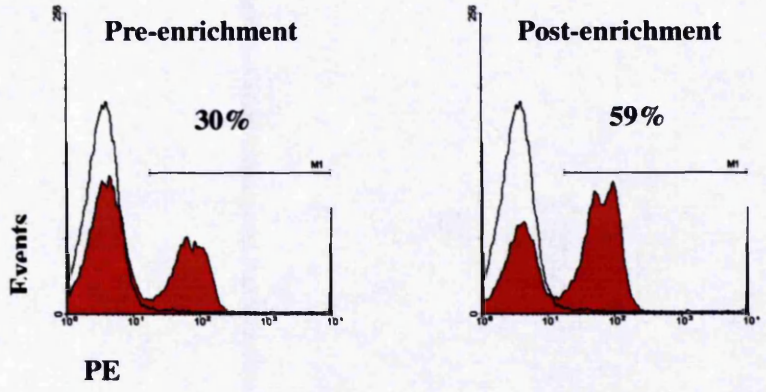


Figure 6.10 Murine T cell enrichment using a nylon wool column - Splenocytes were obtained from C57bl mouse spleens and passed through a nylon wool column as described in section 2.2.6.5. Aliquots of cells taken pre and post nylon wool column enrichment were stained with an anti-CD3 PE-conjugated antibody and analysed by flow cytometry (Isotype control staining is shown as clear line).

In this experiment, splenocytes were obtained from C57Bl/6 mouse spleens and passed through a nylon wool column (methods 2.2.6.5). The number of viable cells obtained in three separate experiments after passage of splenocytes through a column is illustrated in ***Table 6.3***. As anticipated, yields of 14-22% of the initial cell number were obtained.

In the first of these experiments, aliquots of cells taken pre and post nylon wool column enrichment were stained for CD3 expression in order to examine the degree of T cell enrichment achieved by this procedure. *Figure 6.10* illustrates that in this particular experiment, passage through a nylon wool column increased the percentage of CD3-staining T cells in the population from 30% to 59%.

Nylon wool column enrichment was incorporated into subsequent transductions (6.3.3-6.4). Combined with the use of commercial anti-CD3 and anti-CD28 antibodies, this resulted in a significant improvement in lymphocyte expansion and survival post-transduction (compare *Figures 6.7b* and *6.9a* with *Figures 6.11b* and *c*).

6.3.3 Enriched T cell transduction using EcoPack™ retrovirus

The preliminary experiments described in 6.3.1 indicated that co-culture using cloned Gp+e86 packaging cells appeared to be the most efficient method of transducing murine lymphocytes. However, as discussed above, Gp+e86 packaging cells were not immediately available. Experiments using transfected EcoPack cells in a co-culture protocol (Experiment 5, 6.3.1) had also yielded encouraging transduction efficiencies, but poor cell survival, and this was therefore repeated using nylon wool column enriched T cells. In addition, cells were preactivated using anti-CD3 and anti-CD28 antibodies rather than PHA.

In order to determine the viral titre, supernatant harvested from transiently transfected EcoPack™ cells was used to infect the murine 3T3 cell line (methods 2.2.6.3.3). *Figure 6.11a* shows the efficiency of 3T3 transduction using supernatant from cells transfected with the rKat.GFP, rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA .IRES.GFP constructs. Approximate viral titres were then calculated using the formula detailed in 2.2.6.3.3. Viral titres of 2-4.2x10^5 were obtained in this particular experiment.

In the subsequent transduction experiment, cell expansion both during and after co-culture was significantly better than in previous experiments with greater than fifteen-fold expansion over the first 11 days (*Figure 6.11b*).

a

Vector	% GFP Positive		Viral Titre* (cfu/ml)
	Neat	1:10	
GFP	53	7	$2x10^5$
CGS-1	79	14	$4.2x10^5$
C23	67	9	$3x10^5$

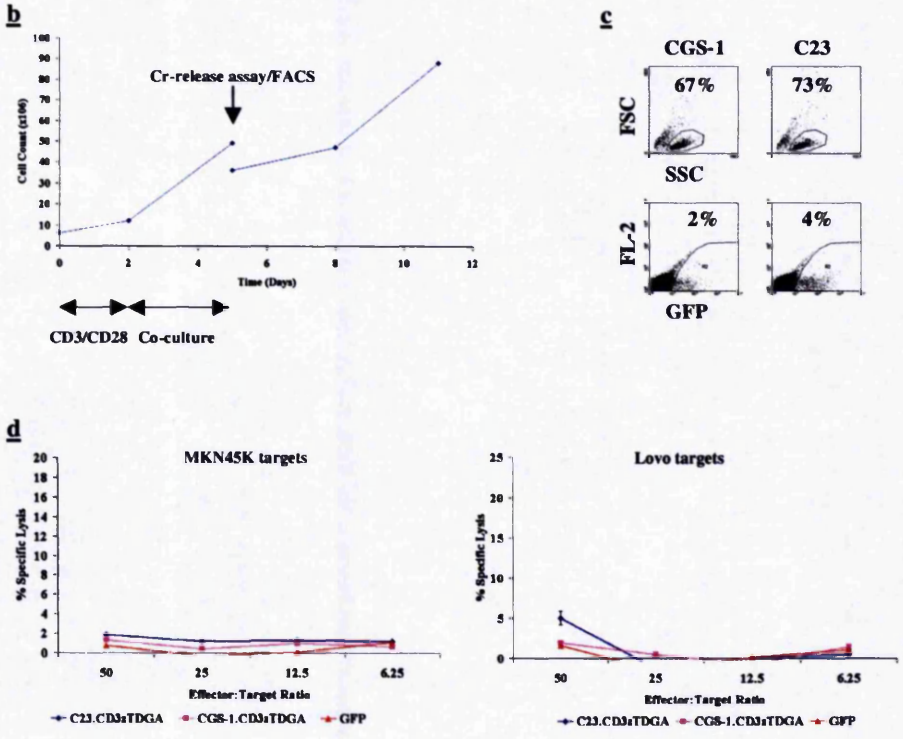


Figure 6.11 Enriched T cell transduction using EcoPack™ retrovirus - (a) Transduction of 3T3 cells using virus harvested from transfected EcoPack cells and calculation of viral titre (see methods 2.2.6.3.3). Viral Titre (*cfu/ml, colony forming units per ml)= (%GFP positive cells (as fraction of cell population)) x (number of target cells plated x2) x (1/amount of viral supernatant used in ml) x (dilution factor), (b) a co-culture protocol was used to transduce enriched T cells; expansion of cells over first 11 days is shown, (c) Immediately following co-culture on day 5, aliquots of cells were analysed by flow cytometry and, (d) functional chimeric receptor expression was tested in a ^{51}Cr-release cytotoxicity assay against the CEA-expressing MKN45K and LoVo cell lines.

Immediately following co-culture on day 5, aliquots of cells were analysed by flow cytometry and functional chimeric receptor expression was tested in a ^{51}Cr-release cytotoxicity assay against the CEA-expressing MKN45K and LoVo cell lines (*Figure 6.11c* and *d*). Lymphocyte viability (67-73%) was significantly improved, however transduction efficiency was disappointing (2-4% GFP positive) and this is the most likely explanation for the absence of any significant cytotoxicity in the ^{51}Cr-release assay.

6.3.4 Enriched T cell transduction using PT67 retrovirus

Recently, transduced PT67 packaging cells (expressing an envelope based on the 10A1 serotype of murine leukaemia virus), produced high titres of retrovirus that gave high efficiency gene transfer in to antigen-specific primary mouse lymphocytes, without the need for cloning high titre producing packaging cell lines (Annenkov, Daly et al. 2002). These polyclonal packaging cell populations retained their viral titre for greater than one year. Potentially using these cells would have the advantages of a stable packaging cell line without the need for long periods of cloning and this approach was therefore examined.

6.3.4.1 Transduction of PT 67 packaging cell line

In order to create retrovirus-producing packaging cell lines, PT67 packaging cells were transduced using ecotropic rKat.GFP, rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat .C23.CD3ζTDGA.IRES.GFP retrovirus (methods 2.2.6.6.1) made by transient transfection of EcoPack™ cells. Transduction efficiencies were analysed by flow cytometry analysis of GFP expression. Two separate transductions were performed using EcoPack viral supernatant obtained from different transfections and high levels of transduction were achieved (*Figure 6.12*).

Although a definitive formal assessment of viral titre of these populations was not completed, at a 1:10 dilution supernatant from the rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP-transduced populations shown in *Figure 6.12b* resulted in transduction efficiencies of 3T3 cells of 97% and 98% respectively.

These polyclonal transduced packaging cell populations were then used in co-culture transduction experiments and to produce PT67 virus for supernatant-based protocols.

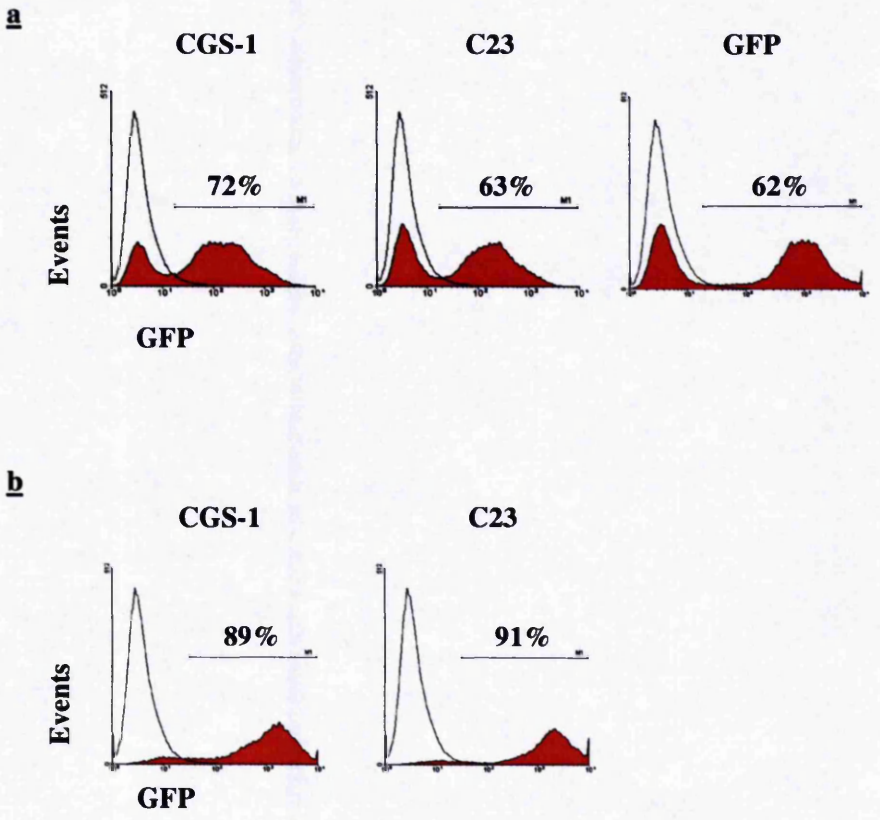


Figure 6.12 Transduction of PT67 packaging cell line - PT67 packaging cells were transduced using ecotropic (EcoPack™) rKat.GFP, rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP retrovirus. Transduction efficiencies were analysed by flow cytometry analysis of GFP expression. Two separate transductions were performed (*Figure 6.12a* and *b*).

6.3.4.2 Comparing methods of enriched T lymphocyte transduction

Enriched murine T lymphocytes were isolated as described in 2.2.6.5 and activated on anti-CD3 and anti-CD28 antibodies. Supernatant harvested from the rKat.GFP-transduced PT 67 cell population (*Figure 6.12*) was compared with amphotropic retrovirus (derived from 293T cells transiently transfected with the pKat packaging plasmid), retrovirus pseudotyped with VSV-G (prepared again by a transient transfection method) and ecotropic (Eco-Pack) retrovirus in fibronectin and centrifugation-based protocols (2.2.6.6.3). The same rKat.GFP vector was used in all groups. In addition, a co-culture protocol using the rKat.GFP-PT 67 cells was tested. The results are shown in *Table 6.4*.

As with previous attempts employing a supernatant-based protocol to transduce non-enriched murine cells, transduction efficiencies were poor, with only amphotropic, retronectin–based transduction reaching double figures at 12%.

In contrast, rKat.GFP-PT 67 co-culture gave a transduction rate of 35% (unfortunately Eco-Pack co-culture was not performed as a comparison in this experiment) and this method of transduction was further investigated in 6.3.4.3-6.3.4.4.

6.3.4.3 Determining optimal period of activation and co-culture for PT67-based protocol

This experiment was performed to determine the optimal period of enriched T lymphocyte pre-activation (on anti-CD3 and anti-CD28 antibodies) and length of co-culture required in order to achieve the highest levels of both transduction and viability. Enriched murine T lymphocytes were isolated as described in 2.2.6.5 and activated on anti-CD3 and anti-CD28 antibodies for 24, 48 and 72 hours respectively. Immediately following activation lymphocytes were co-cultured with rKat.GFP-transduced PT67 packaging cells (*Fig 6.12a*) for 24, 48 or 72 hours respectively, making a total of nine different groups. Samples of cells from all groups were analysed by flow cytometry 72 hours after the final group of lymphocytes had been removed from co-culture and the viability and level of GFP expression in each group is shown in *Figure 6.13*.

Reducing the period of CD3/CD28 activation decreased both cell viability and transduction efficiency. Reducing the period of co-culture did not improve cell viability and reduced transduction rates.

