Studies of Human T cell Costimulation:

Potential for the Immunotherapy of Cancer

By

Alexander Charles Dowell

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CRUK Institute for Cancer Studies
School of Cancer Sciences
University of Birmingham
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Abstract

Costimulation is required for the generation of an effective T cell based immune response. The presentation of tumour associated antigens may occur in the absence of effective costimulation, inducing tolerance. Conversely effective costimulation can overcome immunosuppressive mechanisms present within the tumour. Costimulation may therefore hold significant potential for cancer immunotherapy. Using recombinant adenoviral vectors encoding the costimulatory molecules CD80 and 4-1BBL (CD137L) and the cytokine IL-12 tumour cell lines were transduced to express these molecules individually or in combination *in vitro*. Using PBMC from healthy donors the effect of costimulation in response to pan-T cell stimulation with the anti-CD3 antibody OKT-3 were initially studied. The combination of CD80+4-1BBL supported the proliferation of $CD8^+$ T cells and was superior to either molecule alone. Proliferation was further enhanced by the addition of IL-12 to the combination of CD80+4-1BBL. Unexpectedly in the absence of OKT-3 costimulation with 4-1BBL or IL-12 was observed to predominantly induced the proliferation of natural killer (NK) cells. The effects of 4-1BBL on human NK cells are not clearly defined in the literature. Further experiments were therefore conducted to investigate the ability of 4-1BBL and IL-12 to stimulate NK cells. The combination of 4-1BBL+IL-12 was superior to either stimulation alone for the activation, proliferation and function of NK cells from healthy lab donors. 4-1BBL was also shown to promote the long term expansion of NK cells. Importantly renal cell carcinoma patient NK cells were shown to require a combination of 4-1BBL+IL-12 for short and long term expansion; stimulated NK cells were also shown to be functional. These data highlight the need for understanding of the pleiotropic effects of costimulatory molecules and the necessity to choose optimal combinations for the activation of not only the adaptive

but also the innate immune response. The combined intratumoural delivery of 4-1BBL and IL-12 via adenoviral vectors could potentially stimulate beneficial T cell and NK cell responses, and therefore warrant further investigation as a potential immunotherapy.

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Abbreviations

µm	Micro meter
µg	Micro gram
µM	Micro molar
Ab	Antibody
Ad	Adenovirus
ADCC	Antibody dependent cellular cytotoxicity
ADEPT	Antibody directed enzyme prodrug therapy
AICD	Activation induced cell death
AIRE	Autoimmune regulator
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
ATP	Adenosine-5'-triphosphate
Bcl	B-cell lymphoma
bp	Base pair
BSA	Bovine serum albumin
CA-125	Cancer antigen 125
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxy Fluorescein diacetate, Succinimidyl Ester
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTLR	C-type lectin-like receptor

DC	Dendritic cell
dH2O	De-ionised water
DME	Dulbecco's modified Eagle (medium)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTH	Delayed-type hyper sensitivity
E. coli	Escherichia coli
E1	Adenovirus early region 1
E3	Adenovirus early region 3
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Acceleration due to gravity
GDEPT	Gene directed enzyme prodrug therapy
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
hr	Hour(s)
HS	Human Serum
HSC	Haemopoietic stem cell

IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal ribosome entry site
ITAM	Immune tyrosine based activation motif
ITIM	Immune tyrosine based inhibitory motif
ITR	Inverted terminal repeat
IU	International units
IκB	Inhibitor of κB
JAK-STAT	Janus kinase-signal transducer and activator of transcription
kb	Kilobase
kDa	Kilodalton
KIR	Killer immunoglobulin receptor
LB	Luria broth
M	Molar
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MART-1	Melanoma Antigen Recognized by T-cells 1
mg	Milligram
MHC	Major histocompatability complex
min	Minute
ml	Millilitre
mm	Milli metre

mM	Milli molar
Mock	Mock infected cells
MOI	Multiplicity of infection
MTD	Maximum tolerated dose
NaCl	Sodium chloride
NCR	Natural cytotocity receptor
ng	Nanogram
NK	Natural killer
NKT	Natural killer T
nm	Nanometre
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerytherin
pfu	Plaque forming unit
pg	Picogram
PI3K	Phosphoinositol 3 kinase
PKB	Protein kinase B
PKC	Protein kinase C
pM	Picomolar
pRb	Retinoblastoma protein
RAG	Recombination activating gene
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute

RT	Room temperature
SCID	Severe combined immunodeficiency
SCID	X-linked severe combined immunodeficiency syndrome
SDS	Sodium dodecyl sulphate
SLAM	Signalling lymphocytic activation molecule
$T_{10}E_1$	10 mM Tris, 1 mM EDTA buffer
TAA	Tumour associated antigen
TAE	40 mM Tris acetate, 1 mM EDTA buffer
TAL	Tumour associated lymphocytes
TAP	Transporters associated with antigen processing
TCR	T-cell receptor
TGF	Transforming growth factor
Th1	T-cell helper type 1
Th2	T-cell helper type 2
TIL	Tumour infiltrating lymphocytes
TLR	Toll-like receptor
TNF	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TRAF	TNF Receptor Associated Factor
TRAIL	TNF related apoptosis-inducing ligand
T_{reg}	Regulatory T cell
Tris	Tris(hydroxymethyl) methylamine
Tween	Polyoxyethylene-sorbitan mono oleate
UTR	Untranslated region
UV	Ultraviolet light

V	Volts
v/v	Volume by volume
VDEPT	Virus directed enzyme pro-drug therapy
VEGF	Vascular endothelial growth factor
w/v	Weight by volume
WT	Wild type

1 Introduction

1.1 The immune system

1.1.1 Overview

The immune system contains many cell types which are derived from an original hematopoietic stem cell population, which produces all cells of myeloid and origin as shown in figure

Figure 1-1. The immune system can be viewed as a series of sub-divisions based on function and origin, firstly the division of the innate and the adaptive immune response. In humans the innate immune system is commonly erroneously thought of as a simple system. It is able to respond immediately to generic danger signals by the recognition of conserved microbial products by germline-encoded pattern recognition receptors (PRR) such as Toll-like receptors (TLR). In contrast the adaptive immune system is considered to be superior, only being possessed by higher animals. Through the generation of diverse antigen binding receptors the adaptive immune response is able to respond to and recognise almost any antigen. Most remarkably the adaptive immune response also possesses the ability to form memory and life long immunity.

The view of the immune system as functionally separate subdivisions with their own roles however is flawed. The immune system possessed by humans began to evolve around 500 million years ago with the development of the ability to rearrange antigen binding receptors, facilitated by the formation of recombination-activating genes (RAG); the hallmark of the adaptive immune system. As such all jawed vertebrates (cartilaginous fish to humans) possess lymphocytes, and are able to rearrange antigen binding receptors. Over the following millions of years the immune system evolved, and not in isolation, the adaptive and innate immune systems have developed in

parallel, and therefore have evolved to function together (Flajnik and Du Pasquier, 2004; Litman et al., 2005).

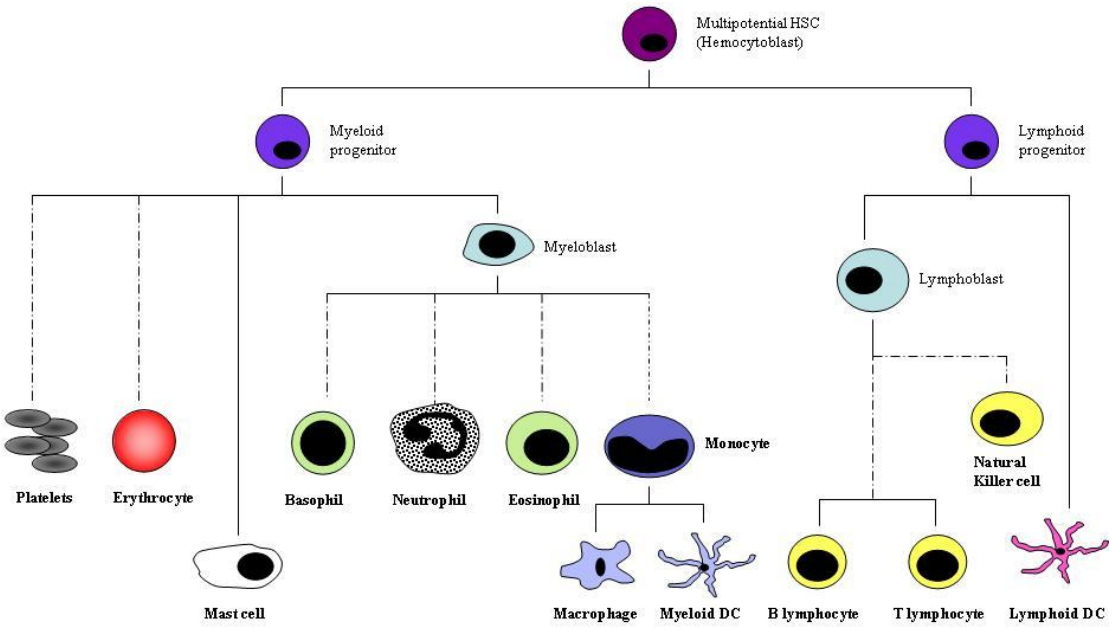

Figure 1-1 Haematopoiesis. A schematic diagram showing the origin and common progenitors of all terminally differentiated hematopoietic cell lineages. Dotted lines represent un-shown intermediaries during differentiation. Adapted from Abbas and Lithcman, (2000).

The innate immune system includes many cell types, such as monocytes, macrophages, neutrophils and eosinophils, however two cell types within the innate immune system are of particular interest firstly the natural killer (NK) cell which have important anti-viral and anti-tumour functions and the dendritic cell (DC) which is the most efficient of professional antigen presenting cells (APC), able to provide signals to promote the strongest of adaptive immune responses or alternately provide signals to dampen down an overactive immune response. The adaptive immune system on the other hand contains two cell types, the B cell and the T cell. The B cell is responsible for the production of antibodies and thus the humoral immune response, which is linked into the innate immune system for example by opsonization mediated by

complement and antibody dependent cellular cytoxicity (ADCC) mediated by NK cells. While the T cell in its many subsets plays a defining role in the B cell led humoural immune response but is also responsible for the generation of the cellular immune response, where NK cells and APC also have important roles. Thus our immune system is interconnected. There is significant crosstalk; the innate immune system is required to activate and alert the adaptive immune response to potential dangers, and likewise the adaptive immune response is capable of fuelling the innate immune response.

Besides the immune systems role in protection from pathogens, the immune system has also been shown to prevent the formation of cancer through a process of immunosurveillance and can also actively impact on the growth of a tumour.

Ultimately the immune system protects us from harm whether from pathogenic viruses, bacteria, fungi or parasites, or from threats within in the form of neoplastic changes. Utilising the immune system as a novel therapy for cancer is a growing area of development of great importance.

1.1.2 T cells

There are two dominant types of T cells in the periphery, those which express the CD8 co-receptor and those which express the CD4 co-receptor. In a textbook model of the immune system these two subsets of T cells can be thought to have two distinct roles, the $CD8^+$ T cell being responsible for cytotoxicity, while the $CD4^+$ T cell is able to provide 'help' for the immune response interacting with antigen presenting cells

and producing cytokines, however in reality the division and functions of these cells are far more diverse.

T cell antigen recognition is restricted to specific peptide epitopes presented in a complex with MHC molecules on the surface of target cells or APC; the MHC-peptide complex is recognised by the T cell receptor (TCR). The recognition of the plethora of epitopes generated from the many pathogens encountered is dependent on the diverse repertoire of TCRs which is generated during T cell development.

Peptide antigen can either be presented via MHC class I to $CD8^+$ T cells, or via MHC class II molecules to $CD4^+$ T cells. This in itself restricts the function of the CD4 T cell since, in contrast to HLA class I which is ubiquitously expressed on all cell types, expression of class II is restricted to specific cell types. However, the recognition of antigen alone is not capable of inducing activation and proliferation of T cells and requires further stimulus, as is discussed in section 1.1.2.3.

1.1.2.1 T cell receptor rearrangement

TCR diversity is generated by the rearrangement of the four T cell receptor genes (*Tcra, Tcrb, Tcrg, Tcrd*) by V(D)J recombination during intrathymic T cell development. The site-specific recombination process directed by recombinase (RAG1 and RAG2) (Bassing et al., 2002), which create double-strand breaks at specific recombination signal sequences (RSSs) flanking the TCR variable (V), diversity (D) and joining (J) gene segments. The subsequent rejoining of these double-stranded breaks creates unique *Tcr* gene sequences (Krangel, 2009).

Along with TCR rearrangement the development of T cells is a series of fate decisions, between B, NK and T cell lineage and subsequently between $CD4^+$ or $CD8^+$ T cell. These choices are controlled at the molecular level by transcription factors. There are also cell fate decisions between αβ-TCR and γδ-TCR usage, both of which arise from a common double negative (DN) precursor, with progression to the $CD4^+CD8^+$ double positive (DP) stage marking the point of irreversible commitment to the αβ lineage, however the control of this process is not understood (Narayan and Kang, 2007). The TCR rearrangement of γδ-T cells is similar to that of αβ-T cells however the selection pressures on these cells during development vary considerably. Unlike αβ-T cells there is no known antigen-presentation mechanism for γδ-T cell ligands nor do antigens require to be peptide–MHC complexes. Interestingly γδ-T cells also do not require prior stimulation to produce cytokines (Jensen and Chine, 2009; Konigshofer and Chine et al, 2006). As these cells are distinct and their exact roles in the immune response are not fully defined the discussion of T cells will focus primarily upon αβ-T cells.

Within the thymus as αβ-T cells develop they progress through a series of defined steps allowing the generation and release of naïve but mature T cells into the periphery. As the naïve T cell develops it moves from the bone marrow to the subcapsular sinus and outer cortical regions within the thymus. Within the thymus appropriate developmental signals are delivered to allow T cell development. Thymocytes first express TCRs within the cortex and begin to mature into $CD4^+$ or $CD8^+$ T cells, followed by the final stages of T cell development in the medulla and release into the periphery (Abbas and Lichtman, 2005; Hayday and Pennington, 2007).