Table 6.4 Comparing methods of enriched T lymphocyte transduction

	Retronectin (%GFP+ve)	Centrifugation (%GFP+ve)	Co-culture (%GFP+ve)
Amphotropic	12%	9%	-
Ecotropic	5%	6%	-
VSV-G	4%	6%	-
PT67	5%	5%	35%

(Figures shown are the % of live cells expressing GFP as assessed by flow cytometry 72 hours post transduction – see text 6.3.4.2)

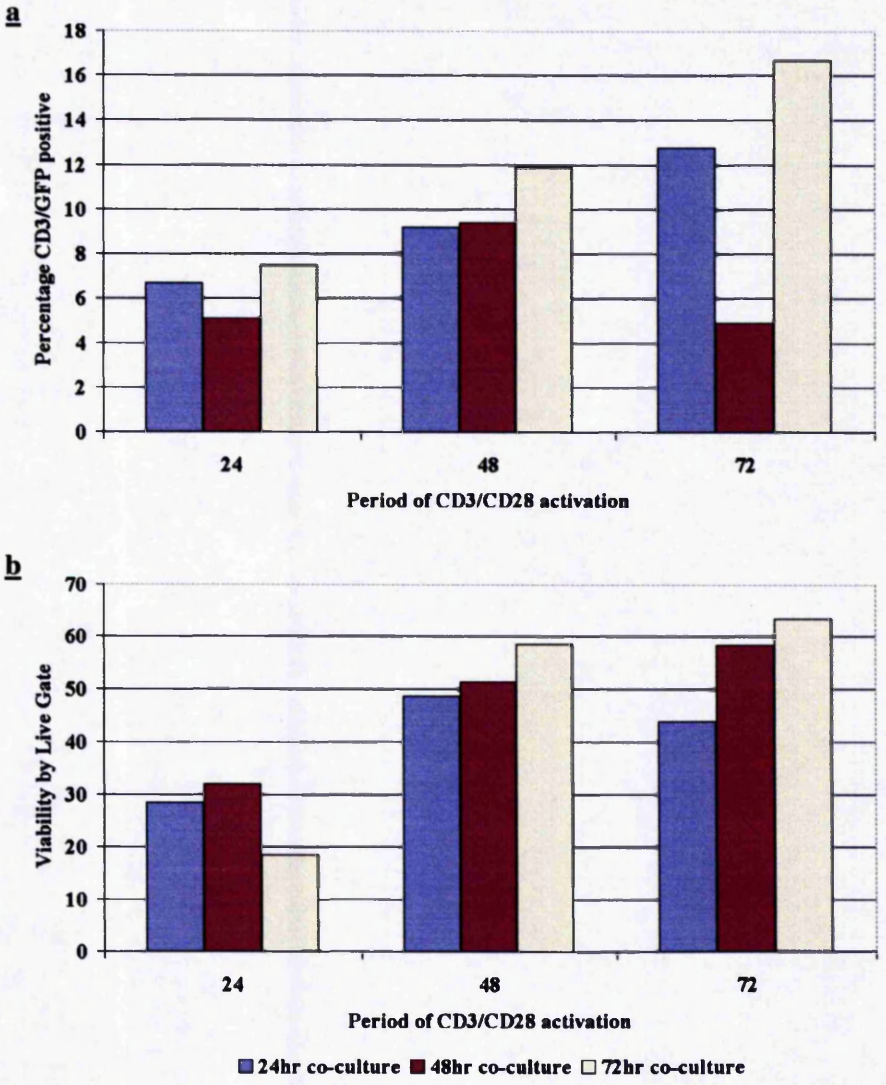


Figure 6.13 Determining optimal period of activation and co-culture for PT67-based protocol - Enriched murine T lymphocytes were isolated and activated on anti-CD3 and anti-CD28 antibodies for 24, 48 and 72 hours respectively. Immediately following activation lymphocytes were co-cultured with rKat.GFP-transduced PT67 packaging cells (*Figure 6.12a*) for 24, 48 or 72 hours respectively, making a total of nine different groups. Samples of cells from all groups were analysed by flow cytometry 72 hours after the final group of lymphocytes had been removed from co-culture.

6.3.4.4 Optimized T cell transduction using PT67 co-culture

Enriched murine T lymphocytes were isolated and activated on anti-CD3 and anti-CD28 antibodies for 72 hours. Lymphocytes were then co-cultured with rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP-transduced PT67 packaging cells (*Figure 6.12b*) for 72 hours.

72 hours post co-culture, aliquots of cells were analysed by flow cytometry (*Figure 6.14a*) and functional chimeric receptor expression was tested in both a murine interferon-γ release assay against immobilised antigen (*Figure 6.14b* - methods 2.2.4.3) and a ^{51}Cr-release cytotoxicity assay against the CEA-expressing MKN45K cell line (*Figure 6.14c* – methods 2.2.6.9). Lymphocyte transduction efficiency was again disappointing with only 3-4% of cells expressing GFP. Despite these low transduction rates, C23.CD3ζ-transduced lymphocytes did produce interferon-γ upon contact with CEA (*Figure 6.14b*), but demonstrated no significant cytotoxicity in the ^{51}Cr-release assay (*Figure 6.14c*). In this assay, CGS-1.CD3ζ-transduced lymphocytes did not produce interferon-γ upon contact with its target, recombinant ED-B protein.

6.4 Expansion of murine lymphocytes by allogeneic stimulation

In this preliminary experiment, CD3/CD28 antibody stimulation was compared with the use of irradiated allogeneic dendritic cells as a method of expanding and maintaining the viability of murine lymphocytes *in vitro*, with a view to potentially using this as a way of activating lymphocytes prior to retroviral transduction and enabling long term survival of transduced cells.

Murine T lymphocytes were isolated and enriched from a fresh C57BL/6 spleen. Cells were then divided into five groups ($5x10^6$/group) and cultured over a period of 50 days. Activation conditions were IL-2 (100u/ml), anti-CD3/CD28 antibody for 72 hours then IL-2 100u/ml), weekly stimulation with irradiated XS52 cells (an allogeneic Balbc dendritic cell line) plus IL-2 (100u/ml), or weekly stimulation with irradiated DC 2.4 cells (an autologous C57BL/6 dendritic cell line) plus IL-2 (100u/ml). Aliquots of cells in each group were counted by trypan blue exclusion every 48-96hrs and the results are shown in *Figure 6.15*.

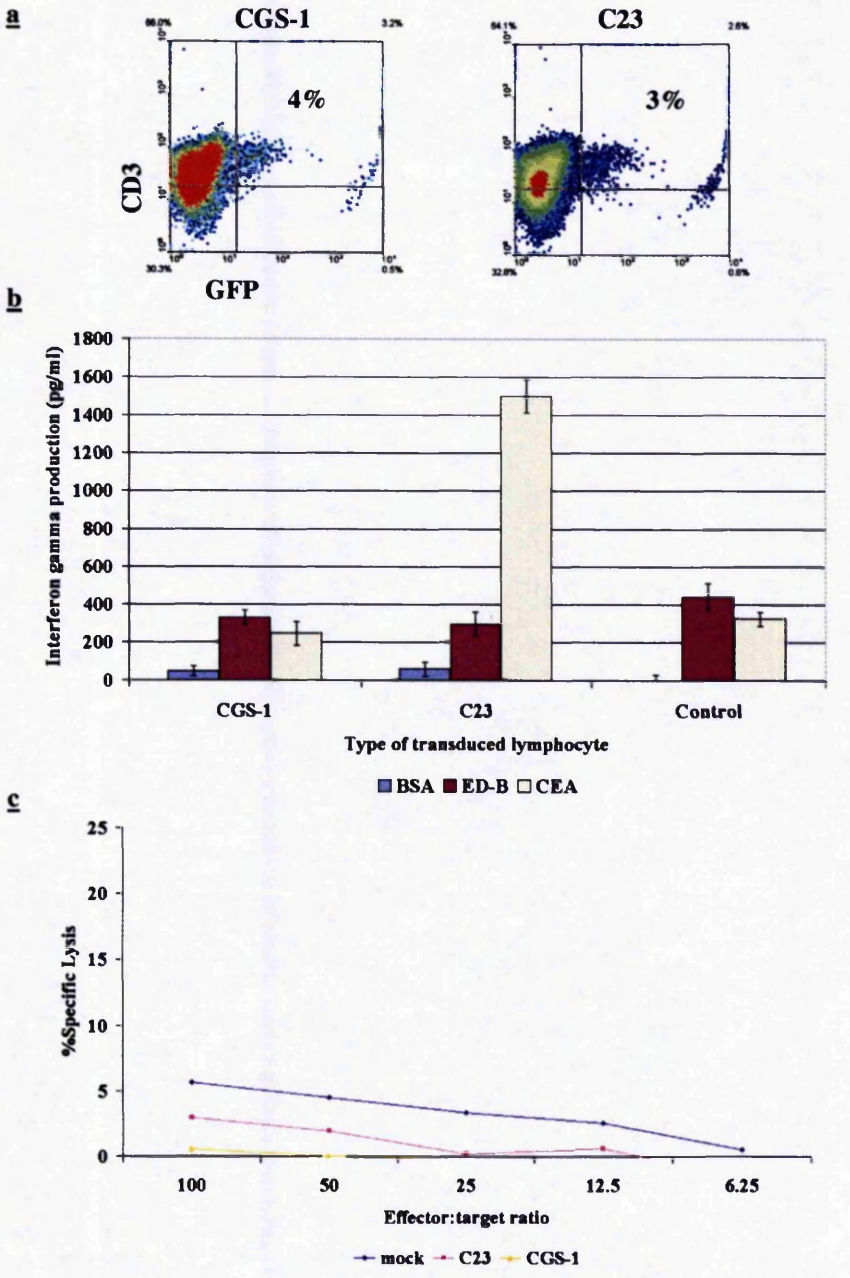


Figure 6.14 Optimized T cell transduction using PT67 co-culture - Enriched murine T lymphocytes were co-cultured with rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23. CD3ζTDGA.IRES.GFP-transduced PT67 packaging cells (*Figure 6.12b*) for 72 hours. Cells were analysed by flow cytometry (*Figure 6.14a*) and functional chimeric receptor expression was tested in both a murine interferon-γ release assay against immobilised antigen (*Figure 6.14b*) and a ^{51}Cr-release cytotoxicity assay against the CEA-expressing MKN45K cell line (*Figure 6.14c*).

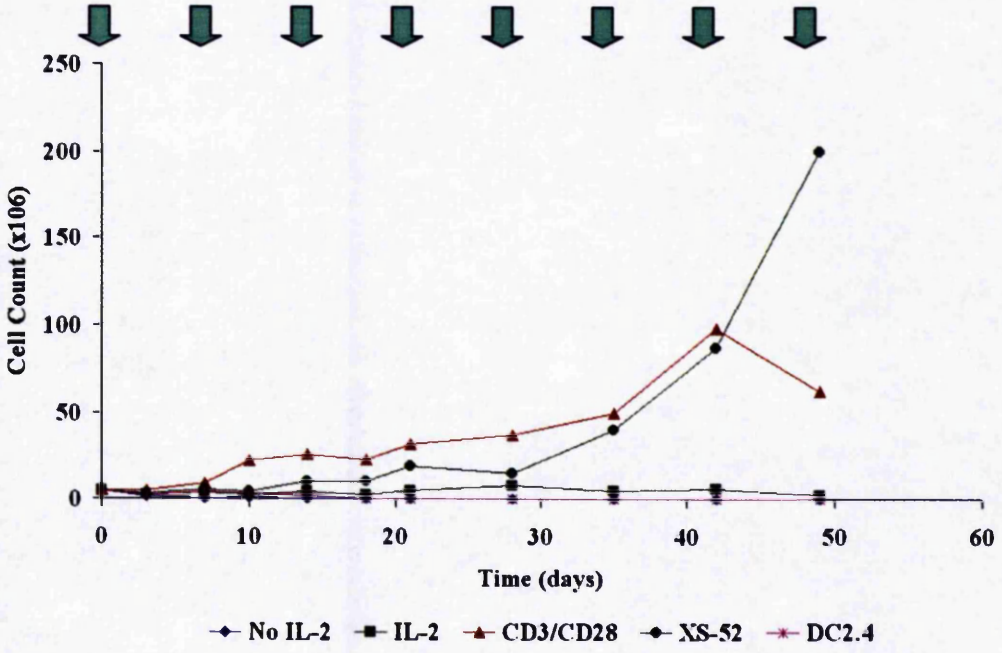


Figure 6.15 – Expansion of murine lymphocytes by allogeneic stimulation - Murine T lymphocytes were isolated and enriched from a fresh C57Bl/6 spleen. Cells were divided into five groups ($5x10^6$/group) and cultured over a period of 50 days. Activation conditions were IL-2 (100u/ml), no IL-2, anti-CD3/CD28 antibody for 72 hours then IL-2 100u/ml), weekly stimulation with irradiated XS52 cells (an allogeneic Balbc dendritic cell line) plus IL-2 (100u/ml), or weekly stimulation with irradiated DC 2.4 cells (an autologous C57B dendritic cell line) plus IL-2 (100u/ml). Aliquots of cells in each group were counted by trypan blue exclusion every 48-96 hours. Green arrows indicate weekly DC stimulation.

Unstimulated lymphocytes and lymphocytes stimulated with autologous dendritic cells died within 20 days. Lymphocytes stimulated with IL-2 maintained viability but did not expand. As might be expected (given that only a proportion of the lymphocyte population would have been alloreactive) over the initial 20 days expansion in the CD3/CD28 activated group was greater than in the allogeneically-stimulated group. However, after 40 days expansion in this group began to decline. In contrast, in the lymphocyte group stimulated with irradiated allogeneic dendritic cells there is a logarithmic expansion in cell numbers between day 30 and day 50. This would suggest that for long-term in vitro culture allogeneic stimulation could be a useful alternative to antibody-stimulation.

6.5 Discussion

In this chapter, the genetic modification of antigen-specific and fresh murine lymphocytes was investigated.

An antigen-specific primary murine T cell line with specificity against a B cell lymphoma idiotype was successfully genetically-modified to over-express Bcl-X_L using ecotropic retrovirus produced from a cloned stable Gp+e86 packaging cell line. Transduced cells underwent a limited expansion and retained *in vitro* cytolytic activity in a ^{51}Cr-release assay. However, despite weekly stimulation using irradiated dendritic cells, expansion of the transduced cells tailed off and in a subsequent *in vivo* lymphoma model adoptively transferred transduced T cells failed to demonstrate activity.

Clearly, this approach had the advantage of only requiring the expression of a single functional transgene (since the target lymphocyte population was already antigen-specific) and appeared to be the simplest method of studying the hypothesis that Bcl-X_L over-expression in T cells may improve *in vivo* survival and function.

There are a number of deficiencies and possible explanations that may explain the failure of the model. Firstly, the transduced T cells were no longer expanding when adoptively transferred suggesting perhaps that the retroviral transduction process and the lengthy *in vitro* culture following this may have affected the condition of these cells. This is supported by the flow cytometry analysis performed immediately prior to the model (*Fig 6.4b*) that indicated only approximately 43% of cells to be viable by FSC vs SSC live cell gating. However, against this argument is the fact that the majority of these cells were transduced (61%) and they retained comparable (if not better) cytolytic

activity (when compared with non-transduced T cells) in ^{51}Cr-release assays again performed immediately prior to the *in vivo* model.

In contrast to the study shown in *Figure 6.1* (where a small but distinct effect was seen at a dose of 10^6 T cells/mouse), in this study the *in vivo* function of the non-transduced A20 idiotype-specific T cells was indistinguishable from control PBS injections. It is possible therefore that the overall cytolytic capacity of these cells was reduced compared to the cells used in the previous study. In retrospect, treating a further group of animals with the higher dose of 10^7 T cells would have clarified this issue.

Another omission was the failure to perform a western blot comparing Bcl-X_L expression in the rKat.Bcl-X_L.IRES.GFP-transduced A20-specific cells with the non-transduced A20-specific T cells. This was due, in part, to the small number of cells available. This information would have been extremely useful however as another possibility is that the prolonged in vitro culturing with repeated antigenic stimulations involved in generating the antigen-specific T cell line either selected for or resulted in an already high level of Bcl-X_L expression in the non-transduced cell population rendering further Bcl-X_L expression unlikely to make a difference.

The alternative simple explanation for the result, of course, is that Bcl-X_L over-expression does not improve the in vivo function of idiotype-specific lymphocytes when used in this model.

In the second half of this chapter the genetic modification of fresh murine lymphocytes was studied with a view to developing the *in vitro* work described in Chapter 7 testing the targeting of lymphocytes through the expression of tumour vasculature-specific chimeric T cell receptors.

Different activation and transduction protocols were studied employing retroviruses that expressed alternate envelope proteins. The use of cloned Gp+e86 packaging cell lines (prepared for the work described earlier in the chapter) resulted in the highest level of transduction of whole splenocyte populations, however this work was hampered by an inability to expand and maintain a viable population of transduced cells (for example, *Fig 6.9a*).

In view of these problems two changes were made to the transduction protocol. Prior to activation and transduction a purer T lymphocyte population was obtained by passing splenocytes through a nylon wool column (*Fig 6.10*) and commercial anti-CD3 (37.51)

and anti-CD28 (145-2C11) antibodies were used to activate the lymphocytes instead of the 'hybridoma' antibodies used until then. These changes resulted in a significantly improved cell expansion and viability post transduction (eg *Fig 6.11b-c*).

As discussed earlier in the chapter, this protocol was then developed by substituting Gp+e86 producers with an alternative (transiently transfectable) ecotropic packaging cell line, EcoPack^{TM}2-293 (Clontech) and subsequently, the amphotropic 10A1 expressing PT67 packaging cell line. Both of these approaches, in theory, had the advantage that reasonable titre virus could be produced without the need for long periods of cloning.

However, at between 2 and $4x10^5$ cfu/ml, the retroviral titre of supernatants harvested from transfected Eco-Pak cells was significantly lower than had been expected ($>10^6$ cfu/ml). As a consequence of this, although cell viability and expansion was good, transduction efficiencies were poor (*Figure 6.11c*) and it may well be that this explains the lack of targeted cytotoxicity seen using these lymphocytes in a ^{51}Cr-release assay (*Figure 6.11d*).

In a recent publication, transduced PT67 packaging cells had produced high titres of retrovirus that gave high efficiency gene transfer in to antigen-specific primary mouse lymphocytes (when combined with centrifugation), without the need for cloning high titre producing packaging cell lines (Annenkov, Daly et al. 2002). In order to test this PT67 cells were transduced with the rKat.GFP, rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP vectors. Use of PT67 rKat.GFP virus was then compared with amphotropic, ecotropic and VSV-G pseudotyped rKat.GFP virus in supernatant-based centrifugation and fibronectin protocols (*Table 6.4*). A low level of transduction was seen in all groups, with PT67 virus giving a transduction efficiency of only 5% when combined with centrifugation (although the speed of centrifugation used in this experiment was significantly lower (1200G) than that described by Annenkov and colleagues (2000G)). In contrast, rKat.GFP-PT 67 co-culture gave a transduction rate of 35% and therefore this method of transduction was further investigated.

An experiment was performed to determine the optimal period of murine T cell pre-activation and PT67 co-culture (*Figure 6.13*). The result of this study were that increasing the period of T cell pre-activation from 24 to 72 hours improved both cell viability and also transduction efficiency. For each group the duration of co-culture did

not affect viability and there was a trend towards poorer transduction rates in the 24 hour co-culture groups as compared with 72 hours.

Disappointingly, when this optimised PT67 co-culture protocol was then used to transduce T cells to express the CGS-1.CD3ζTDGA- and C23.CD3ζTDGA- chimeric receptors very low level transduction was again seen (*Figure 6.14a*). Despite these low levels of transduction, evidence of specific functional C23.CD3ζTDGA- chimeric receptor cell surface expression was demonstrated by the production of high concentrations of interferon-γ when the cells were cultured in the presence of the target antigen, CEA, but not BSA or ED-B controls (*Figure 6.14b*).

Despite this however no significant cytotoxicity was again seen in a ^{51}Cr-release assay against the CEA-expressing MKN45K cell line (*Figure 6.14c*).

It is not clear why, in these later experiments, no chimeric receptor activity was seen in cytotoxicity assays, despite definite evidence (in terms of interferon-γ release) of chimeric receptor expression. The efficiencies of murine lymphocyte transduction obtained, however, were poor and this is likely to be one of the major reasons for the failure to demonstrate activity.

In retrospect (bearing in mind the reasonable transduction rates obtained in section 6.2), an alternative would have been to generate high titre stable Gp+e86 lines producing the chimeric T cell receptor constructs (rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP) under investigation. Unfortunately, empty Gp+e86 packaging cells were not available at the time this later work was performed.

It became apparent during these experiments that a major limiting factor was an inability to maintain murine lymphocytes in *in vitro* culture following genetic modification for periods longer than 10 to 14 days. In contrast, the A20-specific primary cell line used in section 6.2 had been cultured *in vitro* for a period of greater than one year (by Dr Armstrong) by a protocol involving weekly stimulation with irradiated syngeneic dendritic cells that had been transduced to express the A20 antigen.

In the absence of syngeneic dendritic cells expressing CEA or ED-B, in section 6.4 a preliminary experiment was performed studying the feasibility of using irradiated allogeneic dendritic cells (aDC) to maintain transduced lymphocytes post-transduction. As might be expected (given that only a minority of the cell population will be

alloreactive) in the first four weeks of culture, the cell group stimulated by aDCs did not expand as fast as the CD3/CD28 activated group, however there was a dramatic expansion in cell numbers in the aDC group between days 30 and 50 suggesting that for long-term in vitro culture allogeneic stimulation could be a useful alternative to antibody-stimulation. This was a pilot study and is the subject of ongoing work.

7 Results - Targeting lymphocytes to tumour neovasculature

7.1 Introduction

The initial aim of the work in this chapter was to genetically-modify human peripheral blood lymphocytes to express an antibody scFv fragment (CGS-1) on the cell surface that was specific for an antigen found in and around tumour neovasculature (ED-B protein). Such gene-modified lymphocytes could potentially be targeted to the vasculature of tumours where they could also be engineered to secrete a therapeutic protein at locally high concentrations. An approach of this kind could provide an excellent system of *in vivo* protein delivery. Essentially, the lymphocytes would be acting as 'cellular vectors' in order to deliver the desired gene expression to the target tissue (tumour blood vessels) (see *Figure 7.1*).

One obstacle to the success of the above approach was the requirement for high-level expression of two transgenes from the same retroviral vector in order to achieve efficacy. In the latter half of the chapter, in a similar strategy that potentially required the expression of only one transgene, the use of chimeric TCR-modified lymphocytes to target the same vascular antigen, was investigated. As discussed in the introduction (section 1.2.5), this strategy involves fusing the antigen-recognition domain (ie scFv) of a specific anti-tumour antibody with intracellular T cell receptor signaling chains to form so-called 'chimeric' T cell receptors (*Figure 1.4*). Upon contact with tumour antigen cytotoxic T lymphocytes modified to express such receptors are specifically activated without the need for MHC expression. This could, in itself, result in an immune-mediated destruction of tumour vasculature whilst still allowing the targeted delivery of other co-expressed therapeutic gene products.

7.1.1 Targeting Tumour Vasculature

Targeting tumour angiogenesis is appealing for several reasons. The growth and metastatic spread of most solid malignancies is dependent on the formation of new blood vessels (Folkman 1990). Endothelial cells, unlike tumour cells, are genetically stable and do not mutate and resistance to therapeutic agents is therefore less likely to develop. In addition, it is likely that targets on newly formed blood vessels should be readily accessible to therapies given intravenously, overcoming the well-recognised

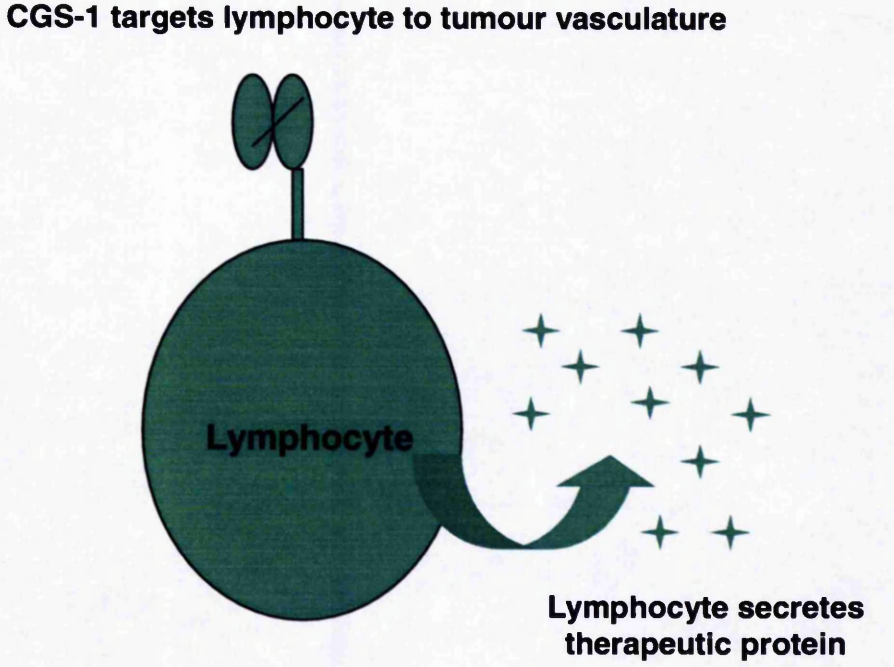


Figure 7.1 - Gene-modified adoptive cellular therapy approach to achieving transgene expression within tumour neovasculature

problem of poor tumour penetration of targeting agents that are specific for actual tumour cells.

Consequently, there has been a great deal of interest in therapeutic anti-angiogenic strategies. 'Cytostatic' approaches have involved the use of small molecules (Boehm, Folkman et al. 1997) or soluble receptors (Aiello, Pierce et al. 1995) to inhibit further development of vasculature. In a classic example of the potential for this approach, it was demonstrated in 1997 that the use of the potent angiogenesis inhibitor endostatin caused regression of experimental lung tumours in mice without the development of resistance to therapy that can occur when using conventional chemotherapy (Boehm, Folkman et al. 1997).

Alternatively, other 'cytotoxic' strategies aim to destroy tumour vasculature through the targeted delivery of toxins or thrombotic agents linked to antibodies or receptor ligands (Ramakrishnan, Olson et al. 1996) (Burrows and Thorpe 1993). Such strategies could potentially provide for prolonged inhibition of tumour growth by both preventing new tumour growth and destroying existing neovasculature.

Potential anti-angiogenic gene therapy employing a cytostatic strategy could involve the local delivery of genes encoding angiogenesis inhibitors (such as endostatin or thrombospondins) (Feldman, Alexander et al. 2001) or genes that inhibit pro-angiogenic cytokines (eg sFLT-1 or VHL) (Mori, Arii et al. 2000). Alternatively, gene therapy could feasibly deliver cytotoxic gene products such as toxins or prodrugs (eg HSV –TK) to the tumour vasculature.

Lymphocytes that were targeted to these vessels would be ideally placed to express either type of gene product, or, through the activation of chimeric TCR receptors, could mediate a cell-based immune response.

7.1.2 The ED-B Oncofetal Domain – A Marker of Angiogenesis

Fibronectins (FN) are large molecular weight adhesive glycoproteins that are found in a soluble form in plasma and other body fluids and in an insoluble form within the extracellular matrix. They are thought to be involved in various biological phenomena including cell adhesion, establishment and maintenance of normal cell morphology, differentiation, wound healing and cell migration (Alitalo and Vaheri 1982; Yamada 1983).

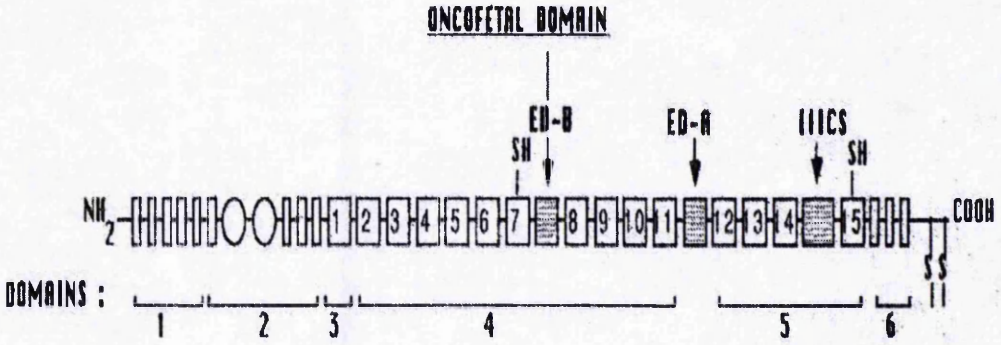


Figure 7.2 Model of the Domain Structure of a Subunit of Human Fibronectin - The ED-B (or Oncofetal) domain is indicated (modified from Castelliani et al, 1994). Also shown are the two other sites of alternative splicing (ED-A and IIICS)

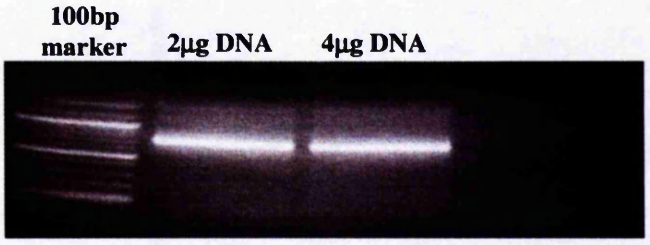


Figure 7.3 CGS-1 pcr - CGS-1 ScFv gene was amplified from a plasmid kindly donated by D.Neri (Cambridge, UK) using a standard polymerase chain reaction (methods 2.2.3.2). As expected a 750 bp pcr product was obtained on gel electrophoresis.

Alternative splicing of the mRNA product of the fibronectin gene results in the existence of several polymorphic isoforms (Kornblihtt, Umezawa et al. 1985). One of these is the B-FN isoform containing the Extra Domain B inserted by splicing (*Figure 7.2*, adapted from Castellani *et al* (Castellani, Viale et al. 1994)). ED-B is a 91 amino acid sequence that has been shown to be identical in mouse, rat, rabbit, dog and man (Zardi, Carnemolla et al. 1987).

Total fibronectin appears to be distributed throughout normal adult human tissues. Using immunohistochemical techniques, Carnemolla *et al* (Carnemolla, Balza et al. 1989) studied the distribution of B-FN in human foetal, adult and tumour tissues. They demonstrated that the B-FN isoform appeared to be present only in the stroma of foetal and neoplastic tissues, with the exception of certain synovial cells and the interstitium of the ovary.

Subsequently, other workers (Castellani, Viale et al. 1994; Tarli, Balza et al. 1999) have shown that whilst the B-FN isoform is undetectable in mature blood vessels, it is highly expressed during angiogenesis in both neoplastic and normal tissues.

The ED-B domain of fibronectin therefore appears to be an excellent marker of angiogenesis. The source of B-FN, however, continues to be debated. Recent work (Midulla, Verma et al. 2000) has shown that both tumour and endothelial cells can express B-FN.

7.1.3 CGS-1 - A Human Antibody with Pan-Species Affinity for B-FN

CGS-1 is a human antibody fragment (ScFv) that has previously been isolated from naïve, human phage display libraries with pan-species high affinity for the ED-B domain (Carnemolla, Neri et al. 1996).

Using infrared photodetection, CGS-1 chemically coupled to a fluorophore has been subsequently shown to bind to and image an experimental murine teratocarcinoma in nude mice (Neri, Carnemolla et al. 1997). Immunohistochemical samples of tumour from animals sacrificed twenty-four hours after injection of labelled CGS-1 ScFv showed antibody localisation in new-forming blood vessels. There was also a small amount of staining of the femur (possibly explained by the low levels of B-FN known to be present in synovial cells).

A similar phage-derived antibody with high affinity for the ED-B domain has been shown to selectively localise to newly formed blood vessels in a rabbit model of ocular angiogenesis (Birchler, Viti et al. 1999). When coupled to a photosensitizer and irradiated with red light, the antibody mediated complete and selective occlusion of the ocular neovasculature and promoted apoptosis of the corresponding endothelial cells.