Following production in the bone marrow and migration to the thymus the initial stages of T cell development begin with the pro-T cell stage, during which the double-negative thymocytes lack CD4 or CD8 expression and TCR, CD3 or ζ chain expression. At this stage RAG-1 and RAG-2 genes are expressed allowing receptor rearrangement to take place. The first rearrangement events to occur are Dβ-to-Jβ involving joining of either the Dβ1 or Dβ2 gene segment to one of six Jβ1 or Jβ2 gene fragments respectively (Abbas and Lichtman, 2005; Hayday and Pennington, 2007).

Following D_β-to-J_β rearrangement the pro-T cell is considered to have become a pre-T cell, which can now undergo V_β-to-DJ_β rearrangements. The DNA sequences between the rearranging elements are deleted during this stage, and the TCR-β chain is transcribed and expressed on the cell surface which along with the invariant pre-Tα chain, CD3 and ζ chain forms the pre-T cell receptor. Signals from the pre-TCR allow transition to the double positive stage of T cell development and down regulation of the RAG genes inhibiting further TCR-β rearrangement (Abbas and Lichtman, 2005; Krangel, 2009).

A second phase of RAG gene expression occurs late in pre-T cell development facilitating the rearrangement of the TCR α-chain; as no D segment is present in the α locus, rearrangement consists of V and J rearrangements. Productive rearrangements may occur on both chromosomes, which may result in a single T cell expressing two TCR-α chains but one β-chain; up to 30% of mature peripheral T cells express two different TCRs however the functional consequences of this are not understood. If no productive TCR α rearrangements occur the thymocyte will undergo apoptosis at this

stage. Following expression of αβ-TCR the thymocyte will enter the medulla prior to release into the periphery (Abbas and Lichtman, 2005; Krangel, 2009).

Following expression of the final αβ-TCR the T cell will also undergo positive and negative selection on the basis of binding self peptide and self HLA, helping to ensure tolerance, the mechanisms by which tolerance is achieved are subsequently discussed.

1.1.2.2 Tolerance

Due to the remarkable ability of the TCR to recognise virtually any antigen there will inevitably be TCRs generated during TCR rearrangement which will be reactive to peptides derived from self proteins. If T cells possessing these self reactive TCRs were allowed into the circulation and were allowed to become activated considerable pathology could result. Therefore there are a number of safeguards to ensure tolerance to self.

1.1.2.2.1 Central tolerance

Central tolerance is the initial mechanism facilitating tolerance by deletion of T cells possessing TCR which recognise self with high affinity (Mathis and Benoist, 2004).

Positive selection is the first step in the process of central tolerance, resulting in the selection of thymocytes with self-MHC restricted TCRs. Within the thymic cortex double positive thymocytes encounter epithelial cells expressing MHC class I and class II, presenting self peptides. If the TCR possessed by a double positive cell can recognise self-MHC with interactions from either $CD8^+$ or $CD4^+$ the cell will receive survival signals and continue to develop into a $CD8^+$ or $CD4^+$ T cell, dependent upon whether Class I or Class II is bound. If no interaction takes place the cell will die

through neglect, therefore producing self-MHC restricted single positive immature T cells. However this process also enriches T cells with TCR which are self reactive (Hogquist et al., 2005).

If a TCR is able to recognise self-MHC loaded with self peptide with high affinity there is a requirement to remove this TCR from the repertoire. To achieve this negative selection functions in the thymic medulla subsequent to the process of positive selection. While the majority of thymocytes are removed through positive selection the remaining population (~10% of the original thymocytes) are subject to negative selection, of these approximately half are deleted (Palmer, 2003).

The process of negative selection is dependent on the expression of the transcription factor known as autoimmune regulator (AIRE) by medullary thymic epithelial cells and DC within the thymus. AIRE allows the promiscuous expression of tissue specific genes within the thymus. Mutation of the *AIRE* gene results in autoimmune polyendocrine syndrome type 1, a multi-organ form of autoimmunity. During negative selection it is believed that a developing T cell will interact with a number of different cells presenting varying combinations of self peptides. T cells possessing TCRs which recognise self peptide with high affinity undergo deletion (Palmer, 2003; Hogquist et al., 2005). However the mechanisms by which the varied avidity of TCR binding can be translated into a decision of cell fate have yet to be defined (McCaughtry [and] Hogquist, 2008). Alternatively some cells may not undergo deletion instead being converted to suppressor T cells which are discussed further in section 1.1.2.4.3 (Jordan et al., 2001; Apostolou et al., 2002).

Interestingly ligation of TCR alone has been shown to be insufficient to promote deletion. Counter to its requirement in the activation of peripheral T cells (as discussed in section 1.1.2.3.2) interaction of CD28 on the T cell with the costimulatory molecules CD80/CD86 has been shown to be required to promote deletion of a self reactive clone (Kishimoto and Sprent, 1999; Gao et al., 2002). Although a consensus on the mechanisms by which deletion is mediated has not been reached (Palmer, 2003; Hogquist et al., 2005), the orphan nuclear steroid receptor Nur77 which is induced following strong TCR stimulation has been implicated to have a role as has the pro-apoptotic Bcl-2 family member Bim (McCaughtry and Hogquist, 2008).

1.1.2.2.2 Peripheral tolerance

While central tolerance is able to remove a significant proportion of potentially pathogenic self reactive T cells it is impossible to ensure complete tolerance to all self epitopes and still retain sufficient TCR diversity, therefore potentially self reactive T cells still remain in the periphery. These T cells therefore must be controlled in order to prevent autoimmune pathology (Caspi, 2006; Mathis and Benoist, 2004).

The mechanisms by which peripheral tolerance is achieved is diverse, firstly some organs such as the testis, central nervous system and the eye are so called 'immune-privileged' sites. Tolerance within these sites may be achieved by a number of differing passive and active strategies, including the prevention of access to specific sites, e.g. blood-ocular barrier. The organ itself may also actively produce an immunosuppressive environment through the production of immunosuppressive cytokines, e.g. TGF-β, and the expression of inhibitory ligands, e.g. FasL and PDL-1,

inducing apoptosis and anergy. Privileged sites may also show a paucity of APC while promoting the development of regulatory T cells (Stein-Streilein, 2008).

Although these mechanisms can protect a select population of organs which are highly sensitive to the detrimental effects of inflammatory immune responses, whether the immune response is valid or otherwise, peripheral tolerance must also be able to control auto-reactive T cells in the wider environment. As will be discussed further in section 1.1.2.3 the encounter of antigen alone by a T cell, such as a self antigen, without further stimulation generally results in the production of a state anergy (hypo-responsiveness), this is particularly relevant to self reactive T cells; having been through the process of central tolerance self reactive T cells should recognise their cognate antigen with low affinity, this process is known as ignorance (Redmond and Sherman, 2005). Repeated exposure to antigen without further costimulation can also lead to activation-induced cell death (AICD) (Walker and Abbas, 2002). However, ignorance of self-antigen is far from complete, under appropriate inflammatory conditions low avidity T cells can be activated (Sacher et al., 2002; O'Sullivan et al., 2006).

A number of further models exist to account for the control of self reactive T cells such as the 'danger' signal hypothesis in which APC require danger signals provided by cell death or microbial products, in order to become activated; APC which have not been activated, such as immature DCs, present antigen in a tolerogenic manner (Dhodapkar et al., 2001; Levings et al., 2001). Likewise there are subsets of APC which actively induce anergy or convert T cells to regulatory T cells; therefore self antigen presented under such conditions will also induce anergy or generate

regulatory T cells. As naïve T cells leaving the thymus circulate between secondary lymphoid tissue and the blood a self-reactive T cell could be most likely to see antigen presented by immature DC, i.e. in a tolerogenic manner, rather than expressed in tissue (Redmond and Sherman, 2005; Walker and Abbas, 2002). Interestingly there is also a growing body of work suggesting that AIRE may be expressed, all be it at low levels, in the secondary lymphoid tissue, however the functional consequences of such expression are not yet understood (Gardner et al., 2009).

The final mechanism by which peripheral tolerance is maintained is through the action of regulatory T cells, in particular $CD4^{+}FoxP3^{+}$ T cells, without which severe autoimmune pathology results. Regulatory T cells will be discussed separately in section 1.1.2.4.3. The stringent requirement for this cell population suggests regulatory T cells play a major role in maintaining tolerance to self in the periphery (Sakaguchi, 2004; Walker and Abbas, 2002).

As will also be discussed in later chapters many of these mechanisms of tolerance are subverted in the course of tumour development and are therefore particularly pertinent to understanding immune responses within a tumour.

1.1.2.3 Antigen presentation and activation of T cells

As stated all cells possess MHC class I and are therefore capable of presenting antigen to $CD8^{+}$ T cells, however only a restricted population also express class II allowing presentation to $CD4^{+}$ T cells. The presentation of antigen alone however will ultimately result in the production of anergy or in AICD unless antigen is presented in an appropriate manner. This is achieved by the provision of an additional signal

(signal two) i.e. costimulation, by professional antigen presenting cells, allowing the activation, proliferation and acquisition of effector functions by T cells (Sharp and Freeman, 2002).

Over the last 15 years the number of receptor:ligand pairs which are known to have costimulatory functions has risen drastically. The majority of costimulatory molecules fall into one of two families. Either the immunoglobulin superfamily including CD28:CD80/CD86 (1.1.2.3.2) or the tumour necrosis factor receptor (TNFR) superfamily including 4-1BB:4-1BBL (1.1.2.3.3). It can also be argued that a number of cytokine:receptor pairs could also be considered to have costimulatory action, such as IL-2R, IL-7R and IL-12R. There is also a suggestion that an additional signal in the form of cytokines may be required (signal three) for the full acquisition of effector functions; cytokines such as IL-12 can provide this additional signal (1.1.2.3.4) (Schmidt and Mescher 2002; Croft, 2003; Curtsinger et al., 2003).

As there is a diverse range of costimulatory molecules and receptors the expression patterns of which vary from constitutive to tightly regulated it is not surprising that the different costimulatory molecules have been shown to have some redundant functions but also to act separately to enhance or generate distinct immune responses in a temporal manner (Bertram et al., 2002; Croft, 2003; Watts, 2005). Therefore investigation of the optimal costimulatory combinations is important to allow a better understanding of their influences within an immune response; ultimately with the aim of allowing the enhancement or inhibition of immune responses for clinical applications.

1.1.2.3.1 Antigen processing

The majority of peptides to be loaded on to MHC class I molecules are generated proteasome degradation of newly synthesised ubiquitinated proteins. Two proteins within the proteasome, LMP-2 and LMP-7, are up-regulated by IFNγ, increasing the generation of 6-30 residue peptides containing carboxyl terminal or hydrophobic amino acids, which are suited to presentation by MHC class I. Peptides which are capable of binding to MHC class I molecules are actively transported from the cytosol to the endoplasmic reticulum by two ATP-binding cassette (ABC) proteins called TAP-1 and TAP-2 (transporters associated with antigen processing-1 and -2). Peptides are then loaded onto newly synthesised class I molecules by a complex of several ER resident chaperons such as tapasin calreticulin (Wright et al., 2004; Cresswell et al., 1999). The loaded MHC-peptide complex is then presented on the cell surface via the Golgi apparatus (see

Figure 1-2).

Breakdown of endogenously synthesised proteins and presentation on MHC class I is an important process in surveillance of viral infection or malignant transformation by $CD8^+$ T cells. As there is also a requirement for costimulation in order to generate a $CD8^+$ T cell response there is a necessity for APC to be able to present antigen from virus infected or tumour cells in order to provide costimulation to a specific T cell. In order to do this a process of cross presentation or cross dressing is undertaken by the DC. Cross presentation involves the uptake of cell debris in phagosomes resulting in processing and presentation of antigen via MHC class I. Alternatively DC can also acquire MHC-peptide complexes from the surface of dead cells, termed cross dressing, allowing presentation by the DC (Dolan et al., 2006).

Presentation of peptides by MHC class II molecules occurs through a distinct pathway. Most Class II peptides are generated from proteins internalised by endocytosis, and degraded by proteases, e.g. cathepsins, within the acidic endosome. Class II molecules are synthesised in the ER, αβMHC class II dimers form with the help of ER-chaperones e.g. calnexin, and associate with a trimer of invariant (I_i) chain, forming a nonamer complex which exits from the ER and passes through the Golgi apparatus and enter the endocytic pathway. Within the acidic compartment of the endosome I_i chains are degraded and MHC class II molecules are loaded with antigenic peptides (generated from endocytosed proteins) under the control of HLA-DM and HLA-DO. The class II-peptide complexes subsequently traffic to the surface of the plasma membrane (see

Figure 1-2) (Abbas and Lichtman, 2005; Banchereau and Steinman, 1998).

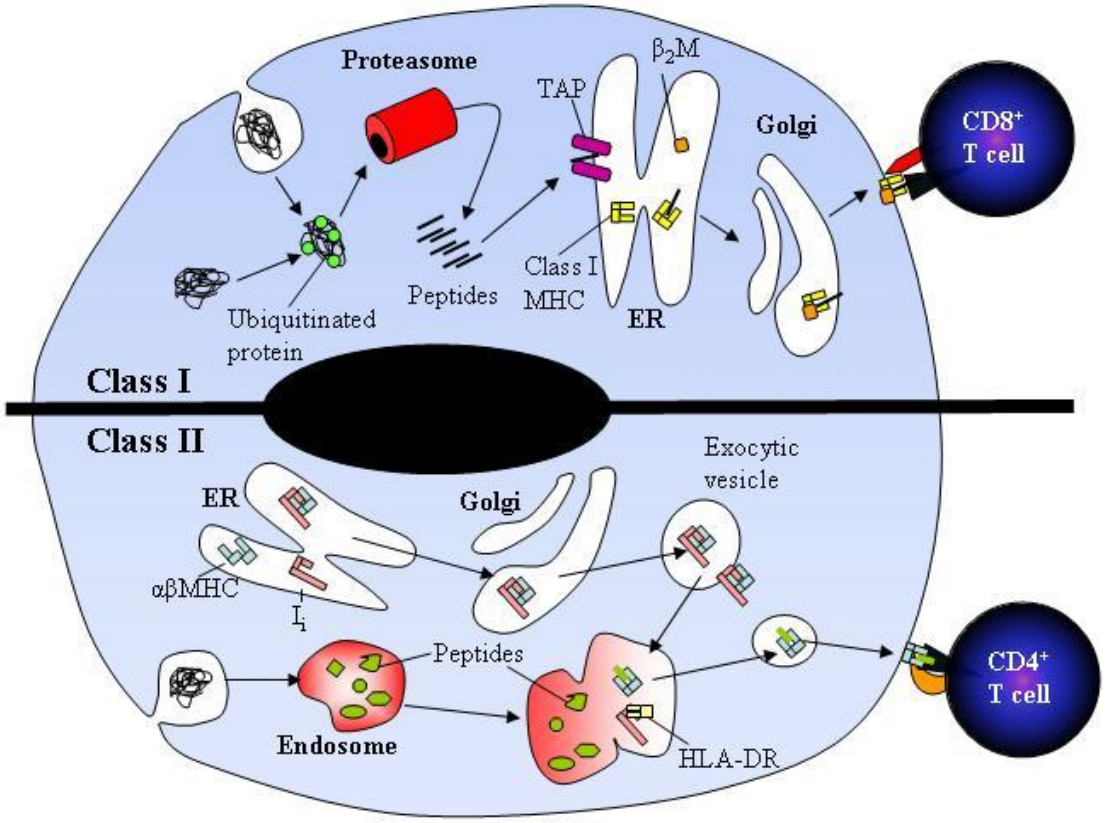

Figure 1-2 Processing of MHC class I and Class II antigens.
Adapted from Abbas and Lichtman, (2005).

1.1.2.3.2 Costimulation: Immunoglobulin superfamily

The immunoglobulin (Ig) superfamily is a diverse selection of proteins in the immune system ranging from immunoglobulin itself to MHC; all contain conserved constant or variable domains. The costimulatory receptor:ligand pairs present in the Ig superfamily include CD27:CD70 and ICOS:ICOSL. The Ig superfamily also includes the coinhibitory receptor PD-1 and its ligands PD-1L and PD-2L. However the most widely studied of all costimulatory receptor or molecules are CD28 and its ligands CD80 or CD86 (that are also members of the Ig superfamily) (Carreno and Collins, 2002; Greenwald et al., 2005).

1.1.2.3.2.1 CD28

CD28 is characterised by a single extracellular Ig variable-like (IgV) domain followed by a short cytoplasmic tail (Aruffo and Seed, 1987). Its ligands CD80 and CD86 also have an IgV and a Ig constant-like (IgC) domain present in the extracellular portion (Freeman et al., 1989; Freeman et al., 1993). CD28 expressed by 90% of $CD4^+$ T cells and 50% of $CD8^+$ T cells in human peripheral blood (Peggs and Allison, 2005).

CD28 lacks inherent signalling capacity, requiring interaction with the SH2 and SH3 domains of a number of intracellular signalling proteins, including phosphatidylinositide 3-kinase (PI3K) and Grb2, via proline and tyrosine-motifs. Ligation of CD28 results in activation of diverse signalling pathways. Briefly, increased tyrosine phosphorylation by src-family kinases (p56lck and p59fyn), PIK3 mediates the activation of phosphoinositide-dependent protein kinase 1 (PDK1) via pleckstrin homology (PH) domains, which in turn activates protein kinase B (PKB/AKT). Phosphorylation of PDK1 and PKB modulate the activity of multiple pathways linked to protein synthesis, cellular metabolism, and cell survival including Bcl-2 and Bcl-X_L. CD28 ligation can also activate NF-κB through phosphorylation and subsequent degradation of IκB by serine/threonine kinases and the IκB kinases (IKKs) (Rudd et al., 2009).

Signalling activated by CD28 engagement induces the expression of cytokines such as IL-2 and chemokines such as MIP-1α by the T cell (Freeman et al., 1989; Herold et al., 1997) and also accelerate entry into and progression through the cell cycle, in a partly IL-2 independent manner including the activation of cyclin-dependent kinases, phosphorylation of retinoblastoma protein (pRb) and degradation of cyclin-dependent

kinase inhibitor (KIP)-1 and PI3K-AKT (Mittnacht, 1998; Appleman et al., 2000; Bonnevier and Mueller, 2002). Ligation of CD28 is also able to increase the survival of T cells, partially through the extrinsic survival effects of IL-2 signalling but also through the increased expression of the anti-apoptotic protein Bcl-2 and Bcl-X_L (Boise et al., 1995).

CD28 signalling can also facilitate further costimulation by promoting the expression of the inducible TNF superfamily costimulatory receptors OX40 and 4-1BB which will be discussed in section 1.1.2.3.3 (Diehl et al., 2002; Walker et al., 1999).

1.1.2.3.2.2 CTLA-4

CTLA-4 was initially identified by Burnet et al. (1987) during cDNA screening as a member of the Ig superfamily and shown to be expressed by activated T cells. An interesting feature in the complexity of costimulation is the inducible expression of CTLA-4 following activation. CTLA-4 is expressed following initial TCR and CD28 engagement, while CD28 is down regulated. CTLA-4 also binds CD80/CD86 with a higher affinity than CD28 (Kd values of approximately 12nM compared with 200nM respectively) (Greenwald et al., 2005; Rudd et al., 2009). Subsequent ligation of CTLA-4 results in negative signals being delivered to the T cell, antagonising the initial stimulatory effects of CD28 ligation , and may also act to out-compete CD28 for the binding of CD80/CD86 (Peggs and Allison, 2005), or reduce the contact time with APC thus reducing the strength of other signals, including TCR (Rudd et al., 2009). CTLA-4 therefore acts as a regulatory mechanism to prevent over zealous T cell activation in the presence of the ubiquitous expression of CD80/CD86 by APC. It is likely that other costimulatory molecules are then required to allow the continuation of an immune response (Bertram et al., 2002; Bertram et al., 2004).

Knockout of CTLA-4 in mouse results in a lethal polyclonal CD4-dependent lympho-proliferation within the first few weeks after birth (Chambers et al., 1997). Likewise a number of autoimmune diseases including insulin-dependent diabetes mellitus, Grave's disease and rheumatoid arthritis have been implicated to have a genetic linkage to the CTLA-4 locus (Ueda et al., 2003).

In contrast to the inducible expression of CTLA-4 on $CD8^+$ and CD4 Th cells, Tregs express CTLA-4 in a constitutive manner. The function of this is not clearly defined, however blocking of CTLA-4 has been shown in a number of studies to antagonise the generation and suppressive functions of Tregs (Horwitz et al., 2008; Read et al., 2006; Tang et al., 2004). As discussed later in section 1.2.6.8 CTLA-4 blocking antibodies are currently being trialled for the treatment of solid tumours with some interesting and encouraging results.

1.1.2.3.2.3 CD80/CD86

The CD28/CTLA-4 ligands CD80 and CD86 are structurally homologous and through knockout studies in mice are known to have similar signalling pathways with very closely overlapping functions (Sharp and Freeman, 2002). The identification of CD86 by Lenschow et al. (1993) was facilitated by the inability of anti-CD80 to inhibit a mixed lymphocyte response while hCTLA-4-Ig inhibited the response.

Both ligands can be expressed by monocytes, B cells and DC, while T cells can also express these ligands (Greenwald et al., 2005). The expression patterns of CD80 and CD86 however vary; CD86 is constitutively expressed at low levels and up-regulated within 6h of stimulation, with maximal levels of expression occurring between 18 and 24h. CD80 is expressed later than CD86 by activated APC; expression is not

detectable until 24h after initial stimulation and does not reach maximal levels until 48 to 72h (Lenschow et al., 1993).

1.1.2.3.3 TNF superfamily

CD40 and its ligand, CD40L (CD154), were the first costimulatory molecules to be identified as members of the TNFR/TNF superfamily of which 19 ligands and 30 receptors have now been identified, including CD27/CD70, CD30/CD30L, OX40/OX40L, 4-1BB/4-1BBL, and glucocorticoid-induced TNF receptor (GITR)/GITR ligand. The TNF super family are separated into three groups; the death domain (DD)–containing receptors, the decoy receptors, and TNF receptor-associated factor (TRAF) binding receptors. The DD containing receptors, e.g. Fas and TRAIL, activate caspases leading to apoptosis. A number of decoy receptors have also been identified, i.e. DcR1, 2 and3, which lack portions of the cytoplasm domain preventing activation of caspases; these receptors therefore compete for DD receptor ligands antagonising their function. In contrast the TRAF binding receptors e.g. 4-1BB, OX40 and CD27 recruit TRAF through a 4-6 amino acid motif. Subsequent signalling following ligation of the receptor, provided by TRAF activation, promotes cellular activation, differentiation, and survival (Ashkenazi, 2002; Croft, 2009; Watts, 2005).

Currently six TRAFs have been identified (Dempsey et al., 2005). All TRAF associated receptors bind TRAF2, while the other TRAFs are differentially to various receptors, as shown in

Figure 1-3. Interestingly although all receptors bind TRAF2, the TRAF2 binding domain of each receptor is divergent; therefore different receptors bind TRAF2 with differing affinities (Ye and Wu, 2000).

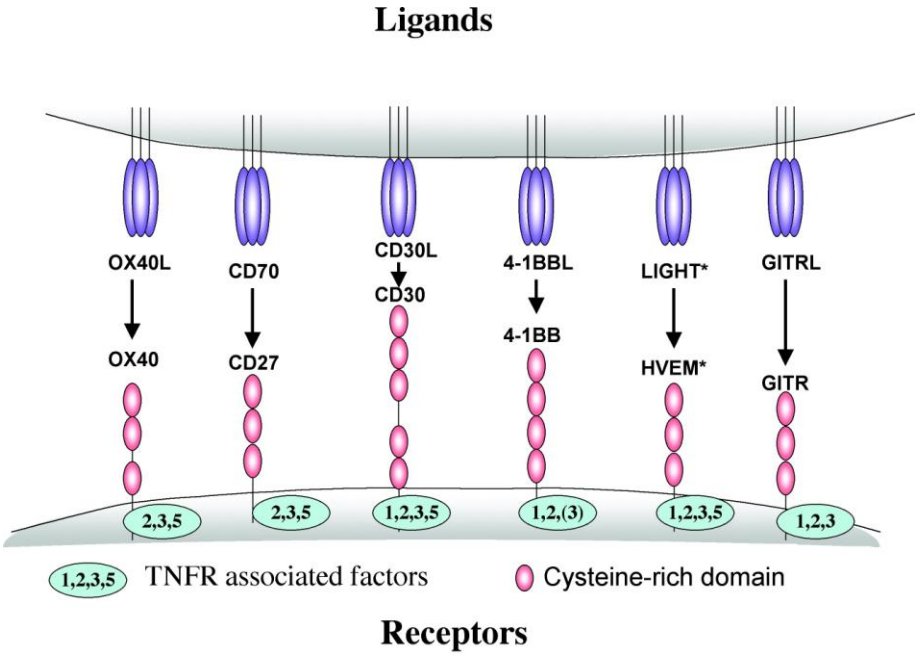

Figure 1-3 TNFR family members and association with TRAFs. Shown are the most prominent TNF receptor:ligand pairs and the variable association with TRAFs (Adapted from Watts, 2005).

The expression of these receptors on naïve T cells is activation induced (with the exception of CD27 which is expressed but up-regulated following T cell activation). In contrast memory T cells and Tregs constitutively express certain family members such as HVEM and GITR respectively, however the expression of OX-40 and 4-1BB remains activation induced, in humans (Pollock et al., 1993; Croft, 2009). The expression of ligands by APC is also regulated; expression can be induced by signals including TLR signalling. Curiously, while $CD4^+$ and $CD8^+$ T cells express receptors, both are also capable of expressing ligands; possibly resulting in T cell:T cell interactions (Croft, 2009; Stephan et al., 2007).

1.1.2.3.3.1 4-1BB Receptor

As a member of the TNFR family 4-1BB (CD137) is a type I membrane protein, the extracellular portion of which is characterized by multiple cysteine-rich pseudo-repeats (Won et al., 2009).

As stated above the expression of 4-1BB is inducible on T cells in response to antigen and/or cytokine stimulation. 4-1BB is also expressed on a number of other cell types including NK cells, NKT cells, mast cells and neutrophils (Wang et al., 2009).

Both $CD4^+$ and $CD8^+$ T cells are capable of expressing 4-1BB following stimulation, likewise Th1 and Th2 cells can both express 4-1BB. *In vitro* T cell antigen stimulation induces 4-1BB expression on the cell surface reaching a maximal level of expression 48hr after initial stimulation, expression then diminishes 4-5 days after initial stimulation (Watts, 2005). However the expression of 4-1BB *in vivo* may vary from that seen *in vitro*, with more rapid and transient expression (Takahashi et al., 1999).

4-1BB associates with TRAF2 and TRAF1 (Watts, 2005), TRAFs associated with 4-1BB complex with inhibitor of NF-κB-α subunit (IκBα), IκB kinase-β (IKKβ) and NF-κB-inducing kinase (NIK), allowing activation of both canonical and non-canonical NF-κB signalling pathways, resulting in decreased cell death through increased expression of the anti-apoptotic proteins Bcl-2, Bcl-X_L and BFL1. In addition 4-1BB signalling down regulates pro-apoptotic Bim and activates PIK3 and PKB and can influence the expression of cyclins. Ligation of 4-1BB also synergizes with TCR-induced signals to induce cell cycle progression and cytokine production.

4-1BB signalling activates JUN N-terminal kinase (JNK) and activator protein 1 (AP1), mitogen-activated protein kinases p38 and ERK, and promotes the nuclear accumulation of nuclear factor of activated T cells (NFAT). These factors promote the production of cytokines, including IL-2, IL-4, IL-5 and IFN and up-regulation of cytokine receptor expression, such as CD25 and IL-12Rβ2 (Croft, 2009; Wang et al., 2009). Additionally 4-1BB stimulation has also been shown not only to prevent but to also break established anergy of T cells (Wilcox et al., 2004).

1.1.2.3.3.2 4-1BB Ligand

4-1BBL (CD137L) is a type II membrane protein. Based on sequence and structural characteristics 4-1BBL can be grouped with other TNF family members including GITRL, and OX40L which are characterized by divergent sequences having relatively low sequence homology (15-34%) with other TNF family members. Like other TNFR family ligands 4-1BBL forms trimeric structures, binding to three receptor molecules. Unlike other TNF Ligand structures which have been solved including GITRL and OX-40L, 4-1BBL shows a novel trimer organization, a 3-fold symmetric, three-bladed propeller as opposed to a canonical bell shape or blooming flower-like structure of other TNF family members (Won et al., 2009).