More recently, cytokine (IL-2 and IL-12)-scFv fusion proteins targeted to ED-B have shown evidence of significant anti-tumour activity in *in vivo* murine models (Carnemolla, Borsi et al. 2002; Halin, Rondini et al. 2002) and a tissue factor-scFv fusion protein has been demonstrated to mediate the complete and selective infarction of three different types of solid tumors in mice (Nilsson, Kosmehl et al. 2001). ED-B targeted scFv-modified liposomes are also under investigation as a method of targeting drug delivery to tumour neovasculature (Marty, Odermatt et al. 2002).

7.2 CGS-1 PCR

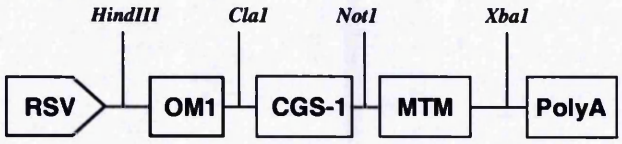
The CGS-1 ScFv gene was amplified from a plasmid kindly donated by D.Neri (University of Cambridge, Cambridge, UK) using a standard polymerase chain reaction (methods 2.2.3.2). Oligonucleotide primers CGS-1-F and CGS-1-R (materials 2.1.3) were designed according to the published CGS-1 sequence in which additional restriction enzyme target sequences (*ClaI* 5' and *NotI* 3') were added. As expected, an approximately 750 base pair PCR product was obtained following DNA separation by gel electrophoresis (*Figure 7.3*).

7.3 Construction of CGS-1 Vectors

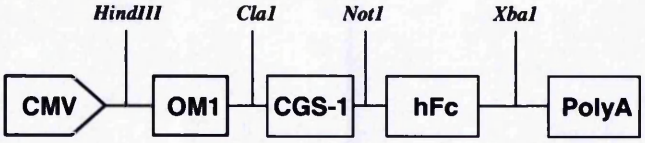
7.3.1 pVOM1.CGS-1.MTM

CGS-1 PCR product and the expression plasmid pVOM1.B1.8.MTM (containing the control scFv, B1.8) were both digested with the *ClaI* and *NotI* restriction enzymes. CGS-1 was subsequently ligated into the pVOM1.MTM vector to make pVOM1.CGS-1.MTM (*Figure 7.4a*). MTM in this construct was the murine MHC class I transmembrane region. Following chemical transformation (methods 2.2.1.3) plasmid DNA was obtained from mini-preps of colonies picked (methods 2.2.1.4). Two diagnostic restriction enzyme digests indicated which plasmids contained the vector pVom1.CGS-1.MTM. This construct linked the scFv CGS-1 to the murine transmembrane region, MTM.

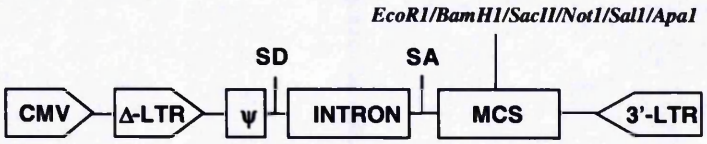
7.4a. pVOM1.CGS-1.MTM



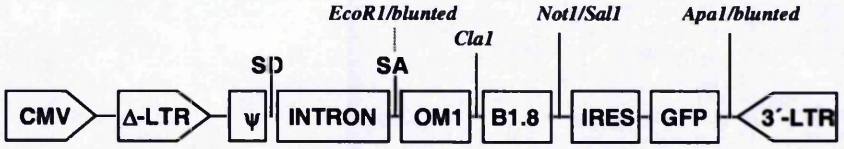
7.4b. pADLOX.CGS-1.hFc



7.4c. rKat.43.267



7.4d rKat.OM1.B1.8.IRES.GFP



7.4e. rKAT.OM1.CGS-1.MTM.IRES.GFP

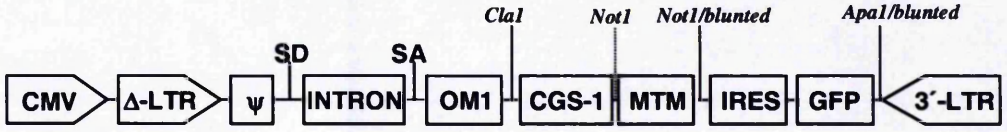
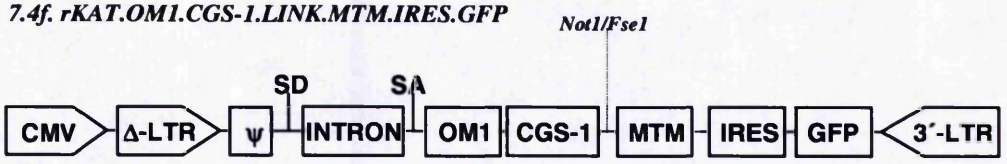
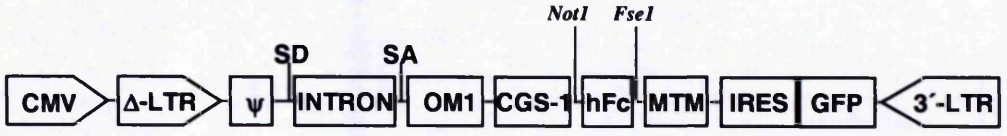


Figure 7.4a-e - Schematic representation of constructs – a) pVOM1.CGS-1.MTM, b) pAdlox.CGS-1.hFc, c) Basic rKat 43.267 vector, d) rKat.OM1.B1.8.IRES.GFP, e) rKat.OM1.CGS-1.MTM.IRES.GFP. ψ = packaging signal, SD, SA are splice donor and splice acceptor sites respectively. MTM is murine MHC class I transmembrane region. hFc, human Fc.

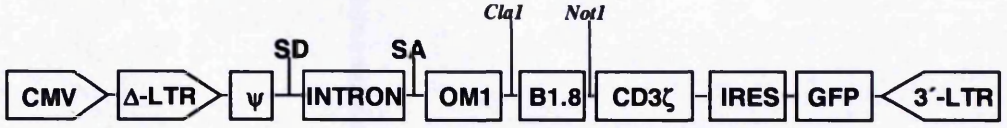
7.4f. rKAT.OM1.CGS-1.LINK.MTM.IRES.GFP



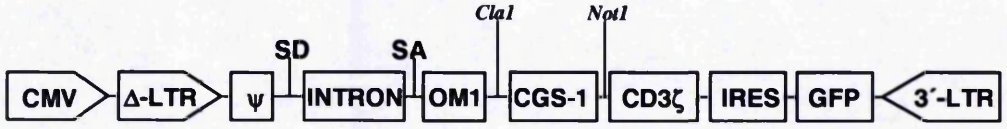
7.4g. rKAT.OM1.CGS-1.hFc.MTM.IRES.GFP



7.4h. rKAT.OM1.B1.8.CD3ζTDGA.IRES.GFP



7.4i. rKAT.OM1.CGS-1.CD3ζTDGA.IRES.GFP



7.4j. rKAT.OM1.CGS-1.hFc.CD3ζTDGA.IRES.GFP

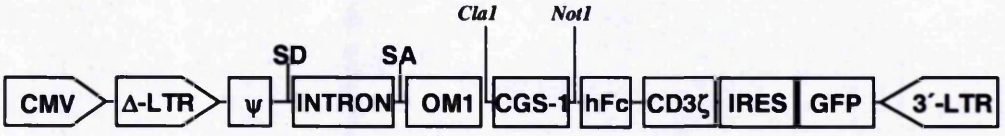


Figure 7.4f-j - Schematic representation of constructs – f) rKat.OM1.CGS-1.LINK.MTM.IRES.GFP, g) rKat.OM1.CGS-1.hFc.MTM.IRES.GFP, h) rKat.OM1.B1.8.CD3ζTDGA.IRES.GFP, i) rKat.OM1.CGS-1.CD3ζTDGA.IRES.GFP. ψ = packaging signal, SD, SA are splice donor and splice acceptor sites respectively. MTM is murine MHC class I transmembrane region. hFc, human Fc.

Prior to further vector construction, the CGS-1 gene was sequenced to check for replication errors occurring during the polymerase chain reaction (section 7.4). An appropriate clone was selected and a maxiprep was prepared (methods 2.2.1.5).

7.3.2 pAdlox.CGS-1.hFc

This construct was made in order to express CGS-1 fused to human Fc as a soluble secreted expression product. Expression vector pAdlox.A20.hFc and pVOM1.CGS-1.MTM were digested with *HindIII* and *NotI* restriction enzymes). The resulting fragment (from the latter vector) containing the oncostatin M leader sequence and CGS-1 was ligated into the vector pAdloxhFc to make pAdlox.CGS-1.hFc (*Figure 7.4b*). Following transformation and miniprep purification, successful ligation was confirmed by diagnostic restriction enzyme digestion.

7.3.3 rKat.OM1.CGS-1.MTM.IRES.GFP

This retroviral vector was a bicistronic construct combining cell surface expression of the CGS-1 scFv (ie linked to the murine transmembrane domain, MTM) with expression of the marker protein, GFP through an IRES.

The Moloney murine leukaemia virus vector rKat 43.267 (Cell GeneSys, CA – introduction section 1.2.2.2) (*Figure 7.4c*) had previously been modified by Dr Ryan Guest (Dept of Medical Oncology, University of Manchester) to contain the oncostatin M leader sequence and the scFv, B1.8 (inserted *blunted EcoR1/Not1* into the multiple cloning site (MCS)). An internal ribosomal entry site (IRES) and the cDNA of enhanced green fluorescent protein (GFP) was also subcloned into the vector by transferring a *Blunted/Sal1* fragment containing IRES-EGFP from pIRES.EGFP (Clontech, California, USA) into the *Apa1(blunted)/Sal1* MCS of rKat 43.267, making the vector rKat.OM1.B1.8.IRES.GFP (*Figure 7.4d*).

In order to construct rKat.OM1.CGS-1.MTM.IRES.EGFP (*Figure 7.4e*), an *Xba1/blunt/Cla1* fragment containing CGS-1.MTM from pVOM1.CGS-1.MTM was ligated into the *Not1/blunt/Cla1* digested rKat.OM1.B1.8.IRES.EGFP vector. Two diagnostic digestions were subsequently performed to confirm successful ligation.

7.3.4 rKat.OM1.CGS-1.hFc.MTM.IRES.GFP

This retroviral vector was identical to rKat.OM1.CGS-1.MTM.IRES.GFP except for a human Fc region inserted in between the CGS-1 scFv and the murine transmembrane

domain. In order to do this an extra *Fse 1* site had to be first introduced into the rKat.OM1.CGS-1.MTM.IRES.GFP vector (*Figure 7.4e*) between CGS-1 and the MTM (3' to the *Not1* site). This was achieved by first designing two linker oligonucleotides (LINK 1 and LINK 2, designed by Dr David Gilham, Department of Medical Oncology) such that when annealed together (37°C for 30mins, 95°C for 15mins, cool slowly to 4°C at 3°C/min) a fragment of DNA was created with *Not1* compatible overhangs and an *Fse1* site. rKat.OM1.CGS-1.MTM.IRES.GFP was then digested *Not1* and dephosphorylated to prevent religation. The linker DNA was ligated into the cut *Not1* site and the new vector was sequenced to ensure that it had inserted in the correct orientation. The linker overhangs were designed such that the *Not1* site 5' to the *Fse1* site was reconstituted, whereas the 3' *Not1* site was not. This created the vector rKat.OM1.CGS-1.LINK..MTM.IRES.GFP (*Figure 7.4f*).

The hFc region was cut out of the vector rKat.OM1.B1.8.hFc.CD3ζTDGA.IRES.GFP as a *Not1/Fse1* fragment. rKat.OM1.CGS-1.LINK.MTM.IRES.GFP was cut *Not1/Fse1* and the hFc was ligated in to create the vector rKat.OM1.CGS-1.hFc.MTM.IRES.GFP (*Figure 7.4g*). This final cloning step and the cloning in section 7.3.5 were performed by Miss Alison O'Neil, Department of Medical Oncology.

7.3.5 rKat.OM1.CGS-1.CD3ζTDGA.IRES.GFP

The retroviral vector rKat.B1.8.CD3ζTDGA.IRES.GFP (*Figure 7.4h*) expressing the control B1.8 chimeric receptor had previously been cloned by Dr David Gilham, Department of Medical Oncology. This vector was digested *Cla1/Not1* and a DNA fragment encoding the CGS-1 scFv (cut *Cla1/Not1* out of rKat.CGS-1.MTM.IRES.GFP) was ligated in in place of the B1.8 scFv. This made the vector rKat.OM1.CGS-1.CD3ζTDGA.IRES.GFP (*Figure 7.4i*).

7.3.6 rKat.OM1.CGS-1.hFc.CD3ζTDGA.IRES.GFP

In a similar way to section 7.3.5, the B1.8 scFv was cut out of the vector rKat.B1.8.hFc.CD3ζTDGA.IRES.GFP that had been previously constructed by Dr David Gilham, Department of Medical Oncology and replaced with the CGS-1 scFv (cut *Cla1/Not1* out of rKat.CGS-1.MTM.IRES.GFP), to make rKat.OM1.CGS-1.hFc.CD3ζTDGA.IRES.GFP (*Figure 7.4j*).

7.4 CGS-1 ScFv Sequencing

Four separate plasmid clones of pVOM1.CGS-1.MTM were used to sequence the CGS-1 ScFv gene (methods 2.2.2.5) using sequencing primers PVACFOR562 and MLMREV1441 (materials 2.1.3). All four clones contained three identical point mutations, as highlighted in *Table 7.1*, at base pair positions 206, 633 and 677 as compared with the published sequence. This results in two amino acid changes, an alanine for a threonine at amino acid position 62 and a proline for an arginine at amino acid position 219. The likelihood of three Taq enzyme replication errors occurring at the same positions in four independent clones would have been very small (in view of the estimated Taq error rate of approximately $1x10^{-5}$). However, in order to fully exclude potential error, primers CGS-1PLASMIDFOR1+2 and CGS-1PLASMIDREV1+2 (Materials 2.1.3) were designed and the original CGS-1 plasmid from which the gene had been amplified was sequenced (*Table 7.1*). This confirmed that the CGS-1 gene in the original plasmid also contained the three mutations. It is possible, that the original published sequence is incorrect.

7.5 CGS-1hFc antibody shows high affinity for recombinant ED-B protein

The adenoviral vector pAdlox.CGS-1.hFc (expressing soluble CGS-1 as an antibody fused to human Fc) (*Figure 7.4b*) was used to transfect 293T cells by calcium phosphate co-precipitation (methods 2.2.6.2). Control transfections were also performed using a similar antibody construct, pAdlox.A20.hFc (expressing hFc fused to a different scFv, A20) and a marker of transfection efficiency, rKat.GFP. Supernatants were harvested at 48hrs.

An indirect ELISA was performed (methods 2.2.4.1) using wells coated with recombinant ED-B protein (10μg/ml) and various dilutions of supernatant. Results shown in *Figure 7.5* are representative of three independent experiments. There was no evidence of antibody binding to ED-B in wells treated with control (GFP) supernatant or supernatant containing the A20hFc antibody. In contrast, *Figure 7.5* illustrates evidence of binding of CGS-1hFc to the ED-B protein at dilutions as low as 1:10000 (with no binding of CGS-1hFc in wells coated with BSA only - right hand columns in *Figure 7.5*). These results confirm that CGS-1.hFc antibody is well expressed from pAdlox.CGS-1.hFc transfected 293T cells and binds specifically and with a high affinity to recombinant ED-B protein.