4-1BBL can be expressed by a number of cell types including DC, macrophage and B cells. In concurrence with the regulated expression of 4-1BB the expression of 4-1BBL is also regulated. In the case of APC 4-1BBL is induced under inflammatory conditions in particular following TLR stimulation (Croft, 2003; Lee et al., 2003).

Interestingly reverse signalling by 4-1BBL can augment the expression of pro-inflammatory cytokines including IL-6, TNF-α and IL-12 by antigen presenting cells (Kang et al., 2007; Lippert et al., 2008).

1.1.2.3.4 IL-12

IL-12 is a 70kDa heterodimer composed of the p35 light chain and the p40 heavy chain, which may also form a heterodimer with p19 to form IL-23. IL-12 is produced mainly by activated macrophages, monocytes and DC *in vivo* in response to TLR stimulation and is enhanced by cytokines such as IFNγ. This creates a positive feedback loop during inflammatory responses, or maturation of DC by CD40-CD40L interactions in which IFNγ is produced stimulating IL-12 production and vise versa.

Our Ad-IL-12 vector is composed of the CMV immediate-early promoter driving p35 and p45 expression, which are separated by an IRES (internal ribosome entry site) (i.e. CMV-p35-ires-p40). In concordance with what may be expected from our adenoviral vector the production of the p35 subunit has been shown to be in excess of the p40 subunit (Kang et al., 2005). The synthesis of excess p40 monomer or homodimer has been reported to result in either IL-12p70 antagonistic effects or independent agonistic stimulatory effects in *in vivo* murine models (Cooper and Khader, 2007).

IL-12 was originally identified in 1989 by Kobayashi et al. from the supernatant of an EBV-transformed human B cell line and termed NK cell stimulatory factor (NKSF), due to its ability to activate NK cells and increase their cytotoxicity; it was also shown to induce IFNγ production and T cell proliferation.

The receptor for IL-12 originally characterised by Presky et al. (1996) is composed of two chains, IL-12Rβ1 and IL-12Rβ2. Expression is primarily associated with activated T and NK cells, however resting NK cells have been shown to constitutively express the IL-12 receptor at low levels (Trinchieri, 2003). TCR ligation induces expression of the IL-12 receptor, which is enhanced by cytokines including IFNγ, TNFα and IL-12 itself, as well as by ligation of CD28. Binding of IL-12 to its receptor mainly signals through the activation of STAT4 (signal transducer and activator of transcription-4), via the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway (Trinchieri, 2003; Kang et al., 2005).

The IL-12Rβ1 subunit is expressed at similar levels on Th1 and Th2 cells (Szabo et al., 1995), commitment to the Th1 cell type is associated with expression of IL-12Rβ2 allowing responsiveness to IL-12. The expression of the β2 chain is dependent on T box transcription factor (T-bet) activation through IFN-γ and Stat1 signalling (Mullen et al., 2001; Afkarian et al., 2002). The Th2-inducing cytokine IL-4 represses IL-12 signalling through inhibition of β2 chain expression, antagonizing Th1 cell differentiation (Szabo et al., 1997).

IL-12 can augment the proliferation of T cells and NK cells in response to a variety of stimuli including mitogenic lectins, alloantigens, CD3-specific antibodies and phorbol diesters, and has a direct proliferative effect on pre-activated T cells and NK cells. IL-12 can also synergise with CD28 ligation to induce proliferation of resting T cells in the absence of antigen, but also facilitates proliferation and IFNγ production by antigen specific T cells. IL-12 can also enhance cytotoxicity by up regulation of perforin and granzyme and expression of adhesion molecules (Trinchieri, 2003).

1.1.2.4 T cell subsets

1.1.2.4.1 T cell polarisation

The identification of two distinct subsets of T helper cells capable of producing different cytokine profiles, differentially polarised from a non-polarised naïve (Th0) precursor cell led to the conceptualisation of Th1 and Th2 subsets. The Th1 subset is characterized by production of IFNγ and is involved in cellular immunity against intracellular pathogens (e.g. viruses) and tumours. While the Th2 subset predominantly produces IL-4, IL-5, and IL-13 and are required for humoral immunity. While the majority of interest in subsets has involved $CD4^+$ T helper cells, $CD8^+$ T cells are also polarised to form Tc1 and Tc2 subsets (Amsen et al., 2009; Zhou et al., 2009). The polarisation of $CD8^+$ T cells to Tc1/Tc2 has similar stimulating factors to the polarisation of $CD4^+$ T cells (Croft et al., 1994).

The polarisation of these subsets involves signalling via mutually exclusive cytokines and transcription factors. Th1 cells are polarised by IL-12 and IFNγ through induction of STAT4, STAT1, and T-bet. Conversely Th2 cell polarisation requires IL-4 mediated induction of STAT6 and subsequent GATA3 expression. Under polarizing conditions stable cytokine production was observed only after a set number of cell divisions suggestive of a requirement for the generation of a stable transcriptional program (Bird et al., 1998; Grogan et al., 2001). Importantly once naive Th0 cells have been polarized into Th1 or Th2 cells, it has been generally accepted commitment is not reversible even under different polarizing conditions (Murphy et al., 1996). However the relationship, cytokine production and commitment to Th subsets may be more plastic than has previously recognised (O'Shea and Paul, 2010).

A number of other T cell subsets have been identified recently based on their cytokine production profiles and induction under varying cytokine milieu including the regulatory subsets discussed in 1.1.2.4.3 and the IL-17 producing proinflammatory Th17 subset. However the exact roles and plasticity of these subsets and which of these subsets are truly representative of differential subset commitment is currently debated (Locksley, 2009; O'Shea and Paul, 2010; Zhou et al., 2009).

1.1.2.4.2 Memory T cells

The ability of T cells to form memory is a defining feature of the adaptive immune response, allowing rapid response to subsequent antigen encounter. Following an initial clonal expansion a contraction phase occurs where ~90% of responding T cells undergo apoptosis, from this a small minority of cells are believed to transition to a memory state. As could be expected the initial size of the T cell response determines the size of the memory pool created. Therefore the quality of the initial stimulation (of which costimulation plays an important role) provided to the primary responding T cells has an impact on the quality of not only initial response but also the formation of memory (Obar and Lefraniçois, 2009; Prlic et al., 2007; Seder and Ahmed, 2003).

The signals and mechanisms governing the formation of T cell memory are not fully understood. $CD4^+$ and $CD8^+$ T cells have been shown to develop memory as a linear process, i.e. naïve to effector to memory. Murine model systems, utilising Cre-mediated recombination to irreversibly mark effector T cells with IFNγ or Granzyme B promoter activity, have shown these effector T cells go onto form long lived memory T cells (Bannard et al., 2009; Harrington et al., 2008). Additionally by transferring a single antigen specific T cell into mouse Stemberger et al. (2007) showed a single $CD8^+$ T cell has the potential to form all memory and effector subsets observed during an immune response. These data suggest memory formation is

governed by signals during the immune response. Interestingly commitment to memory formation has been shown to occur during the initial stages of T cell division as shown by Chang et al., (2007). Therefore suggesting the earliest signalling events may dictate the final outcome of memory and effector T cell development (Obar and Lefraniçois, 2009).

As survival of T cells is an important factor in the generation of memory, and the strength of initial stimulation also impacts upon memory formation costimulatory molecules such as OX40L and 4-1BBL have been implicated in the formation of T cell memory (Croft, 2009; Dawicki et al., 2004; Hendriks et al., 2005).

While the development of memory may be a prerequisite for the efficacy of prophylactic vaccine development, a strong initial response may be as important in cancer immunotherapy (Rosenberg et al., 2004), which may lead to memory however the development of antigen loss variants may mean these do not have a role to play in further anti-tumour responses.

1.1.2.4.3 Regulatory T cells

Suppressive T cells were initially proposed in the 1960s, a seminal paper by Gershon and Kondo (1971) lead to the hypothesis that regulatory T cells were a specific lymphocyte subset. Due to inadequate techniques, lack of specific markers and conflicting data, for a time a consensus developed against the presence of suppressor T cells (Germain, 2008). Nevertheless there was growing evidence that a subset of T cells was able to regulate autoimmunity in murine models following neonatal thymectomy (Sakaguchi et al., 1985). The true renaissance of the field however was led by the work of Sakaguchi et al. (1995) who were able to show that a population of

$CD4^+CD25^+$ T cells were able to prevent autoimmune disease in a mouse model. Following the identification of the selective expression of FoxP3 in the $CD4^+CD25^{hi}$ population in mouse a definitive phenotype and transcription factor was assigned to the regulatory subset (Hori et al., 2003; Fontenot et al., 2003).

The presence of Tregs is vitally important for the correct function of the immune system. While tolerance inducing mechanism may play an important role in controlling pathological immune activation, in the absence of Tregs gross pathology will occur as shown in the scurfy mouse strain or in IPEX (immune dysregulation polyendocrinopathy enteropathy X-linked) syndrome in humans. Both are associated with mutations in the *FoxP3* gene (Hori et al., 2001). Conversely in the cancer setting Tregs have been implicated in the suppression of anti-tumour immune responses and are a major target for novel therapies (Curiel, 2007; Nizar et al., 2009). Tregs are produced in the natural course of T cell development termed natural Tregs (nTreg), however there are also Treg that are induced from T cells in the periphery, so called induced Treg (iTreg), both share functional properties, however they have also been shown to have differences in responses to cytokines and costimulatory signals (Horwitz et al., 2008).

While in mouse the $CD4^+CD25^{hi}FoxP3^+$ phenotype holds true as a marker of Tregs, in humans the phenotype is somewhat less rigidly defined. Currently in humans, many groups define the classical Treg as a $CD4^+CD25^{hi}CD127^{lo}FoxP3^+$ T cell (Liu et al., 2006; Seddiki et al., 2006), other markers may also be used such as LAG-3, however the ability of a diverse group of markers such as these to provide a pure Treg population is disputed (Corthay, 2009). Numerous other T cell subsets with

suppressive functions have also been identified, including $CD8^+$ T cells; the phenotype and regulatory functions of these cells are varied (Shevach, 2006; Wang and Wang, 2007).

From *in vitro* data Tregs have been shown to have the ability to suppress the function of a diverse population of cells including $CD4^+$ and $CD8^+$ T cells, B cells, Macrophage, NKT cells and NK cells (Piccirillo et al., 2001; Ghiringhelli et al., 2005; Lim et al., 2005). The mechanism of suppression by Tregs and other regulatory subsets are as yet undefined *in vivo*. Many contact dependent and independent mechanisms have proposed including the generation of immunosuppressive cytokines, e.g. TGF-β and IL-10, IL-2 consumption and cytolysis, resulting in the induction of anergy, inhibition of proliferation, and suppression of effector functions (Shevach, 2009). There are therefore numerous subdivisions in the phenotypes and mechanisms used by regulatory T cells to suppress lymphocyte activation, involving various combinations of cell contact dependent and independent mechanisms.

While the immune system has clearly developed mechanisms to inhibit or down regulate immune responses which may be harmful there are logically also mechanisms to allow the suppression of the suppressors or allow activation in their presence. Such effects can be mediated by glucocorticoid-induced tumour necrosis factor receptor (GITR) or OX40 mediated suppression of Tregs (Valzasina et al., 2005). While interestingly for this project IL-12 is able to reverse Treg mediated suppression and allow cytokine production and proliferation by $CD8^+$ T cells in the presence of Tregs (King and Seagal, 2005). Likewise 4-1BBL has also been shown to allow T cells to become resistant to Treg suppression (Choi et al., 2004), while TLR9

stimulation has also been shown to impair Treg conversion under Treg inducing conditions (Hall et al., 2008).

There is substantial evidence that the polarisation of this cell type may be more plastic than originally thought (Zhou et al., 2009), for example nTreg and iTreg have been shown to be capable of being converted into IL-17 producing pro-inflammatory Th17 cells in the presence of TGF-β and IL-6 (Xu et al., 2007; Yang et al., 2008; Zheng et al., 2008). However iTreg appear to be resistant to conversion under Th1 inducing conditions (Croxford et al., 2009). It has also been show that Th17 cells can be converted into Th1 cells in the presence of IL-12 (Lee et al., 2009). Therefore in the presence of IL-6 produced by DC following TLR ligation (Pasare and Medzhitov, 2003) it may be possible to repress Treg suppression by conversion to Th17 cells, thereby reducing the suppressive environment. Th17 cells may then be converted to Th1 cells.

1.1.3 Natural Killer T cells

Natural killer T (NKT) cells comprise a small population of peripheral blood lymphocytes (2.5% average). They can be described as unconventional T cells displaying features of both the adaptive and innate immune system, and are proposed to bridge between the two systems (Giroux and Denis, 2005). Classically NKT cells express CD3 and αβ-TCR however rearrangement is restricted producing an invariant TCR, in humans a Vα24-Jα18 rearranged TCR α-chain with a Vβ11-containing β-chain. Expression of this TCR combination results in the TCR no longer recognising peptide bound to MHC but rather recognising glycolipids presented on the unconventional antigen presenting molecule CD1d. The recognition of CD1d by NKT

cell TCR is highly conserved across species with mouse invariant NKT cell TCRs being able to recognise human CD1d (Godfrey et al., 2004).

The invariant NKT (iNKT) cell represents the classical (type I) NKT cell however a second type of NKT has also been defined which does not express the Vα24-Jα18 invariant TCR, although does retain CD1d restriction. In contrast to type I iNKT cells the type II NKT cells do not recognise the archetype NKT cell antigen α-GalCer (α-galactosylceramide). Division of NKT cell subsets has additionally been proposed to be more reliably defined by an NKT cell's ability to recognise this antigen. A third subset of NKT cells has also been suggested which rearrange TCR and are not CD1d restricted, however whether these cell truly represent type III NKT cells or a subset of T cells is debated (Godfrey et al., 2004).

NKT cells also express a number of NK associated markers, most commonly CD161, while other markers including CD56 can also be expressed (Loza et al., 2002; Giroux and Denis, 2005). Like NK cells NKT cells can become activated without the need for additional signals, and are capable of producing large amounts of cytokine, including Th1 cytokines IFNγ and TNFα, and Th2 cytokines IL-4 and IL-13, rapidly upon activation (Loza et al., 2002), possibly allowing these cells to also coordinate the polarisation of adaptive immune responses.