Table 7.1 – CGS-1 Gene Sequence

```
C - ATGCAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGTCTCT 78
D - ATGCAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGTCTCT 78
V - ATGCAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGTCTCT 78
P - ATGCAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGTCTCT 78

C - GGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATT 156
D - GGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATT 156
V - GGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATT 156
P - GGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATT 156

C - AGTGGTAGTGGTGGTAGCACATACTAC-CAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAAC 234
D - AGTGGTAGTGGTGGTAGCACATACTACACAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAAC 234
V - AGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAAC 234
P - AGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAAC 234

C - ACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCAAGAAGTCTTCCTAAGTGG 312
D - ACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCAAGAAGTCTTCCTAAGTGG 312
V - ACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCAAGAAGTCTTCCTAAGTGG 312
P - ACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCAAGAAGTCTTCCTAAGTGG 312

C - GGCCAAGGTACCCTGGTCACCGTGTCGAGAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGTCT 390
D - GGCCAAGGTACCCTGGTCACCGTGTCGAGAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGTCT 390
V - GGCCAAGGTACCCTGGTCACCGTGTCGAGAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGTCT 390
P - GGCCAAGGTACCCTGGTCACCGTGTCGAGAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGTCT 390

C - GAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAAACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA 468
D - GAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAAACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA 468
V - GAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAAACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA 468
P - GAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAAACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA 468

C - AGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCCTGTACTTGTCATCTATGGTAAAAACAACCGGCCC 546
D - AGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCCTGTACTTGTCATCTATGGTAAAAACAACCGGCCC 546
V - AGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCCTGTACTTGTCATCTATGGTAAAAACAACCGGCCC 546
P - AGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCCTGTACTTGTCATCTATGGTAAAAACAACCGGCCC 546

C - TCAGGGATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGG-GCTCAGGCGGAA 624
D - TCAGGGATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGTGCTCAGGCGGAA 624
V - TCAGGGATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAA 624
P - TCAGGGATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAA 624

C - GATGAGGCTGACTATTACTGTAACTCCTCTC-TGTGGTTCTGAATGGGGTGGTATTCGGCGGAGGGACCAAGCTGACC 702
D - GATGAGGCTGACTATTACTGTAACTCCTCTCGTGTGGTTCTGAATGGGGTGGTATTCGGCGGAGGGACCAAGCTGACC 702
V - GATGAGGCTGACTATTACTGTAACTCCTCTCCTGTGGTTCTGAATGGGGTGGTATTCGGCGGAGGGACCAAGCTGACC 702
P - GATGAGGCTGACTATTACTGTAACTCCTCTCCTGTGGTTCTGAATGGGGTGGTATTCGGCGGAGGGACCAAGCTGACC 702

C - GTCCTAGGCGCGGCCGCAGA 722
D - GTCCTAGGCGCGGCCGCAGA 722
V - GTCCTAGGCGCGGCCGCAGA 722
P - GTCCTAGGCGCGGCCGCAGA 722
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C-Consensus, D-Published sequence, V-Sequence in pVOM1.CGS-1.MTM, P-Original plasmid sequence. Mutations highlighted in **bold.**

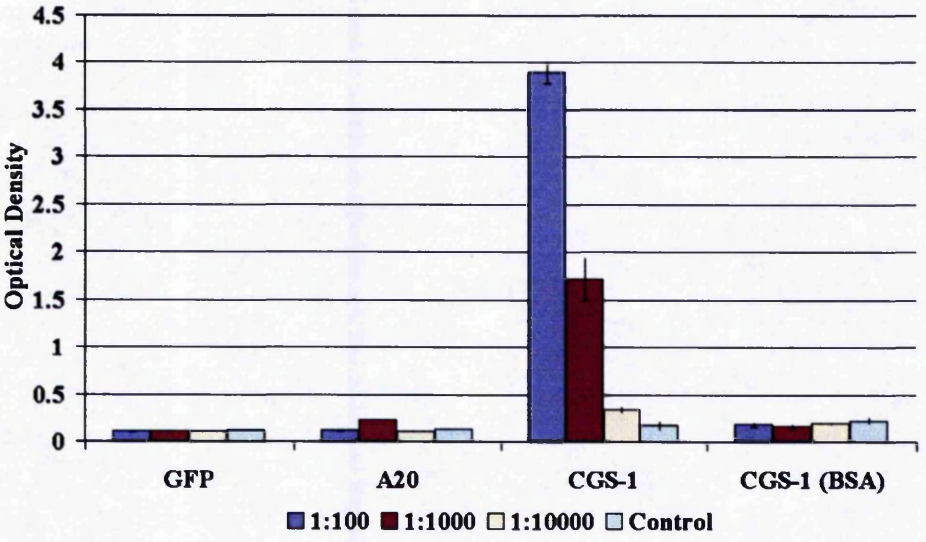

Figure 7.5 Indirect ELISA to detect CGS-1hFc affinity against ED-B - A construct was made which expressed CGS-1 fused to human Fc (pAdloxCGS-1hFc). 293T cells were transiently transfected by calcium phosphate coprecipitation and the supernatant (containing CGS-1hFc antibody) was harvested at 48 hours. Control transfections were also performed using a different antibody construct (pAdloxA20hFc) and a marker, rKatGFP. An indirect ELISA was performed using wells coated with recombinant ED-B protein (10μg/ml) and various dilutions of supernatant from each of the transfected cells. CGS-1hFc-containing supernatant dilutions were also plated onto wells coated with BSA as a control (far right). These results confirm previously published data regarding the high affinity of CGS-1 for ED-B.

7.6 rKat.OM1.CGS-1.MTM.IRES.GFP Jurkat transduction

7.6.1 rKat.OM1.CGS-1.MTM.IRES.GFP transduced jurkat cells show functional binding to recombinant ED-B protein in vitro - 1

rKat viral supernatant was made using the vector rKat.OM1.CGS-1.MTM.IRES.EGFP by transient transfection of 293T cells (methods 2.2.6.2). A spin transduction protocol (methods 2.2.6.6.1) was subsequently used to transduce the Jurkat T cell line. Control cells were transduced with a different (cell surface expressed) scFv, B1.8, which has affinity to the NIP antigen (4-hydroxy-5-iodo-3-nitrophenyl acetyl).

Transduction efficiencies of 45% and 55% respectively, were achieved using the rKat.OM1.CGS-1.MTM.IRES.EGFP and rKat.OM1.B1.8.MTM.IRES.GFP vectors, as measured by flow cytometry analysis of GFP expression seventy-two hours post transduction (*Figure 7.6*). In the absence of an antibody against CGS-1, it was necessary to demonstrate functional cell surface expression of CGS-1 scFv by the binding of transduced cells to recombinant ED-B protein. Transduced cells (10^6/well) were cultured overnight on non-tissue culture plates which had been pre-coated with either recombinant ED-B protein (10μg/ml) or 1% BSA. Wells were then washed with PBS and photomicrographs were taken after each wash. As shown in *Figure 7.6*, B1.8-transduced and control non-transduced cells were entirely washed away after three PBS washes. In contrast, a significant number of CGS-1-transduced cells remained adherent to the ED-B protein. No cells remained in wells coated with 1%BSA after two washes (data not shown). These results suggested that the CGS-1 scFv was indeed being expressed on the cell surface and that in this *in vitro* assay using transduced jurkat cells this expression resulted in specific binding of the cell to the surface of tissue culture wells coated with the ED-B protein target.

7.6.2 rKat.OM1.CGS-1.MTM.IRES.GFP transduced jurkat cells show functional binding to recombinant ED-B protein in vitro - 2

In order to confirm the results of section 7.6.1, CGS-1 and B1.8 expressing Jurkat cells and non-transduced control cells were then plated overnight in a U-bottom ninety-six well plate ($2x10^5$/well) coated with ED-B (10mcg/ml), NIP (10mcg/ml) or 1%BSA.

Figure 7.7 shows cells that efficiently bound antigen were spread over the walls of the plate, whilst those cells that did not bind antigen accumulated as a pellet at the bottom

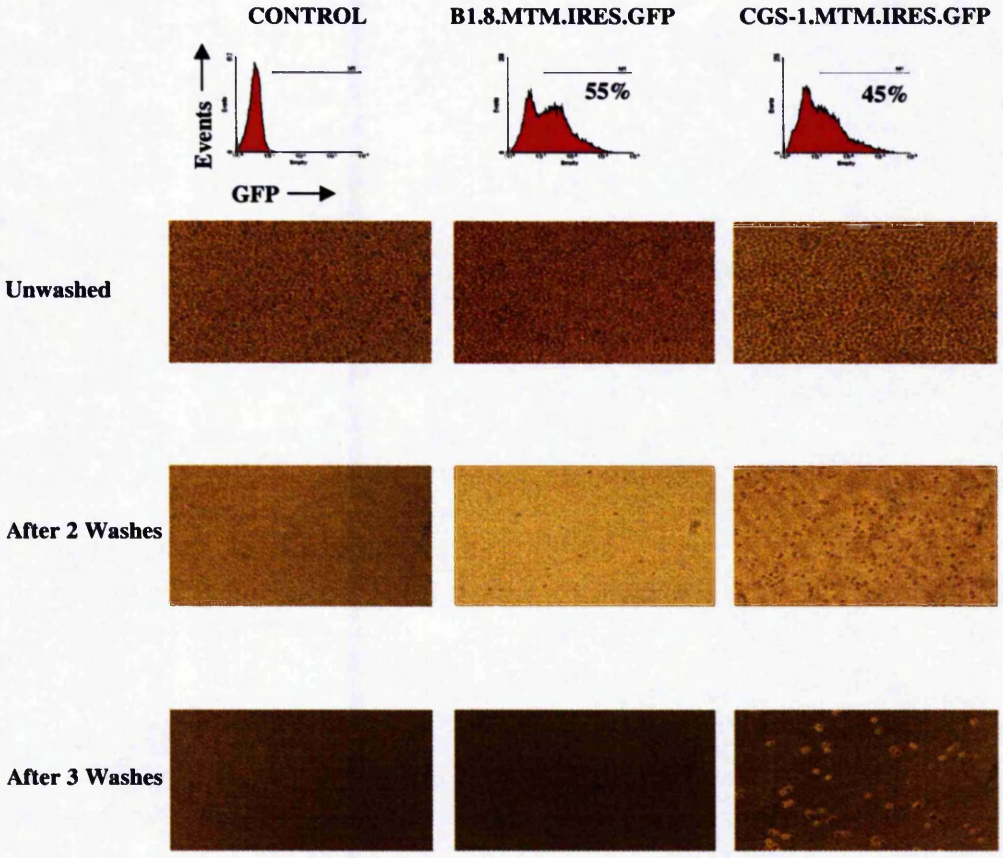

Figure 7.6 CGS-1 transduced jurkat cells show functional binding to recombinant ED-B protein 1
- rKat.OM1.CGS-1.MTM.IRES.GFP viral supernatant was made by transient transfection of 293T cells. A spin transduction protocol was used to infect Jurkat cells. Control cells were transduced with a different scFv, B1.8 (specific to the NIP antigen). Transduction efficiencies of 45-55% were seen, as measured by FACS analysis of GFP expression. Unsorted cells (10^6/well) were left overnight on non-tissue culture plates coated with either recombinant ED-B protein (10mcg/ml) or 1% BSA. Wells were then washed with PBS and photomicrographs were obtained after each wash. No cells remained in wells coated with BSA after two washes (not shown).

of the well. It can be seen that CGS-1 expressing cells bound specifically to the surface of the ED-B protein coated wells. In a similar way, a specific interaction was seen between the control B1.8-expressing cells that were spread over the walls of the wells coated with NIP antigen.

7.7 Human lymphocyte transduction with rKat.OM1.CGS-1.MTM.IRES.GFP

Recombinant retroviral supernatant was generated using the vector rKat.OM1.CGS-1.MTM.IRES.EGFP by transient transfection of 293T cells (methods 2.2.6.2). Human peripheral blood lymphocytes were obtained from a healthy donor and pre-activated on immobilised anti-CD3(iCD3) and anti-CD28(iCD28) monoclonal antibodies with IL-2 (30u/ml) for 72hrs (methods 2.2.6.4). A spin protocol (methods 2.2.6.6) was then used to transduce the lymphocytes. Following transduction cells were further expanded on iCD3/iCD28 antibodies for seventy-two hours at which point transduction efficiency was measured by flow cytometry analysis of GFP expression (methods 2.2.6.11).

Fig 7.8 shows a transduction efficiency of only 5.4% was achieved. As in section 7.6, an assay was then performed to test for functional expression of the CGS-1 scFv on the cell surface of these lymphocytes. Unfortunately, no evidence of specific binding of transduced lymphocytes to the ED-B protein was found in two separate experiments. Repeat human lymphocyte transductions using the rKat.OM1.CGS-1.MTM.IRES.GFP construct failed to result in improved transduction efficiency.

7.8 Human lymphocyte transduction with rKat.OM1.CGS-1.CD3ζTDGA.IRES.GFP

In view of the difficulty demonstrating CGS-1 cell surface lymphocyte expression using the CGS-1.MTM vector and the problems involved with achieving high level dual gene expression as illustrated in Chapter 4, it was decided to switch to targeting tumour vasculature using lymphocytes gene-modified to express an ED-B-specific chimeric T cell receptor (ie transduced with rKat.CGS-1.CD3ζTDGA.IRES.GFP, section 7.3.5). The chimeric receptor combined targeting with functional cytotoxicity, thereby removing the need for expression of two genes. In addition, protein expression could potentially be confirmed by western blotting for the ζ-chain and functional specific CGS-1 scFv binding could be detected by measuring release of interferon-γ.

rKat.CGS-1.CD3ζTDGA.IRES.GFP retroviral vector was made by transient 293T

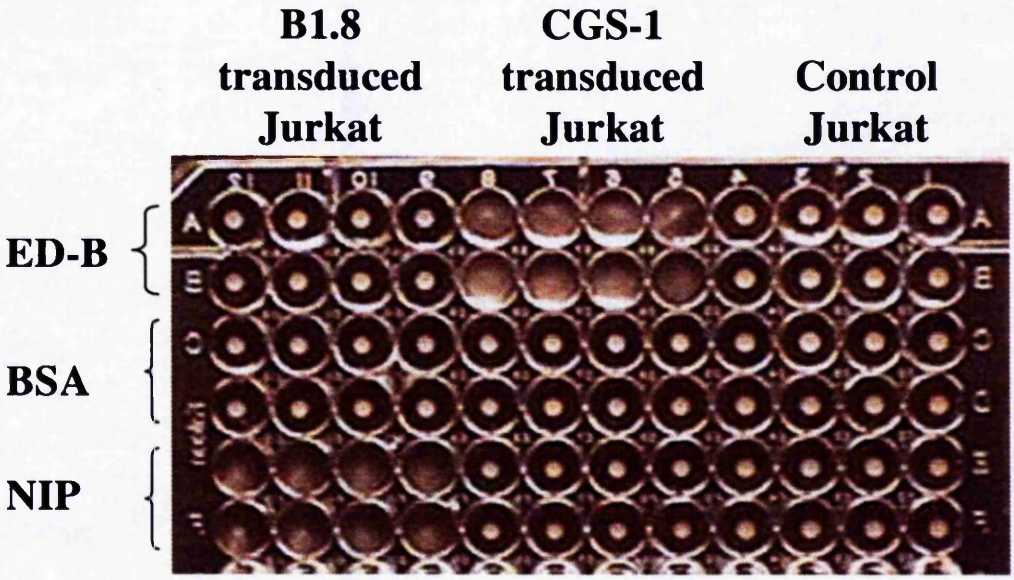

Figure 7.7 CGS-1 transduced jurkat cells show functional binding to recombinant ED-B protein 2 - A spin transduction protocol was used to transduce the Jurkat T cell line. Control cells were transduced with a different scFv, B1.8 (specific to the NIP antigen). Transduced cells ($2x10^5$/well) were then plated overnight in a U-bottom 96-well plate coated with either ED-B (10µg/ml), NIP (10µg/ml) or 1%BSA. *Figure 7.7* shows adherent cells spread out over the sides of the well with non-adherent cells clumped in the base.

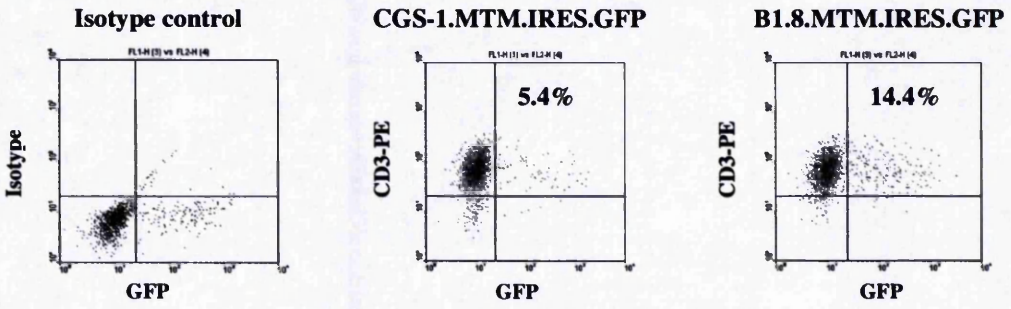

Figure 7.8 Human peripheral blood lymphocyte transduction using rKAT.OM1.CGS-1.MTM.IRES.GFP - Human peripheral blood lymphocytes were obtained from a healthy donor and preactivated on immobilised anti-CD3(iCD3) and anti-CD28(iCD28) monoclonal antibodies with IL-2 (30u/ml) for 72hrs. A spin protocol was then used to transduce the lymphocytes. Following transduction cells were further expanded on iCD3/iCD28 antibodies for seventy-two hours at which point transduction efficiency was measured by flow cytometry analysis of GFP expression after direct staining with an anti-CD3 monoclonal antibody.

transfection and, as before, a spin transduction method (methods 2.2.6.6.2) was then employed to transduce primary human T lymphocytes. Control transductions were performed in the same way using rKat.C23.CD3ζTDGA.IRES.GFP and rKat.GFP retrovirus. C23 is a scFv specific for the carcinoembryonic antigen (Chester, Begent et al. 1994), thus rKat.C23.CD3ζTDGA.IRES.GFP transduced lymphocytes express a chimeric receptor with specificity for this antigen. 72 hours later, transduction efficiency as measured by GFP expression was analysed by flow cytometry (*Figure 7.9a*). Efficiencies of 51%, 21% and 27% respectively were achieved in the rKat.GFP, rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP groups.

Transduced cells were then analysed for chimeric receptor expression by western blot and for functional activity by measuring specific interferon-γ release.

7.8.1 Western blot for ζ-chain expression in rKat.CGS-1.CD3ζTDGA.IRES.GFP-transduced lymphocytes

Seven days post transduction rKat.GFP, rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.C23.CD3ζTDGA.IRES.GFP transduced cells were lysed in RIPA buffer. Protein lysates (10^6 cells/lane) were subjected to SDS/PAGE and Western blot analysis with a monoclonal anti-CD3ζ antibody (Pharmingen) (*Figure 7.9b*).

Although clearly showing an anti-CD3ζ immunoreactive band at the expected molecular weight of the C23-chimeric receptor (approximately 45kD), the Western blot was unable to detect evidence of CGS-1 chimeric receptor expression in the rKat.CGS-1.CD3ζTDGA.IRES.GFP-transduced cells. The intense bands seen at approximately 14kD and 28kD represent the wildtype CD3ζ chain alone and when homo-dimerised (ie the blot was not fully reduced). This western blot was repeated and the same result was obtained.

7.8.2 CGS-1.CD3ζ-transduced human lymphocytes produce interferon-γ on contact with recombinant ED-B protein

In order to examine whether T lymphocytes expressing the CGS-1.CD3ζ receptor could be specifically activated upon contact with the target antigen, ED-B, an *in vitro* interferon-γ release assay was performed.

Quadruplicate wells of a 96-well non-tissue culture plate were coated with either CEA, ED-B (both diluted to 20ng/ml in borate buffer) or 1% BSA, incubated at 37°C for 3

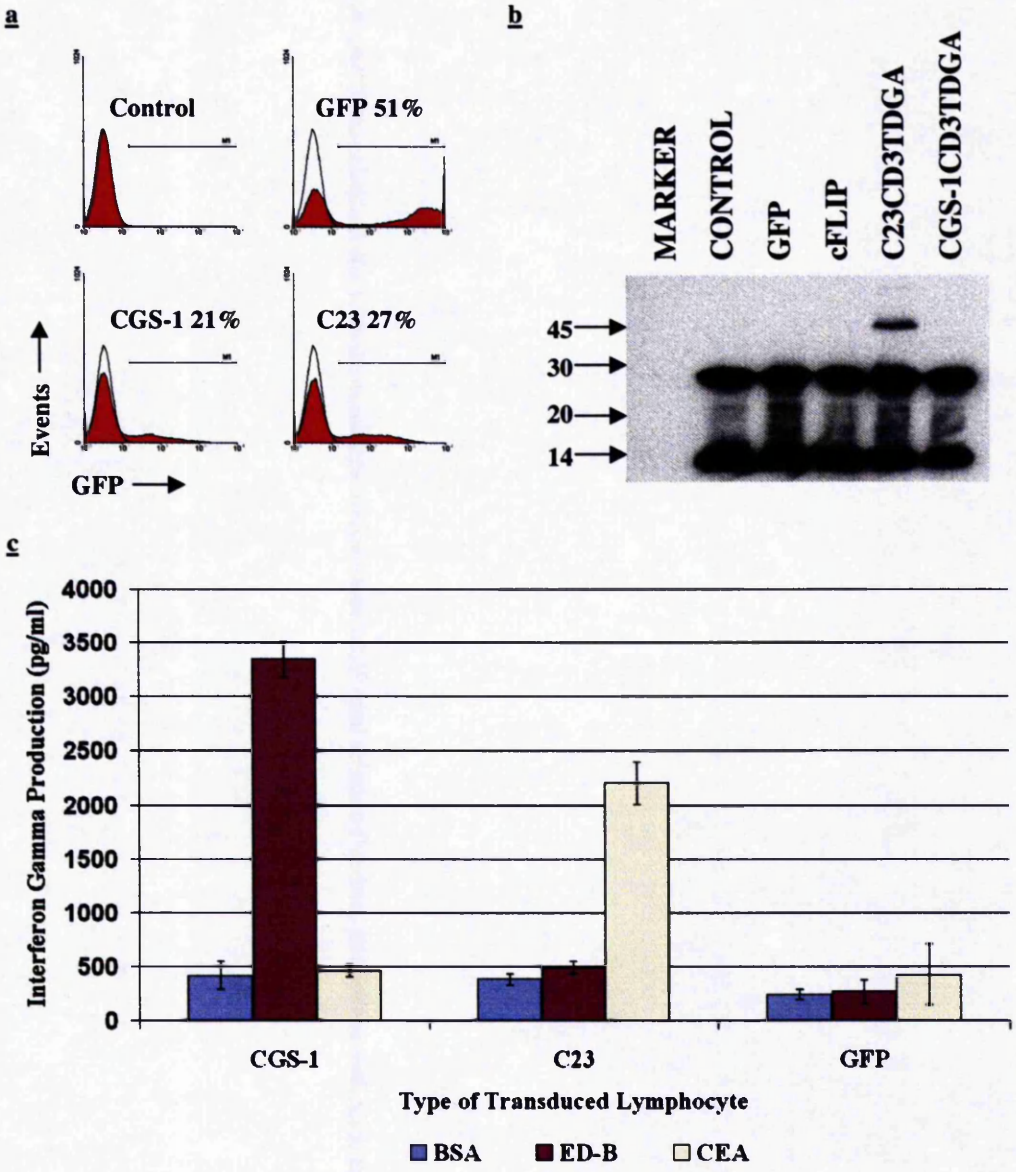

Figure 7.9 CGS-1.CD3z-transduced human lymphocytes produce interferon-γ on contact with recombinant ED-B protein - Human peripheral blood lymphocytes were obtained from a healthy donor and transduced using the rKat.GFP, rKat.CGS-1.CD3zTDGA.IRES.GFP and rKat.C23.CD3zTDGA. IRES.GFP vectors. Transduction efficiencies of 51%, 21% and 27% respectively were achieved (*Figure 7.9a*). Western blot of transduced cell lysates was unable to detect evidence of CGS-1 chimeric receptor expression (*Figure 7.9b*), however CGS-1.CD3ζ-expressing lymphocytes were specifically activated to produce interferon-γ upon contact with the target antigen, ED-B (*Figure 7.9c* – see text).

hours, and residual binding capacity blocked with 1% BSA. rKat.CGS-1.CD3ζTDGA.IRES.GFP, rKatC23.CD3ζTDGA.IRES.GFP and rKat.GFP-transduced human lymphocytes (section 7.8), were plated out at 10^5 cells/ well and incubated in 200µl T cell media. After 24 hours, 100µl media was collected from each well and replaced with fresh media. A 100µl sample was harvested after a further 48hours culture. Interferon-γ concentration in each sample was either determined immediately by ELISA (as described in methods 2.2.4.2), or samples were stored at –20°C until the assay could be performed.

Despite the apparent absence of CGS-1-chimeric receptor expression by Western blotting (*Figure 7.9b*), upon contact with recombinant ED-B protein the CGS-1.CD3ζ-transduced cells were specifically activated to produce high concentrations of interferon-γ (3345+/-167pg/ml) that were approximately seven-fold higher than when the same cells were cultured in the presence of the alternate tumour antigen, CEA (461+/-60pg/ml), or BSA (415+/-128pg/ml) (*Figure 7.9c*). In a similar manner, lymphocytes expressing the C23.CD3ζ chimeric receptor were specifically activated to produce interferon-γ on contact with CEA (2205+/-196pg/ml), but not ED-B (492+/-55pg/ml).

These results suggest that the concentration of CGS-1.CD3ζ-chimeric receptor protein expression required to detect a functional T cell response (ie interferon-γ production) was probably lower than could be detected by western blotting. Nethertheless, the specific interferon-γ production detected in these assays confirmed that the CGS-1.CD3ζ-chimeric receptor was being expressed and, indeed, was functionally active despite the unsuccessful attempts to detect expression of the receptor by Western blotting.

7.9 CGS-1.CD3ζ-transduced human lymphocytes do not lyse F9 tumour cells in a ^{51}Cr-release cytotoxicity assay

Human peripheral blood lymphocytes were transduced using the rKat.CGS-1.CD3ζTDGA.IRES.GFP, rKat.CGS-1.hFc.CD3ζTDGA.IRES.GFP, rKat.C23.CD3ζ TDGA.IRES.GFP and rKat.Bcl-X_L.IRES.C23.CD3ζ (see Chapter 4) vectors. Transduction efficiency as measured by GFP expression was analysed by flow cytometry (*Figure 7.10a*) and ranged between 5 and 14%.

In an assay identical to that performed in section 7.9, CGS-1.hFc.CD3ζ-expressing lymphocytes were specifically activated to produce interferon-γ upon contact with the target antigen ED-B, however, on this occasion CGS-1.CD3ζ and C23.CD3ζ expressing cells were not (*Figure 7.10b*). Control non-transduced cells and rKat.Bcl-X_L.IRES.GFP transduced cells were also tested in this assay and did not produce interferon-γ.

CGS-1.CD3ζ-chimeric receptor-transduced lymphocytes were subsequently tested in a standard ^{51}Chromium-release cytotoxicity assay (methods 2.2.6.9) against the (ED-B producing) F9 murine teratocarcinoma cell line. No significant cytotoxicity was demonstrated (*Figure 7.10c*) (see discussion 7.13).

7.10 F9 tumour *in vivo* growth kinetics model

The interferon-γ cytokine-release assay (*Figure 7.9*) had indicated evidence of specific functional *in vitro* activity of the CGS-1.CD3ζ-receptor transduced lymphocytes upon contact with ED-B. A subsequent cytotoxicity assay against the murine F9 murine teratocarcinoma cell line failed to show activity. However, although this cell line is known to produce ED-B protein when grown *in vivo*, there is debate as to whether tumour endothelial cells or the tumour cells themselves are the source of this production (Midulla, Verma et al. 2000). In view of this it was not clear as to whether an *in vitro* cytotoxicity assay would necessarily be representative of *in vivo* activity and it was planned to test this in an immunodeficient murine model, using human CGS-1.CD3ζ-receptor transduced lymphocytes.

In a preliminary experiment the *in vivo* growth kinetics of the ED-B-producing F9 murine embryonal teratocarcinoma cell line was studied in athymic (BALB/c) mice.

In order to test the optimal route of injection and dose of tumour cells, 4 groups of athymic (BALB/c) mice (n=4/group) were injected either subcutaneously into the right flank (2 groups) or intravenously (2 groups) with two cell doses, $5x10^5$ or 10^6, F9 tumour cells. Mice were subsequently observed daily and weighed/any tumours measured on Monday, Wednesday and Friday each week. Tumour volume was estimated using calipers (length x width^2 x 0.52) and mice were sacrificed when tumour volume was greater than one cubic centimetre, or if mice were showing any signs of distress.

Figure 7.11b shows the overall survival of each group of mice over time and

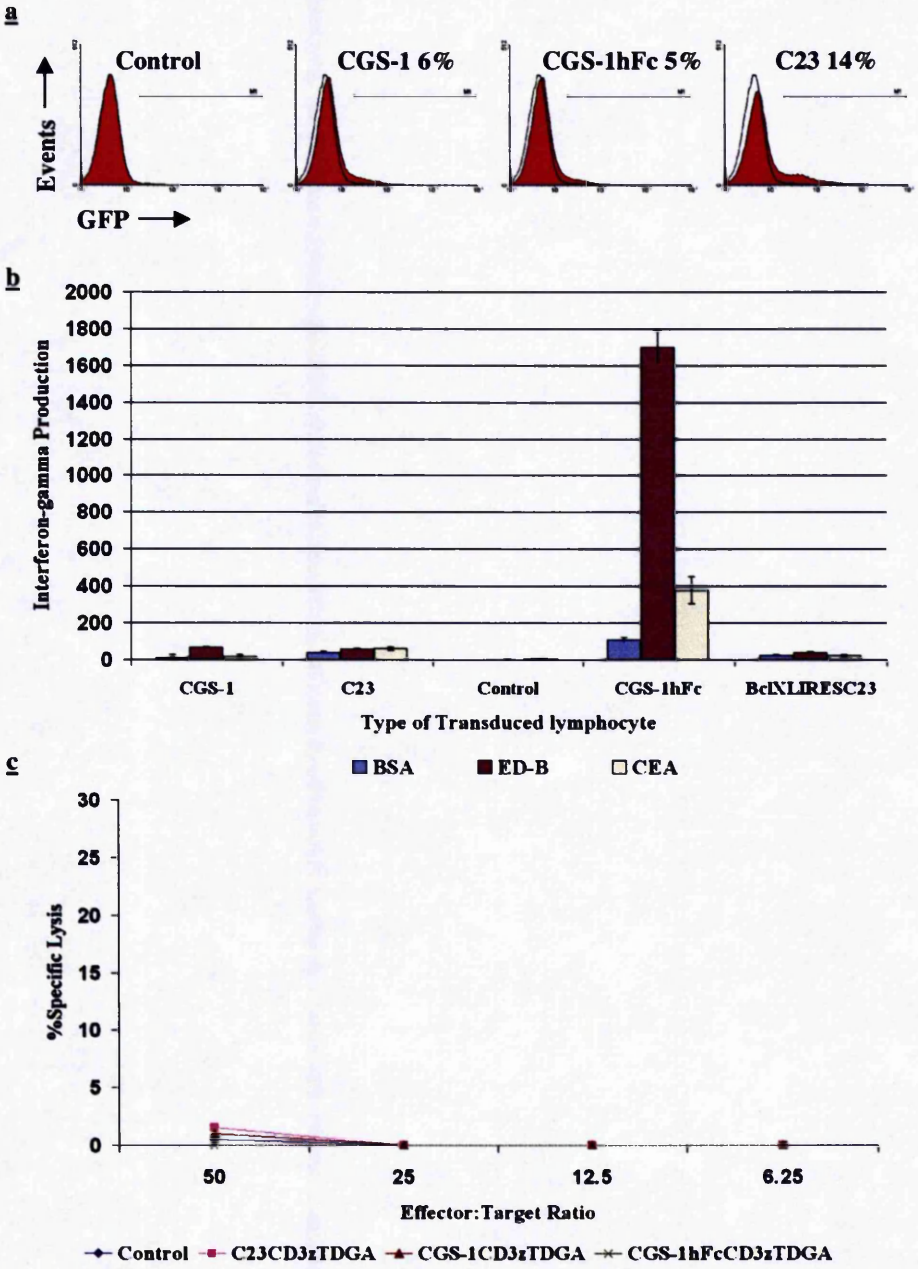

Figure 7.10 CGS-1.CD3z-transduced human lymphocytes do not kill F9 tumour cells in a ^{51}chromium-release cytotoxicity assay -Human peripheral blood lymphocytes were transduced using the rKat.CGS-1.CD3zTDGA.IRES.GFP, rKat.CGS-1.hFc.CD3zTDGA.IRES.GFP, rKat.C23.CD3ζ TDGA.IRES.GFP and rKat.Bcl-X_L.IRES.C23.CD3z vectors. (a) illustrates transduction efficiency by analysis of GFP expression. (b) CGS-1.hFc.CD3ζ-expressing lymphocytes were specifically activated to produce interferon-γ upon contact with target antigen, but not CGS-1.CD3ζ expressing cells. Control non-transduced cells and Bcl-XL.IRES.GFP transduced cells were also tested. (c) Neither type of chimeric receptor induced cytotoxicity against the ED-B-producing F9 murine teratocarcinoma cell line in a ^{51}Chromium-release assay.

demonstrates that in this experiment both cell doses were too low to reliably produce tumours in the mice. At the $5x10^5$ cell dose, one of the mice administered cells intravenously became sick after 50 days and was sacrificed, with no clear tumours at post-mortem. No other mice in either group developed any visible tumours and, when sacrificed after 3 months there were no abnormalities detected at post-mortem.

At the 10^6 cells dose, three of the mice treated subcutaneously developed tumours that resulted in them being sacrificed 16, 16 and 30 days post injection respectively. Growth of these tumours was rapid as illustrated by the tumour growth curves for this group shown in *Figure 7.11a.* However, not all mice in this group developed tumours, with the final mouse well with no evidence of tumour formation at post-mortem after 3 months. When treated with 10^6 cells intravenously, again one mouse was well with no evidence of tumour formation at post-mortem after 3 months. The other three mice were found dead after 30, 35 and 54 days. Although it is likely that the tumour cells resulted in the death of these mice, confirmatory post-mortems were not possible due to cannibalisation.

The conclusion from this preliminary experiment was that intravenous injection of F9 tumour cells resulted in unpredictable sudden death, and that the optimal model would be likely to involve a higher dose of cells given subcutaneously. Development of this model is now the subject of ongoing work by other members of the group.

7.11 Discussion

In this chapter an antibody scFv fragment (CGS-1) specific for an antigen found in and around tumour neovasculature (ED-B protein) was utilised in an attempt to ultimately target gene-modified human peripheral blood lymphocytes against these vessels. A vector was initially cloned that expressed CGS-1 fused to human Fc as a soluble secreted protein product and this antibody was shown to bind specifically and with a high affinity to recombinant ED-B protein when expressed by transfected 293T cells.

The initial aim of this work was to express the CGS-1 scFv fragment on the cell surface thereby targeting lymphocytes to the vasculature of tumours where they could be engineered to secrete a therapeutic protein at locally high concentrations. In order to achieve this the vector rKat.OM1.CGS-1.MTM.IRES.GFP (expressing cell surface CGS-1 joined to the murine transmembrane domain) was cloned. Jurkat T cells transduced with this vector were shown to bind to recombinant ED-B protein in two

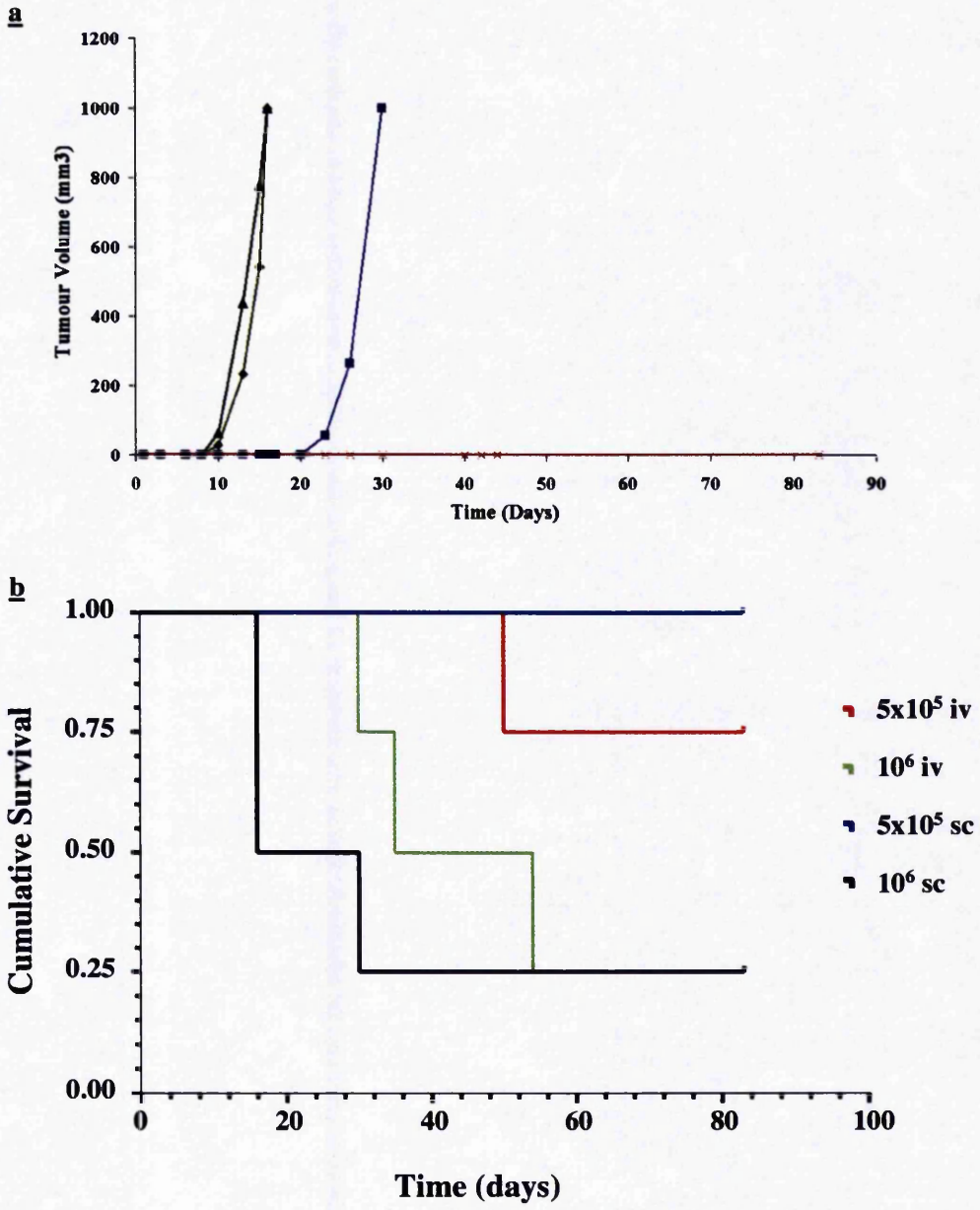

Figure 7.11 F9 in vivo tumour growth kinetics study -4 groups of athymic (BALB/c) mice (n=4/group) were injected either subcutaneously into the right flank (2 groups) or intravenously (2 groups) with two cell doses, $5x10^5$ or 10^6, F9 tumour cells. Mice were observed daily and weighed/any tumours measured on Monday, Wednesday and Friday each week. Tumour volume was estimated using calipers (length x width^2 x 0.52) and mice were sacrificed when tumour volume was greater than one cubic centimetre, or if mice were showing any signs of distress. *Figure 7.11a* illustrates the tumour growth curves for mice given 10^6 cells subcutaneously. *Figure 7.11b* shows the overall survival of mice in all groups as a Kaplan-Meier survival curve.

alternative *in vitro* assays, however when this vector was used to transduce human lymphocytes, transduction efficiencies were poor and no evidence of binding to ED-B could be demonstrated in further *in vitro* assays.

Although this 'binding' assay was a somewhat artificial method of assessing expression of CGS-1 scFv on the cell surface, when used to study CGS-1-expressing Jurkat cells a clear effect was noted, confirming an antibody-antigen interaction. In the absence of any definite evidence of functional expression in human lymphocytes, however, it was felt that proceeding to an *in vivo* tumour targeting experiment at this stage could not be justified.