A number of potential CD1d restricted TCR ligands derived from microbial sources have been defined for NKT cells, the classical α-GalCer is a marine sponge derived glycolipid (Kawano et al., 1997). Interestingly there has been shown to be a differential production of cytokines dependent on the ligand used, for example

synthetic modification of α-GalCer can skew cytokine production towards either IFNγ or IL-4 (Balato et al., 2009). Additionally the cytokine milieu has also been shown to have effects on cytokine production by NKT cells with IL-12 able to induce high levels of IFNγ (Brigl et al., 2003). While NK cells are major responders to IL-12 NKT cells have been shown to be able to respond more strongly in response to low levels of IL-12, due to the higher expression of IL-12R by NKT cells (Kawamura et al., 1998).

NKT cells have been implicated in a number of disease states including autoimmune and allergic diseases. In contrast NKT cells have also been implicated to have regulatory or protective roles in some autoimmune diseases, and have been shown to be important in anti-microbial defence. NKT cells have also been proposed to be important in anti-tumour responses; however the exact role of NKT cells is unclear. While NKT cells can express perforin and FasL, NKT cells have also been shown to activate NK cells and cytotoxic $CD8^+$ T cells by production of IFNγ (Berzofsky and Terabe, 2008), therefore NKT cells may mediate direct and indirect anti-tumour effects.

1.1.4 Natural Killer cells

Human natural killer cells are defined as a population of $CD3^-CD56^+$ lymphocytes; NK cells are a major population of lymphocytes comprising ~10-15% of the total circulating lymphocytes. The initial identification of the NK cell followed the observation of a murine lymphoid cell which was able to kill certain target cells *in vitro* without prior activation which led to the coining of the name natural killer cells in 1975 (Kiessling et al., 1975; Herberman et al., 1975). However it was not until the subsequent proposal of the missing self hypothesis by Karre et al. (1986) that the

mechanisms behind the recognition of target cells by NK cells began to be understood. While originally regarded as a homogeneous population, the complexity of NK cell biology is beginning to be appreciated, with a diverse array of roles and the ability to recognise and respond to differing targets through the mosaic expression of a series of inhibitory and activatory receptors.

1.1.4.1 Development

As lymphocytes NK cells share a common $CD34^+$ hematopoietic progenitor cell T and B cells as show in

Figure 1-1. NK cells develop in the bone marrow however the complexities of NK cell development are currently being elucidated. Five distinct development stages have been proposed for human NK cells, through which NK cell markers and function are acquired and lineage commitment obtained. NK cell development may also occur in secondary lymphoid tissue, or NK cells may undergo a developmental stage within the secondary lymphoid tissue, however this has yet to be confirmed. Currently the γ-chain receptor cytokine IL-15 and interactions with bone marrow stromal cells are considered to have importance in the differentiation of NK cells. The acquisition of self tolerance during developmental and the role for distinct signals between each stage of development have not been defined (Farag and Caligiuri, 2006; Freud et al., 2006; Williams et al., 2006).

1.1.4.2 Activatory and inhibitory receptors

Unlike T cells which recognise target cells through a specific receptor and utilise additional (costimulatory) ligand:receptor interactions in order to become activated NK cells rely on the expression of a diverse array of receptors to recognise and become activated by target cells, and also to achieve tolerance of healthy cells. Also

while T cells recognise one specific peptide, NK cells rely on the balance between inhibitory and activating signals. Triggering of a single activating signal will in general (with the exception of CD16) be insufficient to allow activation (Bryceson et al., 2006), likewise the lack of inhibitory signals is detrimental to the ability of natural killer cells to become activated (Fernandez et al., 2005).

NK receptors can be divided into two main categories the inhibitory and activatory receptors, the diversity of these receptors is shown in Figure 1-4. The inhibitory receptors as the name suggests hold back the activation of cytotoxicity classically through interactions with MHC class I molecules although other non-MHC ligands have also been identified, while activatory receptors are able to bind specific ligands, typically being associated with viral or malignant transformation.

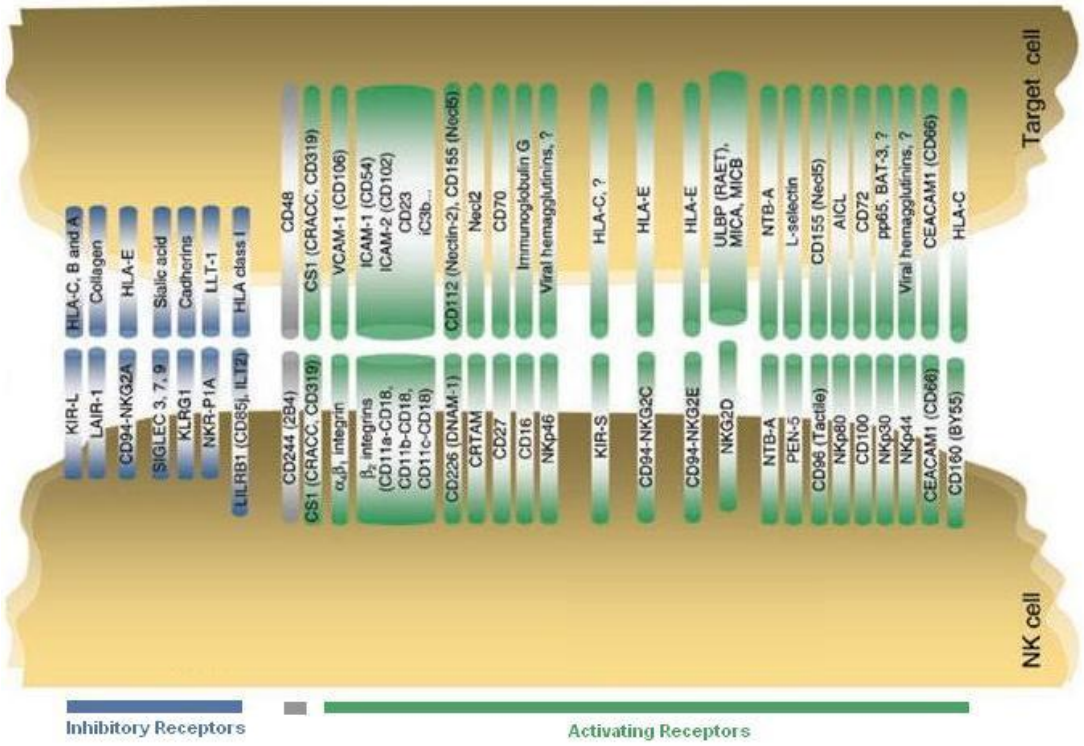

Figure 1-4 Natural killer cell receptor repertoire. The diverse inhibitory (blue) and activating receptors (green) expressed by NK cells are shown. The receptor 2B4 is also shown (in grey) which can have inhibitory and activating roles. NK cells have variegated expression of the shown activating and inhibitory receptors allowing response to a diverse range of targets. Adapted from Vivier et al. (2008).

1.1.4.2.1 Inhibitory ITIM associated receptor signalling

There are numerous inhibitory receptors including, killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs) both of which are type I glycoprotein members of the immunoglobulin superfamily, whereas the CD94-NKG2A receptor is a type II glycoproteins with a C-type lectin-like scaffold. While there is a diverse set of inhibitory receptors and ligands, all share a common signalling motif in the cytoplasmic domain, the immunoreceptor tyrosine-based inhibitory motif (ITIM) which is defined by the sequence (I/L/V/S)XYXX(L/V) (where X represents any amino acid and forward slashes denote alternative amino acids which may be in that position) (Lanier, 2008).

Upon ligation of an inhibitory receptor the tyrosine residue is phosphorylated (by Src family kinases) leading to the recruitment of the phosphatases SHIP-1, SHP-1 or SHP-2. By recruiting these phosphatases to the immunological synapse between the NK cell and its prospective target these phosphatases can inhibit Ca_2^+ influx, degranulation, cytokine production and proliferation by dephosphorylating the substrates of the tyrosine kinases linked to activating NK receptors. Interestingly unlike the permanent tolerance or anergy instilled in T cells to avoid activation, inhibition of NK cell responses by ligation of inhibitory receptors is transient and spatially localised, resulting in an NK cell which has encountered inhibition subsequently being able to become activated upon encounter with a valid target lacking inhibitory ligands and/or expressing adequate activatory ligands (Lanier, 2008).

1.1.4.2.2 Activating ITAM associated receptor signalling

All mature NK cells constitutively express FcεRIγ, CD3-ζ and DAP12 signalling subunits as hetero and homodimers. These interact with NK cell activating receptors through oppositely charged amino acids in the transmembrane regions of the activating receptor and signalling subunits. In a similar manner to the inhibitory receptors FcεRIγ, CD3-ζ and DAP12 are characterised by the presence of specific motifs in the cytoplasmic domain, the immunoreceptor tyrosine-based activation motif (ITAM) which is defined by the sequence (D/E)XXYXX(L/I)X_{6-8}YXX(L/I) (Lanier, 2008).

Similarly to the consequences of inhibitory receptor ligation, ligation of the activating receptor allows the ITAM tyrosine to become phosphorylated by Src family kinases, leading to binding of the tyrosine kinases Syk and Zap70, resulting in a signalling

cascade that is very similar to that of T and B cells. Interestingly there appears from knockout studies in murine NK cells to be a separation of cytotoxicty and cytokine production dependent on differential regulation of downstream signalling events (Huntington et al., 2005; Hesslein et al., 2006), likewise it has been suggested that different adaptor molecules or kinases may be utilised by different receptors although this has yet to be confirmed (Lanier, 2008).

1.1.4.2.3 Non-ITIM/ITAM signalling

While the majority of inhibitory and activatory NK receptors function through ITIM or ITAM motifs respectively, there have also been a number of other signalling systems defined. These include most importantly the activating receptor NKG2D and the inhibitory/activatory receptor 2B4 (CD244) (Garni-Wagner et al., 1993; Schatzle et al., 1999; Billadeau et al., 2003).

NKG2D is a member of the C-type lectin-like receptor (CTLR) superfamily, with diverse expression on NK cells, γδ-T cells, activated $CD8^+$ T cells and macrophages. NKG2D has been of great interest due to its role in recognition of stressed cells such as those undergoing viral infection or malignant transformation. In contrast to other activating receptors NKG2D does not associate with ITAM containing subunits but rather exclusively associates with a DAP10 homodimer, in humans. DAP10 contains the signalling motif YINM, the downstream signalling events associated with activation of this receptor are therefore distinct from ITAM associated receptors utilising PI3K and Grb2 rather than Syk family kinases (Billadeau et al., 2003).

2B4 is a member of the signalling lymphocytic activation molecule (SLAM) family of receptors (members of the immunoglobulin superfamily), which in humans also

includes NK, T- and B-cell antigen (NTB-A) and CD2-like receptor-activating cytotoxic cells (CRACC), the expression of which is present on the majority of mature NK cells (Veillette, 2006).

The signalling pathway of 2B4 is the most widely studied. SLAM-related receptors, such as 2B4, bind members of the SAP family of adapters; SAP or Ewing's sarcoma-activated transcript-2 (EAT-2), through interactions of the SH2 domain of SAP family adapters and the tyrosine-based motif TIYXXV/I, an immunoreceptor tyrosine-based switch motif (ITSM) of 2B4. It has been suggested that the differential binding of either SAP or EAT-2 may control the ability of 2B4 to act as an inhibitory or activatory receptor, expression of which may depend on the maturational status of the NK cell (Lanier, 2008; Veillette, 2006).

2B4 has been shown to act as an inhibitory receptor during NK development prior to expression of ITIM associated inhibitory receptors, possibly also explaining the widespread expression of the 2B4 ligand CD48 on hemopoietic cells. Alternatively the prior activation of NK cells by receptor ligation or cytokines may influence the ability of 2B4 to be activating or inhibitory (Sivori et al., 2002; Bryceson et al., 2006; Veillette, 2006).

1.1.4.3 NK Education, Arming and Licensing

While early experiments indicated NK cells recognise ‘Missing self’, a paradox exists in that NK cells derived from TAP-2 deficient patients are tolerant of autologous cells, although they lack MHC expression, likewise it has been shown that NK cells from healthy individuals which lack inhibitory receptors are also tolerant of self;

however both these groups are also hyporesponsive to missing self (Yawata et al., 2008). This suggests that there is a complex mechanism behind the endowment of NK cells with the ability to kill, allowing the tuning of NK responses to the individual.

The phenomenon of 'hybrid resistance' in mice where parental grafts are rejected by F1 hybrid offspring was originally observed in 1971 by Cudkowicz and Bennettin, it was not until much later however that the significance of these finding could be explained. Yawata et al. (2008) elegantly showed that there is a chimeric expression pattern of inhibitory receptors associated with human NK cells with every possible combination being present to varying degrees in every donor. As the expression of KIR genes is hypervariable, and not linked to HLA inheritance, individuals may also possess KIR for which they do not possess cognate ligands (Parham, 2005). There is therefore a requirement for a process of 'education' of NK cells during their development to allow self tolerance to be achieved. Hence in hybrid resistance a population of F1 NK cells will recognise parental cells as having 'missing self' as they do not possess the appropriate ligands for the inhibitory receptor with which those NK cells have been educated to achieve self tolerance.

NK cells lacking inhibitory receptors are hyporesponsive, likewise NK cells which only possess an inhibitory receptor for which the individual does not possess a corresponding HLA ligand cannot experience self and are therefore also hyporesponsive. In order to acquire cytotoxic potential NK cells must achieve tolerance through the expression of at least one inhibitory receptor to become 'armed'; the 'at least one' model (Anfossi et al., 2006). While there is evidence to support the expression of all inhibitory receptors encoded by an individual's genotype

within the human NK cell population, there is also a suggestion that the expression of inhibitory receptors may be influenced by the presence of appropriate ligand (Yu et al., 2007). Therefore NK cells express every combination of inhibitory receptor possible for each individual, but this expression may be skewed to allow the production of the biggest repertoire of functional NK cells, allowing the highest chance of recognition of appropriate targets.