There are several possible explanations for these results. Undoubtedly, the proportion of Jurkat cells that were transduced and the degree of expression on each transduced cell was far higher than in the transduced lymphocyte population. It may simply be that in order to demonstrate functional binding a certain level of scFv expression was required and this was not achieved when human lymphocytes were transduced. Transduction efficiency may well have been improved by cloning a high titre PG13 packaging cell line producing rKat.OM1.CGS-1.MTM.IRES.GFP (as in section 3.6), however this was not performed.

A potential alternative explanation was that when the CGS-1 scFv was expressed at the cell membrane of lymphocytes, it was effectively 'crowded out' by other cell surface receptors and molecules. In order to test this theory the vector rKat.OM1.CGS-1.hFc.IRES.GFP (section 7.3.4) was constructed, essentially inserting a human Fc domain between the scFv fragment and the murine transmembrane region. This 'spacer' region might facilitate an improved antibody-antigen interaction allowing greater flexibility, and has been shown to be an important way of improving the function of certain chimeric TCR constructs (Moritz and Groner 1995). The presence of the hFc within the construct would have also allowed quantification of cell surface expression of the scFv by flow cytometry using anti-hFc antibodies. Unfortunately, this vector was not completed in suitable time to test its' activity in primary human T cells; however this work is ongoing.

In a similar manner to this, the use of lymphocytes as 'cellular vectors' for gene therapy has previously been investigated using tumour-infiltrating lymphocytes (TILs) transduced with the tumour necrosis factor (TNF) gene (Hwu, Yannelli et al. 1993). TNF is known to have potent anti-tumour activity in murine models but humans can

only tolerate 2% of the equivalent systemic dose due to dose-limiting hypotension (Feinberg, Kurzrock et al. 1988). With this strategy the investigators attempted to deliver locally high levels of TNF to tumours without the systemic toxicity that had previously limited the use of TNF. In a Phase I study 12 patients were treated with TNF-transduced TILs plus IL-2 encountering no significant toxicity, however the investigators concluded that the levels of TNF produced by the lymphocytes were probably insufficient to be clinically effective and that for this sort of approach to be valuable better retroviral vector promotor/enhancer regions were needed to obtain higher levels of gene expression in primary lymphocytes (Devita Jr 2001).

Bearing this in mind, and given the difficulties already encountered when attempting to co-express two genes at high levels as a bicistronic construct (see Chapter 4), it was felt that even if scFv could be expressed on the cell surface at levels sufficient to target the lymphocyte, using the current retroviral vector system it would be unlikely that enough co-expressed 'therapeutic protein' could also be secreted to exert a significant anti-angiogenic effect.

It was therefore decided to switch to targeting tumour vasculature using lymphocytes gene-modified to express an ED-B-specific chimeric T cell receptor.

'Chimeric' T cell receptors link scFv antibody fragments with T cell receptor intracellular signalling chains. Upon contact with tumour antigen, cytotoxic T lymphocytes modified to express such receptors are specifically activated without the need for MHC expression (Gross, Waks et al. 1989). In this case the CGS-1 fragment was linked to the cytosolic signalling domain CD3ζ. In comparison to the previous approach, rather than just acting as cellular 'delivery vehicles' to target gene expression, in this strategy the lymphocytes are also endowed with a killing specificity for the tumour neovasculature. Whilst this does not preclude the co-expression of another therapeutic gene, it means that it is not a prerequisite for efficacy.

Furthermore, use of the CGS-1.CD3ζ chimeric receptor rather than CGS-1.MTM enabled the potential to confirm protein expression by western blotting for the ζ-chain and allowed a sensitive functional assay of specific CGS-1 scFv binding by measuring release of interferon-γ.

Other members of the Department of Medical Oncology are currently studying the targeting of chimeric receptors to other tumour antigens and the vector construction was

therefore a simple cloning step replacing a different scFv with CGS-1, to make the vectors rKat.CGS-1.CD3ζTDGA.IRES.GFP and rKat.CGS-1.hFc.CD3ζTDGA.IRES .GFP (sections 7.3.5 and 7.3.6).

Flow cytometric analysis of GFP expression demonstrated that variable transduction levels were obtained when these vectors were used to transduce human lymphocytes (5-21%). Although CGS-1.CD3ζ receptor protein expression was not demonstrated by western blotting, upon contact with recombinant ED-B protein CGS-1.CD3ζ- receptor-expressing cells were specifically activated to produce high concentrations of interferon-γ suggesting that the CGS-1-chimeric receptor was being expressed and, indeed, was functionally active.

There is no obvious reason why the CGS-1.CD3ζ receptor could not be detected by western blotting. The receptor transduction efficiency of the lysed cells was reasonable and, in an attempt to rule out the possibility of a technical error having occurred, the procedure was repeated twice with the same result. The presumable conclusion was that the concentration of CGS-1.CD3ζ-chimeric receptor protein expression required to detect a functional T cell response (ie interferon-γ production) was lower than could be detected by western blotting.

Neither the CGS-1.hFc.CD3ζ,-, nor the CGS-1.CD3ζ-receptor-transduced lymphocytes induced cytotoxicity against the F9 murine teratocarcinoma cell line in a standard ^{51}Cr-release cytotoxicity assay. The cells used in this assay were only 5-6% GFP positive, and, indeed, the CGS-1.CD3ζ-transduced lymphocytes did not produce interferon-γ in a cytotoxicity assay performed concurrently (*Figure 7.10*), however, there was clear evidence of cytokine release from the CGS-1.hFc.CD3ζ,-transduced lymphocytes despite the low levels of transduction, and, in the presence of target antigen this would have been expected to translate into at least some tumour cell lysis. As discussed in the introduction to this chapter however (section 7.1.2), ED-B protein is found in the stroma and neovasculature of malignant tissues but it remains unclear whether it is produced by the tumour cells themselves or the tumour endothelial cells (Zardi, Carnemolla et al. 1987; Midulla, Verma et al. 2000). Whilst the F9 tumour cell line is known to contain ED-B when a tumour, it is less clear whether ED-B is present when the cells are cultured *in vitro* and this could explain this result. The alternative explanation is, of course, that ED-B was present on the cells but that the CGS-1.hFc.CD3ζ,-transduced lymphocytes were not active.

The optimal quantity and route of administration of F9 cells to reliably produce tumours in nude mice, was then examined in a preliminary model. The use of adoptively transferred CGS-1.CD3ζ-transduced human lymphocytes (administered intravenously) to prevent growth of these tumours is the subject of ongoing work. In similar, recently published work, Neiderman and colleagues have demonstrated significant *in vivo* anti-tumour activity in a syngeneic model using murine CD8 lymphocytes transduced to express a similar CD3ζ-based chimeric receptor targeted against vascular endothelial growth factor receptors (Niederman, Ghogawala et al. 2002). Interestingly, whilst IL-2 administration resulted in no inherent anti-tumour activity in this study, its co-administration along with the gene-modified cells was crucial to demonstrating anti-tumour *in vivo* activity emphasising the importance of cell survival factors in such approaches.

8 Final Discussion

Gene therapy approaches aimed at modifying T lymphocytes to target tumour cells are an attractive means of generating large numbers of autologous T cells with anti-tumour specificity. For such an approach to have clinical efficacy it is critical that following adoptive transfer the T cells survive and maintain a persisting anti-tumour function *in vivo*.

In this thesis, retroviral vectors containing two critical regulators of T cell apoptosis, Bcl-X_L and $cFLIP_S$, were constructed and used to transduce primary human T cells and T cell lines. *In vitro* assays confirmed that over-expression of Bcl-X_L promoted the survival of human T lymphocytes cultured in pro-apoptotic conditions but did not increase proliferation. T cell lines over-expressing $cFLIP_S$ were also shown to be protected from anti-Fas antibody-induced apoptosis, but poor transduction efficiencies limited the assessment of over-expression of this gene in human lymphocytes.

These results suggest that co-expression of Bcl-X_L, and potentially $cFLIP_S$, in allogeneic donor lymphocyte infusions or in conjunction with a therapeutic gene could significantly improve the long-term survival and persistence of transduced cells *in vivo* thereby potentially enhancing the clinical outcome of gene-modified adoptive cellular therapy.

Evidence from another field of medicine supports the hypothesis that preventing apoptosis of lymphocytes can improve functional efficacy. Overwhelming septicaemia is also known to result in extensive lymphocyte apoptosis. Hotchkiss and colleagues have recently demonstrated that the use of either specific caspase inhibitors, or Bcl-2 over-expressing T cells can significantly improve survival in murine models of severe sepsis (Hotchkiss, Chang et al. 2000).

It is now evident from a number of studies that co-stimulation through the CD28 receptor (and other, more recently described co-stimulatory molecules such as 4-1BB (Shuford, Klussman et al. 1997)) both during the T cell expansion phase prior to adoptive transfer, and also *in vivo*, can have a significant effect on the survival and efficacy of adoptively transferred T cells in cancer immunotherapy (Bai, Bender et al. 2001; May, Chen et al. 2002; Brentjens, Latouche et al. 2003).

What remains unclear however is the optimal method of providing this signal to the lymphocyte and a variety of approaches are currently under investigation.

In terms of ex-vivo expansion, proliferation and survival of polyclonal and antigen-specific T cells has been shown to be significantly improved by the use of genetically engineered artificial antigen presenting cells (based on either the K562 or 3T3 tumour cell lines) expressing the ligands for CD28 +/- 4-1BB and the relevant T cell receptors (Latouche and Sadelain 2000; Maus, Thomas et al. 2002). In the paper by Maus and colleagues, the addition of 4-1BB co-stimulation resulted in a significant increase in Bcl-X_L gene expression in the T cells when compared with lymphocytes expanded using CD3 and CD28 beads only (as measured by real-time quantitative rt-pcr).

In a further very recently published paper, Brentjens and colleagues expanded human chimeric receptor-modified T cells in the presence of CD80 and IL-15 and demonstrated that these T cells uniquely persisted in tumour-bearing immunodeficient mice and eradicated disseminated intramedullary tumours (Brentjens, Latouche et al. 2003). On further analysis, they concluded that this improved survival was probably due, at least in part, to the up-regulation of the anti-apoptotic proteins Bcl-2, cFLIP and, most markedly, Bcl-X_L.

As discussed in section 1.2.5, another approach being studied is the use of single chimeric T cell receptors that transmit both CD3ζ and CD28 signals (Maher, Brentjens et al. 2002). Adoptive transfer of murine T lymphocytes expressing such receptors has recently been reported to be more effective in controlling the growth of established syngeneic and xenogeneic tumours when compared with their CD3ζ chain alone equivalents, without any requirement for exogenous IL-2 administration (Haynes, Trapani et al. 2002; Haynes, Trapani et al. 2002; Maher, Brentjens et al. 2002).