To add to the complexity of the process of NK cell education, the process appears to be dynamic, in contrast to T cell anergy. Using a mouse transgenic model on a H-2^d background Johansson et al. (1997) created a mosaic transgenic line expressing H-$2D^d$ on 10-80% of cells. Despite the mosaic expression of ligand and inhibitory receptors, mice were tolerant of cells lacking trangene expression. However tolerance could be broken by separating the $H2D^d$ negative and positive cells *in vitro*, suggesting NK cell tolerance is also regulated by interactions with surrounding cells during education. This is similar to the breakdown of tolerance associated with the *in vitro* cytokine stimulation of NK cells derived from TAP-deficient patients (Zimmer et al., 1998). Likewise NK cells lacking inhibitory receptors as previously mentioned are hyporesponsive, however these cells are still capable of responses to PMA and ionomycin, suggesting a block in activatory signalling (Anfossi et al., 2006).

At present however there has not been an agreement on the model which best fits to the *in vivo* process of NK cell education, and so far the molecular mechanisms governing NK cell education and tolerance have not been defined (Raulet and Vance, 2006; Colonna, 2008).

1.1.4.4 CD56^{dim} and CD56^{bright} subsets

Human NK cells identified by their lack of CD3 and expression of CD56 can also be subdivided into two subsets (CD56^{bright} and CD56^{dim}) on the basis of the level of CD56 expression. These two subsets have been shown to have different distribution and functional abilities as shown in **Error! Reference source not found.**.

Table 1-1 Differential markers and functions of the CD56^{bright} and CD56^{dim} NK cell subsets.

	CD56^{dim}	CD56^{bright}
CD56	+	++
CD16	++	-/+
CD25	-	+
CD62L	-/+	++
CCR7	-	++
CXCR3	-/+	+
CD27	-/+	+
KIR	++	-/+
CD94	-/+	++
NKG2A	-/+	+
Cytokine production	+	++
Natural cytotoxicity	++	+

++ High level expression; + expressed; +/- variable expression, low or absent; - not expressed. Adapted from Cooper et al. (2001) and Vossen et al. (2008).

The CD56^{dim} subset is the dominant subset in the periphery accounting for ~90% of the circulating NK cell population. This subset has been shown to be the more cytotoxic subset when resting, expressing higher levels of perforin and granzyme, compared with CD56^{bright} NK cells. The CD56^{bright} NK cell population in contrast to the CD56^{dim} population is enriched in secondary lymphoid tissues. While basal cytotoxicity of CD56^{bright} NK cells is lower than for CD56^{dim} cells pre-activation with IL-2 results in an increased cytotoxic ability (Robertson et al., 1992). While the CD56^{bright} NK cells may be less capable of conducting NK cell mediated cytotoxic functions they are however superior in the production of a plethora of

immunoregulatory cytokines such as IFNγ, TNFα, and GM-CSF (Cooper et al., 2001).

It has also been suggested that the $CD56^{bright}$ subset represents an immature form of NK cell from which $CD56^{dim}$ cells are derived, conversely it has been suggested that $CD56^{bright}$ NK cells are an activated from of $CD56^{dim}$ cells, whereas there is an argument that these two subsets are distinct being derived from a common progenitor. As yet it is unclear which of these models is correct or equally whether *in vivo* a combination of these models may function (Cooper et al., 2001).

The general definition of the $CD56^{dim}$ population relies on the differential expression CD16 (FCγRIII), expression of which defines the classical $CD56^{dim}$ NK cell, allowing the ADCC function of this cell population. The $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells also display major differences in the expression patterns of many other molecules, firstly associated with the homing phenotype of these cells, but also suggestive of the functional differences of these cell populations. The $CD56^{bright}$ population lacks expression of CD16, there is exclusive expression of CD25 (the IL-2 receptor α-chain) allowing the formation of the high affinity IL-2 receptor in complex with CD122 (β-chain) and CD132 (γ-chain), suggestive of differential roles and responsiveness of these different subsets. This population is also well placed to utilise IL-2 produced by activated T cells in the secondary lymphoid tissues (Caligiuri et al., 1990; Fehniger et al., 2003). The $CD56^{bright}$ population also typically expresses higher levels of the inhibitory CD94-NKG2A receptors complex while expressing low levels of KIR in contrast to the $CD56^{dim}$ subset (Cooper et al., 2001; Jacobs et al., 2001).

Given these differences the CD56^{bright} subset may be more suited to coordinate an immune response, which could potentially be a powerful tool for the immunotherapy of cancer.

1.1.4.5 Interactions with the adaptive immune system

As stated the CD56^{bright} NK cell subset is present in secondary lymphoid tissue. These cells have been shown to localise to the parafollicular T-cell region of lymph nodes, facilitating interactions with DCs and T cells (Fehniger et al., 2002). Besides the localisation of CD56^{bright} NK cells to lymph nodes both subsets of NK cells may be recruited to inflammatory sites following pathogen entry or tumour formation through expression of the chemokine receptors CXCR1 and CXC3R1 by CD56^{dim} NK cells and CXCR3 by CD56^{bright} NK cells (Qin et al., 1998; Lazzeri and Romagnani, 2005; Marcenaro et al., 2006).

The interaction of CD56^{bright} and CD56^{dim} NK cells with DCs has been shown to promote activation of the NK cell, it is however the CD56^{bright} population which are subsequently able to proliferate (Vitale et al., 2004). Mature DC are able to produce a variety of stimulatory cytokines, including IL-12 and IL-15 which are capable of inducing NK activation and proliferation, however there is not a detailed understanding of the mechanisms responsible for the induction of NK cell proliferation by DCs (Trinchieri, 2003; Fehniger and Caligiuri 2001).

While resting NK cells are able to recognise and kill target cells without prior or additional activation, NK cells are not capable of producing immunostimulatory cytokines (including IFNγ and TNFα) in response to target cells without additional

stimulus. The production of cytokines can be induced upon interaction with target cells by type 1 IFNs, IFNα and IFNβ, (which are produced by cells in response to viral infection). The production of cytokines by NK cell co-activation with type 1 IFN (or the pro-inflammatory cytokines IL-18 and IL-12) are a prerequisite for the NK cell induced enhancement of DC function and stable polarisation of the DC towards Th1 induction (Mailliard et al., 2003; Marcenaro et al., 2005; Garcia-Sastre and Biron, 2006). The production of IFNγ has also been shown to directly influence the Th1 polarisation of naïve T cells (Afkarian et al., 2002; Morandi et al., 2006). The stimulation of TLR3 and TLR9 on NK cells in the presence of IL-12 without further activation also induces the production of immunostimulatory cytokines and promotes cytotoxicity against immature DC and also tumour cell targets (Sivori et al., 2004). The engagement of TLRs on DC and NK may provide the initial stimulus to drive an effective immune response.

The process of DC editing, killing of immature DC by NK cells, may seem paradoxical to the ability or necessity of NK cells to drive an effective immune response, however immature DC are less well equipped to drive effective immune responses and may induce tolerance (Dhodapkar et al., 2001; Levings et al., 2005), particularly in the cancer setting (Ghiringhelli et al., 2005). The ability of NK cells to recognise immature DC has been shown to be dependent on the differential expression of HLA-E (the ligand for the CD94/NKG2A) by mature and immature DC. Interestingly it has been shown that the population of NK cells responsible for DC editing functions are CD94/NKG2A^{+}KIR^{-}, i.e. the CD56^{bright} subset, which are also well placed to interact with DC during an immune response (Chiesa et al., 2003).

The process of reciprocal activation of DC and NK cells has been shown to have both contact dependent and independent components. In contrast to mouse, human NK cells separated from DC by transwells are able to induce the maturation of immature DC, however the ability of NK to induce maturation of immature DC has also been shown *in vitro* to be dependent on the ratio of NK:DC where high NK ratios result in the inhibition of DC function (Fernandez et al., 1999; Nishioka et al., 2001; Gerosa et al., 2002; Piccioli et al., 2002). The NK cell mediated process of DC editing may be responsible for this paradoxical relationship, whereby killing of immature DC may allow the activation of NK cells and associated cytokine production and provision of additional danger signals aiding DC maturation.

In a series of experiments using an *in vivo* murine model system, Krebs et al. (2009) administered OVA expressing cells with or without 'missing self' as an antigen source to mice. $CD8^+$ and $CD4^+$ T cell and B cell responses showed that killing of antigen expressing target cells by NK cells was necessary to promote the generation of an effective adaptive immune response. Antigen specific responses driven by NK cells mediated killing of target cells was also very sensitive; generating a response to as few as 10^4 target cells.

There is therefore a reciprocal interaction between NK, APC and T cells during the progression of an immune response. APC may become activated to produce monokines, activating NK cells. Activated NK cells produce immunostimulatory cytokines and provide DC editing functions, enhancing the antigen presenting capacity of APC and promoting the generation of Th1 T cells. NK cells could also

provide antigen for APC to present to T cells by killing targets. Activated T cells in turn produce IL-2 for the further activation of NK cells.

1.2 Interaction of the immune system and cancer

1.2.1 Tumour development

The development of a malignant carcinoma is a progressive multi-step process analogous to Darwinian evolution, requiring the production of many mutations. On average 90 mutated genes are present per tumour in colorectal and breast cancer tumours, however only a subset of these are likely to be 'driver' mutations promoting tumorigenesis (Sjöblom et al., 2006). Insightful work performed by Shah et al. (2009) showed the accumulation of mutations during the progression of a primary breast tumour over a period of nine years with biopsies from the same patient. In the course of progression not only had low frequency mutations come to fixation in the tumour but new mutations had also occurred and become prevalent. Similarly, following on from earlier work by Hunter et al. (2006) showing prevalence of a mutation in a specific mismatch repair gene (*MSH6*) in glioma tumours following treatment with the alkylating agent temozolomide, work by Yip et al. (2009) showed the occurrence of this mutation during therapy due to the selective pressure exerted by temozolomide. The occurrence of *MSH6* mutations were also shown to have a causal relationship with the occurrence of resistance.

There are a number of distinct prerequisite properties required for the development of a cancer; in order for these 'Hallmarks of cancer' to develop there is a requirement to overcome multiple cellular mechanisms and checkpoints (Hanahan & Weinberg,

2000). The progression to malignancy bypasses such controls due to the plasticity of the cancer cell genome resulting from defects in nucleotide-excision repair, and chromosome and microsatellite instability. Work by Sjöblom et al. (2006) and also Wood et al. (2007) has shown the presence of mutations in an assortment of genes proposed to drive cancer development from different tumour samples, however there are a minority of mutations which are present in a high proportion of tumours, demonstrating that certain mutations must be selected for during tumour progression. Cancer cells become self sufficient in growth signals, and insensitive to growth inhibitory signals, whether this be through the corruption of signalling pathways, the autonomous production of growth factors or the coercion of accessory neighbouring cells to generate growth signals. Evasion of apoptosis represents an important facet of tumour development, allowing the continuation of growth and thus increasing the potential for further mutations to take place, ultimately leading to the development of limitless replicative potential. Tumours also spread and recruit a nutrient source through sustained angiogenesis, tissue invasiveness and metastasis facilitated by changes in the expression of adhesion molecules and extracellular proteases by the cancer cell or by recruited stromal cells. Ultimately the development of metastases is the final step in the progression of cancer as this results in the cause of 90% of deaths from cancer (Hanahan and Weinberg, 2000; Lengauer et al., 1998).

While there is a requirement for the development of sustained growth and the subversion of tumour suppressor mechanisms to facilitate the growth of a tumour, tumour development does not occur in an unperturbed environment, there is evidence to suggest the immune system is also a controlling factor in this process (Zitvogel et al., 2006).

1.2.2 Recognition of tumour cells by the immune system

There is substantial evidence to suggest a role for the immune system in the development of cancer. However evidence as to the effector cells which may be mediating this protective effect is mixed, in murine models both elements of the innate and adaptive immune responses have been implicated. Given the complex interactions of the immune system it may be likely that multiple cell types are involved during anti-tumour immune responses; dependent on the tumour cells origin, mechanism of transformation, and its ability to be recognised by the immune system (Dunn et al., 2004).

1.2.2.1 T cell antigens

Multiple methods have been utilised to identify the tumour antigens recognised by T cells. This includes initial autologous typing experiments using the recognition of tumour cells versus normal tissue by autologous cells or serum. The identification of specific antigens is now possible utilising cDNA libraries to express antigens in target cells which possess the HLA class of interest. The SEREX (serological expression) cloning technique is an alternative high-throughput method relying on the generation of humoral responses to tumour antigens with T cell help, whereby cancer patient serum is used to detect proteins expressed by prokaryote cDNA libraries (Rosenberg, 2001).

These techniques have allowed the identification over 1000 putative tumour antigens, not including antigens from contributing pathogens. These antigens can be grouped

into four main categories, differentiation antigens, mutated protein antigens, over-expressed proteins, and cancer testis antigens (Dunn et al., 2004; Rosenberg, 2001).

1.2.2.2 Innate recognition

As NK cells were initially defined by their ability to kill target cells lacking HLA class I (HLA-I) molecules, the inability of NK cells to recognise tumours lacking HLA-I was hard to reconcile until the identification of the diversity of NK activating and inhibitory receptors. More recently the identification of the activating Natural Cytotoxicty Receptors (NCRs) NKp30, NKp44 and NKp46 and the CTLR NKG2D, which have been strongly implicated in the lysis of tumour cells by NK cells, has sparked much interest in the recognition of tumour cells by NK cells (Moretta et al., 2000; Waldhauser and Steinle, 2008).

Ligands for the NCRs NKp30, NKp44, and NKp46 have yet to be defined. However a number of ligands have been identified for NKG2D, of most interest are the MHC class I chain related proteins A and B (MICA/MICB) and the UL16-binding protein (ULBP). The expression of the NKG2D ligands is generally absent in healthy cells; however expression is induced by various forms of cellular stress such as UV radiation, genotoxic and oxidative stress, and has been shown to be expressed on a wide variety of tumour cell types (Groh et al., 1999; Waldhauer and Steinle 2008). Despite the expression of these activating ligands there is still a lack of tumour regression. There are likely to be multiple reasons for this: firstly, as subsequently described, many immunosuppressive mechanisms operate within the tumour, increased expression of inhibitory ligands, the suppression of activating receptors and ligands by TGF-β, and also suppression of NKp30 mediated DC editing functions by

TGF-β, the secretion of soluble ligands may attenuate the response of NKG2D expressing cells as may the presence of Tregs (Ferlazzo et al., 2002; Ghiringhelli et al., 2006; González et al., 2008). Furthermore NK cells derived from cancer patients have been shown to fail to effectively infiltrate the tumour site, and show a generalised dysfunction and increased susceptibility to apoptosis (Kadish et al., 1981; Costello et al., 2002; Bauernhofer et al., 2003). Thus again while the immune system is able to recognise and respond to transformed cells the response is ineffective due to immunosuppressory mechanisms manifested or induced by the tumour.

1.2.3 Immunosuppression by the tumour

The vast array of mechanisms employed by a tumour to suppress the anti-tumour immune response is likely to be the biggest hurdle to the success of cancer immunotherapy. Most simplistically the tumour may lose expression of HLA-I, such as is common in lung cancer. Likewise there is also often a loss of elements of the antigen presentation machinery, such as TAP1 or LMP2. Alternatively expression of specific antigens may be lost. Besides the loss of antigen presentation or antigen there may be active processes by which the immune system is suppressed by the tumour. Tumours create intricate suppressive and tolerising conditions, which range from the tolerising conditions of the tumour microenvironment to systemic immune suppression (Willimsky and Blankenstein, 2005; Zitvogel et al., 2006; Zou, 2005).

TAA specific T cell priming may occur in the tumour microenvironment itself, where antigen may be presented ineffectively either by the tumour itself or by APCs which have entered or been recruited to the tumour where they may become dysfunctional or tolerogenic (Zou, 2005). Tumour cells are able to actively recruit many regulatory cell

types, for example the chemokine CCL2 expressed at high levels in tumours incite the recruitment of myeloid suppressor cells (MSC) (Huang et al., 2007). Found in many tumour types (head and neck squamous-cell carcinoma, non-small cell lung carcinoma, metastatic adenocarcinoma of the pancreas, colon and breast), MSC are able to produce nitric oxide. This interferes with the IL-2 signalling pathway and inhibits arginase-1 (ARG1) which is essential for function and development of T cells and responses to IL-2. Likewise plasmacytoid DC (pDC) have been shown to be abundant in many tumours. Expression of CXCL12 by the tumour recruits pDC where they induce $CD4^+$ and $CD8^+$ IL-10 producing regulatory T cells. In contrast the presence of mature myeloid DC has been shown to be rare in tumours. Furthermore, many factors in the tumour microenvironment e.g. VEGF, IL-10 and TGF-β actively inhibit DC maturation and also promote the generation of tolerogenic DC by down regulating CD80 and CD86 and inducing B7-H1 and B7-H4. B7-H1 has been shown to further inhibit DC function through reverse signalling with Treg, and also induce apoptosis of effector T cells. B7-H4 also suppresses T cell function. Besides the induction of Tregs and suppression of effector functions the presence of TGF-β may also have an impact on the DC editing functions of NK cells, as TGF-β has been show to induce the down regulation of NKp30, which has been implicated in the recognition of immature DC by NK cells. B7-H1 has also been shown to be expressed by a number of tumour types (Ferlazzo et al., 2002; Li et al., 2006; Zou, 2005).

Tryptophan is essential for T cell proliferation and differentiation. Tolerogenic DC and tumour cells may also produce indoleamine-2,3-dioxygenase (IDO) which catalyses the breakdown of tryptophan, preventing clonal expansion and encouraging AICD and anergy (Zou, 2005). TGF-β and IL-10 produced by the tumour favour the

development of Treg (Chen et al., 2003; Levings et al., 2005), as well as tolerogenic APC. Cancer patients have been shown to have increased levels of Treg within the tumour and circulating in the periphery. The presence of increased levels of Treg within tumours has been associated with a decrease in patient survival (Curiel et al., 2004; Zhou et al., 2009a; Zou, 2005). TGF-β reduces antigen presentation by DC, inhibits the activity of IFNγ, inhibits the proliferation of T cells and suppresses the activation of NK cells (Gorelik and Flavell, 2002).

Although there may be a selective pressure to actively suppress the immune system, suppression of the immune response may also be mediated as a by product of the gain of the other prerequisite hallmarks of cancer. IL-10 can also act as an autocrine growth factor. Resistance to apoptosis is contributed to in many cancers by the over expression of MUC1, however MUC1 is also able to inhibit the differentiation DCs, and promote IL-10 producing DCs. The over expression of COX-2, and production of proangiogenic PGE-2 and VEGF also suppress macrophage and T cell mediated cytotoxicity and may also suppress the maturation of DC (Zitvogel et al., 2006).

Therefore tumours exert significant immune-suppression; from an evolutionary standpoint the acquisition of such diverse immunosuppressive features alludes to the selective pressure the immune system must exert on the developing tumour, and thus the potential of immunotherapy.

1.2.4 Prognostic value of tumour infiltrating lymphocytes

The presence of tumour infiltrating lymphocytes has been shown to be a significant predictor of prognosis and survival in a variety of tumour types including melanoma,

colon and breast cancer (Naito et al., 1998; Hadden, 1999; Piras et al., 2005). However some of the most thorough work has been performed in ovarian cancer. Zhang et al. (2003) showed that the presence of tumour infiltrating T cells independently correlated with delayed recurrence and improved survival. The five-year overall survival rate for patients whose tumours contained T cells was 38.0% while survival was 4.5% for patients whose tumours contained no infiltrating T cells. The prognostic significance of tumour infiltrating T cells was even more striking in a subset of 74 patients who had a complete clinical response after surgical debulking and platinum-based chemotherapy, the five-year survival rate was 73.9% compared with 11.9% for patients whose tumours contained no T cells. These results have been confirmed and furthered by Sato et al. (2005) who showed frequencies of intraepithelial $CD8^+$ T cells to be a prognostic factor for improved survival compared with patients with lower frequencies, the subgroups with high versus low intraepithelial $CD8^+/CD4^+$ TIL ratios had median survival of 74 and 25 months respectively. This unfavourable effect of $CD4^+$ T cells on prognosis was found to be due to $CD25^+$ $FOXP3^+$ regulatory T cells. Han et al. (2008) also reported heterogeneous expression of components of antigen presentation pathway components (TAP1, TAP2, HLA-I heavy chain and β_2 microglobulin), with defects in antigen presentation being associated with a poorer prognosis.

The activity of NK cells have also been shown to be a prognostic factor in gastric and hepatocellular carcinoma (Taketomi et al.,1998; Takeuchi et al., 2001) while the absence of NK cells within renal cell carcinoma tumours has been shown to be associated with poor survival (Donskov and von der Maase, 2006). Furthermore low

activity of NK cells towards NK cell targets *in vitro* has also been shown to be predictive of increased risk of cancer (Imai et al., 2000).

1.2.5 Immunosurveillance and immunoediting

It has been a century since Ehrlich initially suggested that the immune system was able to repress the development of cancer, which ultimately led to the formation of the theory of immunosurveillance in 1957 by Burnet and Thomas. Initially there was considerable controversy surrounding this theory. The limitations of the murine model systems of the 1960s and 70s disproved a role for the immune system in the prevention of tumourigenesis. However with the use of pfp (perforin) -/- and IFNγ insensitive mouse models in the 1990s and the subsequent use of RAG-2 -/- immunodeficient mice interest in the immune control of tumour formation was renewed, and confirmed to have a role in the prevention not only of carcinogen induced tumour formation, but also in the suppression of spontaneous tumour growth (Dunn et al., 2004).

As this theory has been investigated and confirmed it has become clear that the immune system's role in cancer development is far from the initial black and white concept of immunosurveillance. Not only does the immune system act in the early stages of tumorigenesis either protecting an individual from cancer or not, but also in the event that immunosurveillance may fail there is continued interaction between the immune system and the developing tumour, sculpting its development.

There is now a vast body of literature to corroborate the role of the immune system in tumour development: firstly there is an increased risk of cancer seen in the

immunocomprimised and the immunosuppressed, secondly cancer patients have been shown to develop spontaneous immune responses to autologous tumours, and finally the presence of tumour infiltrating lymphocytes has been shown to be an independent predictor of prognosis and survival in multiple cancer types. The process of immunosurveillance/immunoediting has been suggested to be composed of three phases, if the tumour cell is able to avoid initial elimination by the immune system, it may then enter a phase of latency where it may stay indefinitely in equilibrium with the immune system until it escapes control by the immune system and goes on to cause clinical disease with limited intervention from the immune system.

1.2.5.1 Elimination, equilibrium and escape

The immunosuppression of transplant patients predisposes those individuals to a range of cancers with viral etiology including Kaposi's sarcoma, non-Hodgkin's lymphoma and cervical cancer. However, more importantly other cancers including colon, pancreas, lung and malignant melanoma all without viral causes have also been shown to be increased (Domhan et al., 2008; Dunn et al., 2004). Likewise Chediak-Higashi syndrome a genetic disorder which causes abnormal NK cell cytotoxic function results in a 200 fold increased risk of developing cancer, while genetic traits such as differences in NKG2D haplotypes have also been shown to result in an increased risk of cancer (Imai et al., 2000; Hayashi et al., 2006; Nakachi et al., 2004; Dunn et al., 2004). These data suggest that without the presence of a fully functional immune system there is an increased risk of cancer development, therefore the immune system must be eliminating tumour cells prior to the clinical development of cancer.

The principles of the equilibrium phase may represent the most intense period of immunoediting by the immune system, whereby the tumour may be actively recognised by the immune system; although unable to eliminate the tumour the immune system constrains the development of the tumour. An interesting case in support of this was published by MacKie et al. (2003) whereby two patients receiving an allogenic kidney transplant from a donor who had previously been diagnosed with melanoma but who had been in remission for 16 years prior to death, both developed metastatic melanoma within two years of transplant. There are numerous other examples of donor derived tumours (Shiel, 2001), which may suggest that the donors immune system was suppressing sub-clinical metastasis, which when transplanted to new hosts under immunosuppressed conditions were able to escape to clinical significance.

In keeping with Darwinian evolution in order for a tumour to escape the selective pressure exerted by the immune system during equilibrium a tumour cell variant must lose the ability to be recognised by the immune system. This may be achieved by the loss of a specific antigen targeted by the immune system, for example following clinical responses recurrence of tumours has been reported to be associated with the outgrowth of antigen loss variants following peptide vaccination and adoptive T cell therapy for advanced melanoma. Likewise the global loss of antigen presentation has also been reported, for example 40-90% of human tumours are seen to completely or selectively lose HLA-I expression, suggesting a selective pressure has been exerted (Boon et al., 2006; Khong et al., 2004; Yee et al., 2002) while the tumour may also gain the ability to suppress the immune response as discussed. These variations

ultimately allow the tumour to overcome the bottleneck imposed by the immune system.

1.2.6 Immunotherapy

The observation of an association of bacterial pathogen associated diseases and a regression of tumours was made in the 1700s. This subsequently led Coley in 1897 to develop Coley's toxins (a mixture of heat killed Streptococcus and *Serratia marcescens)* for the treatment of Sarcomas. Long before the advent of conventional chemotherapy and radiotherapy this strategy provided clinical success (Starnes, 1992). The mechanism of action for Coley's toxins remains unknown however it is assumed to be through the systemic activation of the immune system, and as such represents the first immunotherapy to be developed (Davis et al., 2003).

Currently there are a number of immunotherapies licensed for use in cancer, and a number of more conventional therapies which have been shown to depend partially on the actions of the immune system for their efficacy. Below are a few examples of immunotherapy in the clinic which have had significant effects or are showing promising and interesting proof of principle.

1.2.6.1 Non-specific immune activation: Bacillus Calmette-Guerin Therapy

Similarly to Coley's toxins, Bacillus Calmette-Guerin (BCG) is an attenuated strain of Mycobacterium bovis used primarily as a vaccine for the prevention of tuberculosis, BCG has also gained acceptance as an effective treatment for cancer. The use of intravesical BCG therapy for the treatment of bladder cancer was first proposed by Morales et al. for the treatment of high risk Non-Muscle-Invasive bladder cancer

(NMIBC) in 1976, and has now become the gold standard treatment along with surgery for high risk NMIBC. The efficacy of BCG therapy is drastic with remission rates averaging 70% over 5 years (Alexandroff et al., 1999; Horvath and Mostafid, 2008).

The administration of BCG to the bladder is capable of inducing the recruitment of an assortment of cell types including $CD4^+$ and $CD8^+$ αβ-T cells, γδ-T cells, NKT cells, monocytes, DC, and NK cells, and induces the release of a substantial assortment of cytokines (Patard et al., 1998). However the specific mechanism by which BCG is having anti-tumour efficacy has yet to be defined.

A possible mechanism may however be the activation of NK cells, it has been shown that BCG stimulation of PBMC results in the activation of NK cells and that these NK cells are able to recognise and kill autologous tumour cells following stimulation (Patard et al., 1998). It has also been shown by Suttmann et al. (2004) that the activation of NK cells was dependent on the production of IL-12 and IFNα by monocytes. Importantly it has also been shown that the expression of higher levels of activating ligands for NKp30, NKp44 and NKp46 had a significant influence on the disease free survival of patients treated with BCG (Yutkin et al., 2007). The role of NK cells in anti-tumour effects of BCG therapy were further strengthened by Brandau et al. (2001) who, using a murine model of bladder cancer, were able to show that the dominant anti-tumour effects of BCG required NK cells both *in vitro* and *in vivo*.

These data suggest that BCG therapy is able to significantly activate the whole immune response allowing anti-tumour activity which is likely to be at least partially mediated by NK cells.

1.2.6.2 Cytokine therapy: IL-2

The first clear evidence that manipulation of the immune system through the use of recombinant cytokines as an effective therapy for the treatment of cancer in humans came from the initial work of Rosenburg et al. (1985a) who administered high dose IL-2 to cancer patients with metastatic disease, including melanoma and renal cell, colon and lung cancer, who had failed standard therapy. This work followed on from initial animal studies showing the efficacy of IL-2 against established implanted tumours (Rosenburg et al., 1985b). High dose IL-2 was administered with autologous *ex vivo* IL-2 activated Lymphokine Activated Killer (LAK) cells to 25 patients. Objective regression of tumour (more than a 50% decrease in tumour volume) was observed in 11 of the 25 patients with a sustained complete response observed in one patient with metastatic melanoma, and partial responses in nine patients with pulmonary or hepatic metastases (Rosenburg et al., 1985a). However it was later shown that the transfer of LAKs did not enhance responses compared to IL-2 alone (Rosenberg et al., 1993).