In this thesis, rather than achieve co-stimulation through the CD28 receptor itself, the constitutive over expression of what were considered to be the major anti-apoptotic genes upregulated by CD28 signalling, from within a retroviral vector, has been studied. Incorporating genes such as Bcl-X_L or $cFLIP_S$ into retroviral vectors used to modify T cells potentially had the advantage of removing the requirement for further CD28 co-stimulation *in vivo* rendering the transduced lymphocytes more resistant to apoptosis in the tumour microenvironment. In a clinical setting such an approach could also remove the requirement for additional systemic IL-2 and the toxicity with which this is associated.

However there are also potential disadvantages to this approach. The downstream signalling effects of CD28 ligation are complex affecting the expression of many genes (Sperling and Bluestone 1996) and remain incompletely understood. Although genes such as Bcl-X_L and cFLIP are clearly important regulators of lymphocyte apoptosis, it is possible that in this setting over-expression of a single gene may well not be sufficient to improve the *in vivo* function of the lymphocyte.

The work presented in Chapter 4 indicated that a relatively high level of Bcl-X_L expression was required to achieve a significant pro-survival effect. This level of expression was not achieved by IRES-dependent expression of Bcl-X_L as the second gene in a bicistronic construct in our vector. It may well be, however, that ongoing improvements in retroviral promotor and vector design will result in improved transgene expression that could in the future allow a higher level of dual gene expression. An example of a future approach that could potentially increase IRES-dependent gene expression might be the incorporation of scaffold attachment region (or SAR) elements into the retroviral vector (Agarwal, Austin et al. 1998) as discussed in section 4.7. Alternatively, a vector could be constructed containing two separate heterologous promotors.

Another issue, previously discussed in chapters 3 and 5 (sections 3.14 and 5.8), is the potential for oncogenesis or autoimmunity of constitutively expressing anti-apoptotic genes in T cells for immunotherapy and this would need to be fully investigated prior to the use of such genes in a clinical gene therapy vector. Key to addressing such concerns, and also to further testing the efficacy of such an approach would be to test this hypothesis in a long-term syngeneic murine model. It is important to note that if Bcl-X_L expressing T cells did show efficacy in such a model then the induction of low-level auto-immunity may be acceptable to cancer patients who otherwise face the probability of dying from their disease. A more widespread autoimmune reaction however could limit the use of these T cells. In addition, there are several mechanisms that might allow controlled protein expression and thereby minimise potential side-effects. Tumour-specific or pharmacologically-controllable promotors (Alvarez-Vallina, Agha-Mohammadi et al. 1997) could be incorporated to control gene expression. Alternatively, additional safety features such as the inclusion of suicide gene such as herpes simplex thymidine kinase in the vector could potentially be employed.

On this note, another safety issue concerning the whole field of retroviral gene therapy is the risk of insertional mutagenesis. It was previously considered that although this was theoretically possible the risks were extremely low (about 10^{-7} per insertion (Stocking, Bergholz et al. 1993)). However, the recent development of T cell malignancies arising in 2 out of 10 children with severe combined immuno-deficiency (SCID) who had been successfully treated with retroviral gene modified stem cells in a French trial has led to significant concerns (Marshall 2003). This follows a short report documenting leukaemogenesis in mice transplanted with stem cells transduced with a retroviral vector encoding the truncated nerve growth factor receptor (Li, Dullmann et al. 2002). Over the last 12 years a number of human gene-modified T cell trials have been undertaken with no reports of malignancy. In the SCID trial, the high rate of mutagenesis is thought to have arisen because of the selective advantage and expansion of a limited number of transduced stem cell clones (Marshall 2003). Clearly this raises concerns about such stem cell genetic modification. T cell genetic modification is likely to carry a lower risk but it is not clear what this risk is. As with autoimmunity, the risks of insertional mutagenesis need to be weighed against the significant potential clinical benefits of retroviral gene therapy approaches.

As an alternative to the work discussed in this thesis there are a number of other strategies that are currently being investigated aimed at improving *in vivo* gene-modified T cell proliferation and survival. Clinical studies have demonstrated that EBV-specific T cells generated *in vitro* can expand, persist and function for more than six years *in vivo*. EBV infection of B lymphocytes is near universal in humans and stimulates large numbers of EBV-specific cytotoxic T cells. Rossig and colleagues have proposed the use of tumour-specific chimeric T cell receptor-modified, EBV-specific T cells as a method of improved adoptive immunotherapy. Such cells would recognise EBV-infected targets through their conventional T cell receptor (providing long-term *in vivo* co-stimulation) and tumour targets through their chimeric receptor (Rossig, Bollard et al. 2002).

Approaches aimed at blocking the immunosuppressive effects of transforming growth factor-β have also been shown to mediate improved CD8+ T cell-mediated tumour elimination in transgenic mice (Gorelik and Flavell 2001).

Another factor that is known to have a marked effect on the efficacy of adoptively transferred T cells is the immune status of the patient prior to infusion of the T cells. In

a recently published clinical trial, patients with melanoma were treated with a non-myeloablative chemotherapy conditioning regime prior to the infusion of highly selected tumour reactive T cells directed against self-derived differentiation antigens (Dudley, Wunderlich et al. 2002). Clonal repopulation and proliferation of T cells was observed in these patients with subsequent objective responses seen in six out of the thirteen patients treated. One possible explanation for these encouraging results was that the chemotherapy conditioning could have eliminated regulatory 'suppressor' T cells thus allowing the *in vivo* proliferation of the transferred cells.

Unfortunately, in this thesis, further investigating the strategies of both anti-apoptotic gene over-expression and vascular targeting in syngeneic *in vivo* murine models was hindered by a failure in the time available to adequately optimise fresh murine T cell transduction. Initially total splenocyte populations were activated using 'in house' hybridoma-derived antibodies. Cell survival and expansion was improved after incorporating a lymphocyte-enrichment step (using a nylon wool column) and puchasing commercial anti-CD3 and anti-CD28 antibodies, however transduction efficiencies remained poor despite the use of a variety of packaging cell lines.

An antigen-specific primary murine T cell line with specificity against a B cell lymphoma idiotype was successfully genetically-modified to over-express Bcl-X_L using ecotropic retrovirus produced from a cloned Gp+e86 packaging cell line. Transduced cells underwent a limited expansion and retained *in vitro* cytolytic activity in a ^{51}Cr-release assay. However, expansion of the transduced cells tailed off and in a subsequent *in vivo* lymphoma model adoptively transferred transduced T cells failed to demonstrate anti-tumour activity. This was a disappointing result, however there were a number of deficiencies in this experiment (see section 6.5) that make it difficult to conclude on the basis of this alone that there is definitely no *in vivo* effect of Bcl-X_L over-expression in murine T lymphocytes. After additional work to improve murine T cell transduction efficiencies through the development of higher titre packaging cell lines, further *in vivo* studies involving both Bcl-X_L and cFLIP$_S$ are certainly warranted.

In Chapter 7 an antibody scFv fragment (CGS-1) specific for an antigen found in and around tumour neovasculature (ED-B protein) was utilised in an attempt to ultimately target gene-modified human peripheral blood lymphocytes against these vessels. After successfully confirming the high affinity of CGS-1 antibody for ED-B, a retroviral construct was produced that resulted in cell surface expression of CGS-1. Jurkat T-cells

expressing CGS-1 appeared to bind to plates coated with recombinant ED-B in *in vitro* assays demonstrating the potential for such an approach. However, efforts to confirm this in primary human lymphocytes were limited by poor efficiencies of transduction.

For reasons that are not clear, during this thesis it was apparent that there was a significant variation in the ability of different retroviral vector constructs to transduce T cells efficiently. As has previously been discussed (section 1.2.2.3), one method of improving transduction efficiency of this vector may have been to create a PG13 GALV-pseudotyped stable producer line, however this was not performed and this work was not continued.

The concept of lymphocytes acting as 'cellular vectors' in order to deliver a desired protein to target tissues is an attractive one. In a recent publication, Chester and colleagues genetically modified Jurkat T cells to express a chimeric T cell receptor that upon contact with target tumour antigen resulted in the specific induction of transcriptionally targeted retroviral vectors. In this way the Jurkat cells were converted into tumour targeted retroviral producer cells, that were effective in reducing disease burden in immunodeficient tumour models involving both local and systemic delivery (Chester, Ruchatz et al. 2002). Of note in this paper was the use of Jurkat cells in *in vivo* models to confirm the principle under investigation. When compared with primary human T cells, as is apparent in Chapter 7, the genetic modification and culture of T cell lines such as the Jurkat line is generally much easier. This may well be an approach that could be utilised to test the hypothesis that CGS-1 expressing T cells can localise to tumour neovasculature.

After further cloning, the use of chimeric CGS-1.CD3ζ-modified lymphocytes to target ED-B was investigated. Upon contact with recombinant ED-B protein, CGS-1.CD3ζ-receptor-expressing cells were specifically activated to produce high concentrations of interferon-γ confirming chimeric receptor expression and functional activity. Unfortunately this result was not consistently reproducible with further *in vitro* experiments giving results that were more inconclusive. Further *in vivo* work studying this approach is ongoing.

However, the strategy of targeting tumour vasculature using chimeric T cell receptor-modified T cells holds great promise and it is too soon yet to rule out ED-B as a potential target for these cells. Future developments, such as those proposed in this

thesis, aim to reduce the effect of anergising or pro-apoptotic signals on the T lymphocyte and can only further improve the anti-tumour effect of such a strategy.

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