Subsequent studies confirmed the efficacy of IL-2 for treatment as a single agent, with work by Fyfe et al. (1995) showing that while an albeit modest (5%) complete response rate was achieved this response was durable (to the limit of the study) facilitating the approval of IL-2 in 1992 by the United States Food and Drug Administration (FDA) for the treatment of renal cell carcinoma. Subsequently IL-2

was also licensed for the treatment of metastatic melanoma in 1998, having been shown to have similar response rates (Atkins et al., 1999). The durability of the complete responses achieved by renal cell and metastatic melanoma patients was later confirmed by Rosenburg et al. (1998) with a median follow-up time of 7.1 years, 82% of the complete response patients remained in complete remission from three to over 12 years, with no patient seen to relapse after sustaining a response for three years.

Due to the pleiotropic effects of IL-2 on the immune system the mode of action of IL-2 therapy has not been elucidated. However evidence for both T cell and NK cell mediated effects have been produced (Parton et al., 2006). It is most probable that therapy with IL-2 is having effects on many cell types to mediate clinical efficacy.

1.2.6.3 Adoptive transfer: Autologous Natural Killer cells

Following on from the success of systemic administration of IL-2 there has been much interest in the expansion and activation of autologous NK cells *ex vivo* for adoptive transfer back to the patient. The majority of trials in humans have been conducted using IL-2 based expansion and activation protocols, with mixed success. Escudier et al. (1994) for example expanded autologous CD3 depleted non-adherent PBMC from 10 RCC patients who had achieved a partial response with initial systemic IL-2 therapy. Cells were expanded with 200U/ml IL-2 for 13-21 days, achieving a median 55 fold expansion of NK cells. These activated cells were then infused back into patients along with high dose IL-2. Out of the 10 patients four patients showed a complete response following infusion, and a further two patients achieved a complete response following surgery. Ishikawa et al. (2004) also activated and expanded NK cells from 9 glioma patients with 200U/ml IL-2. Following 6-7

days *ex vivo* culture cells were re-infused along with IFNβ, resulting in a modest response rate.

While these studies are encouraging a number of other studies have failed to show efficacy associated with the expansion and activation of autologous NK cells. For example Burns et al. (2003) combined prolonged systemic IL-2 therapy with the periodic transfer of autologous NK cells which had been activated overnight with 200U/ml IL-2 *ex vivo* in 37 patients (19 patients with lymphoma, 18 with breast cancer), however despite an increase in cytotoxicity and cytokine production *in vitro*, no clinical efficacy could be shown compared with matched controls. Likewise Lister et al. (1995) activated NK cells *ex vivo* from 12 patients (11 lymphoma, 1 breast cancer) for 14-18 days with 6000U/ml IL-2, however again despite *in vitro* activity clinical efficacy was not shown.

1.2.6.4 Adoptive transfer: Allogeneic Natural Killer cells

Barnes and Loutit (1956) first noted an association between a Graft versus Leukaemia (GvL) effect at the time mice were experiencing Graft versus Host Disease (GvHD) in a murine Leukaemia model following allogenic bone marrow transplant. Subsequently Weiden et al. (1979) were able to show an association between GvHD and a decreased risk of relapse in human leukaemia patients, this was attributed to a GvL affect.

Following on from these data and the demonstration of successful engraftment of fully mismatched T cell depleted stem cell transplants in terminal leukaemia patients by Aversa et al. (1994), Ruggeri et al. (1999) went onto examine the potential of

mismatched NK cells to provide a GvL effect in 20 leukaemia patients. Following transplant no GvHD was observed, however at 6 months no AML or CML patient had relapsed, while 5/7 ALL patients relapsed. Despite the lack of GvHD there was shown to be an initial graft versus host effect, which could not be detected following the four months post-transplant. Likewise allo-reactive NK cells were shown to recognise all host myeloid leukaemia targets. In contrast leukaemias of lymphoid origin were less susceptible to killing by NK cells, mirroring the clinical effects of this study.

Subsequent analysis of further haploidentical transplants showed that the presence or absence of a KIR mismatch in the direction GvH was the only independent predictor of survival or poor outcome respectively in AML but not in ALL. The requirement for NK cell KIR mismatch was then confirmed in a murine model using transfer of human leukaemia cells. While KIR matched NK cells resulted in no survival advantage over untreated controls, transfer of KIR mismatched NK cells resulted in survival of all mice (Ruggeri et al., 2002).

These data show that NK cell KIR mismatches have the ability to exert a powerful clinical effect in certain haematological malignancies. Further trials involving solid tumours or AML have also provided some evidence of efficacy, for example Miller et al. (2005) tested the use of *ex vivo* activated, CD3 depleted haploidentical haematopoietic cell transplants in which NK cells were enriched (~40%) and activated with 1000U/ml IL-2 overnight prior to transfer. 43 patients in total were treated, 5/19 poor prognosis AML patients achieved complete remission using this regime, while 4/10 metastatic melanoma patients and 2/13 RCC patients showed stabilisation of disease (although the melanoma patients subsequently progressed 5-10

months after initial treatment). The disparity in the efficacy of this treatment may not come solely from the different disease types (i.e. solid tumour vs. haematological) as AML patients received a high intensity conditioning regime prior to infusion while other patients received low intensity conditioning. It was shown that only the patients undergoing prior high intensity conditioning had a significant *in vivo* expansion of NK cells. KIR mismatched NK cells have been shown to have significant cytotoxicty against RCC and melanoma cell lines *in vitro*, but not against KIR matched targets (Igarashi et al., 2004). Earlier work by Childs et al. (2000) also suggests that allogenic stem cell transplants in RCC can provide an anti-tumour response, with 3/19 patients achieving a complete response and 7/19 achieving a partial response. While this study used high intensity conditioning, grafts were not T cell depleted; therefore GvHD was seen in many patients, but was associated with response. Unfortunately NK cells were not monitored in this study. So while clinical efficacy was shown the mechanism by which this was achieved was not defined.

1.2.6.5 Therapeutic antibodies: ADCC

Since the mid-1990s the field of therapeutic antibodies has increased drastically with more than 20 antibody therapies now approved for use in a number of disease settings including autoimmune disease and cancer. Predominantly these antibodies have been humanised, the constant and Fc regions replaced with human domains. In the cancer setting there has been suggestion that a number of antibodies are partially achieving efficacy through eliciting the ADCC function of NK cells. This has been shown in *in vitro* models and in mouse xenograph models (Kubota et al., 2009). Importantly there is an association with CD16 polymorphisms associated with the improved ability of CD16 to bind Fc domains and responses to Rituximab and Trastuzumab (Herceptin)

(Treon et al., 2005; Varchetta et al., 2007). Also the response of breast cancer patients to Trastuzumab has been shown to correlate with ADCC and NK cytotoxicity in short term responses, while longer term progression-free survival correlates with sustained NK cells activity (Beano et al., 2008).

1.2.6.6 Tumour antigen and Dendritic cell vaccines

The use of prophylactic vaccination for the prevention of infectious diseases has been very effective in a number of cases (Banchereau et al., 2009), and has including the development of the human papilloma virus (HPV) vaccine for the prevention of cervical cancer (Kahn, 2009). The experience gained from prophylactic vaccinations and the identification of TAA has encouraged the development of vaccines for use in the treatment of various cancers, most notably melanoma. Strategies have included the use of TAA peptides, DNA or viral delivered TAA and *ex vivo* modified tumour cells, mixed with various adjuvants or cytokines including incomplete Freund's adjuvant or IL-12 (Sun et al., 1998; Lee et al., 2001; Marshall et al., 2005; Hamid et al., 2007). While there has been a great deal of success in the prevention of infectious disease there has been relatively little success in the treatment of cancer. A number of clinical trials have shown evidence of immune responses to the vaccine in terms of sensitive ELISPOT or tetramer assays, or systemic immune responses in delayed hyper-sensitivity tests, however in general there are poor objective clinical response rates (2.6%) (Rosenburg et al., 2004). This could be for a number of reasons including the general immune suppression of cancer patients, and presumable previous encounter and tolerisation to antigens derived from the tumour.

This has led to the development of DC vaccines; DC matured *ex vivo* and returned to the patient to prime tumour antigen responses. Again a number of strategies have been used to provide antigen for DC, including the use of peptides, recombinant proteins, autologous and allogenic tumour cells, and DNA or RNA encoding tumour antigens. Likewise a number of strategies have been used to produce DCs for transfer, including generation from $CD34^+$ progenitor cells and maturation from monocytes, leading to heterogenatity in the DCs used in trial. It has been shown however after proof of principle experiments in healthy donors and patients that in order to generate an effective immune response there is a requirement for generation of mature DCs. The use of DC vaccines in a number of trials has resulted in an overall response rate of 9.6%, which is higher than other vaccination strategies including viral vectors (1.9%) and tumour cells (4.6%). The ability to generate immune responses *in vivo* has also been associated with improved survival (Davis et al., 2003; Banchereau and Palucka, 2005; Paluka et al., 2007).

Ultimately the use of vaccine strategies must overcome prior encounter of the antigen by the immune system and must also overcome immune suppression orchestrated by the tumour to be effective, many new strategies are under investigation to achieve these aims (Paluka et al., 2009).

1.2.6.7 Adoptive transfer: T cells

In order to circumvent the problems associated with previous encounter and tolerisation to TAA and the necessity to provide sufficient effective *in vivo* priming a number of strategies have been used to generate tumour specific T cells for adoptive

transfer to allow the generation of high numbers of functional tumour specific T cells (Offringa, 2009; Rosenberg and Dudley, 2009).

The first evidence that the *in vitro* manipulation and generation of tumour specific T cells was possible came from Muul et al. (1987), who were able to expand tumour specific T cells from the autologous TIL of melanoma patients. Following on from these studies Rosenberg et al. *ex vivo* expanded TIL with 6000U/ml IL-2 from melanoma patients and returned un-selected cultures to patients. Despite no selection taking place objective clinical response rates were achieved in approximately a third of patients (Rosenberg and Dudley, 2009). Later protocols have utilised selection of cells based on the *in vitro* antitumour reactivity (Dudley et al., 2003). Initial studies, while encouraging, provided only short term responses and short term persistence of transferred T cells *in vivo* (Rosenberg et al., 1990). Subsequent studies corroborate the observation that the persistence of transferred T cells *in vivo* may be a limiting factor in the ability to sustain long term clinical response (Robbins et al., 2004).

Other methods for the expansion of tumour specific T cells such as the use of alternative cytokines and DCs pulsed with tumour antigen are being investigated (Dudley and Rosenburg, 2003; Gattinoni et al., 2006). However the most significant response rates have been seen with the use of non-myeloablative conditioning prior to transfer. In murine models a requirement for lymphodepletion had been identified (North, 1982; Rosenburg et al., 1986) which led Dudley et al. (2002) to test the use of high intensity conditioning prior to adoptive T cell transfer to 13 melanoma patients with tumours refractory to standard therapies, including high dose IL-2. Patients were treated with cyclophosphamide and fludarabine prior to adoptive transfer. Six of 13

patients achieved objective clinical response, four of which were sustained. This trial was subsequently extended to treat 35 patients, 51% of whom achieved an objective clinical response. Furthermore these studies provided evidence showing the long term persistence of transferred cells was obtained in four of five patients, who could be tested (Dudley et al., 2005). Adoptive transfer following lymphodepletion now represents the most effective treatment for late stage melanoma patients (Rosenberg and Dudley, 2009).

The mechanisms behind the ability of lymphodepletion to improve responses following adoptive transfer of T cells are still being elucidated, however a number of non-mutually exclusive mechanisms have been proposed. Conditioning may remove immune suppression allowing greater T cell expansion and expression of effector functions, likewise depletion could remove the 'cytokine sink' present in non-depleted patients allowing homeostatic expansion (Gattinoni et al., 2006).

While adoptive transfer has been successful tumour specific T cells are in general a rare population and require extensive manipulation *ex vivo* to provide sufficient numbers for adoptive transfer. There has therefore been a growing interest in transgenic TCR (tTCR) technology. This involves the *in vitro* identification of TCRs capable of recognising specific TAA epitopes. The identified TCR can then be cloned into retroviral vectors and used to transduce non-specific PBMC populations, resulting in the ability to produce a large population of TAA specific T cells (Morgan et al., 2006; Geiger et al., 2009). The use of tTCRs also allows greater control to produce T cells with high avidity for TAAs, which has been shown to be important for *in vitro* and *in vivo* activity (Zeh et al., 1999).

1.2.6.8 Subverting suppression

Cyclophosphamide which was used in the conditioning of adoptive transfer patients (section 1.2.6.7) has long been used in the clinic as an anti-angiogenic chemotherapy (Kerbel and Kamen, 2004). However cyclophosphamide is also capable of reducing immune suppression. In the 1980s the use of cyclophosphamide was revealed to specifically inhibit an unidentified regulatory cell population in both murine cancer models and cancer patients (Berd and Mastrangelo, 1987; Awwad and North, 1989). Following these initial reports and with increased knowledge of immunosuppressive mechanisms, Ghiringhelli et al. (2006) were able to show cyclophosphamide selectively depletes putative Treg in late stage cancer patients. These data corroborate the earlier studies of Berd and Mastrangelo, (1987) who were able to show a specific effect on a suppressive cell population. Cyclophosphamide has also been tested in combination with other therapies and has been shown to enhance the immune response in melanoma patients, as shown by increased DTH reaction, and increase survival in breast cancer patients when combined with tumour vaccination, compared to vaccine alone (Berd et al., 1986; MacLean et al., 1996).

The ability of standard treatments to provide a specific inhibition of regulatory cells is important. At the same time a number of targeted therapies have also been developed including the IL-2-diphtheria toxin fusion protein denileukin diftitox (ONTAK). ONTAK was originally developed to target haematological malignancies expressing CD25, but is also capable of depleting Tregs due to the constitutive high level of expression of CD25 by this population (LeMaistre et al., 1992; Mahnke et al., 2007). ONTAK has been tested in a number of clinical trials and has been shown to reduce circulating Treg levels in cancer patients. The administration of ONTAK prior to

vaccination with peptide or DC based vaccines has also been show to allow the induction of vaccine specific responses and increase the response compared with vaccine alone. While ONTAK can enhance vaccine responses, convincing clinical responses have not yet been achieved (Mahnke et al., 2007; Morse et al., 2008).

Arguably the most exciting of the immune modulators are the CTLA-4 antagonistic antibodies Ipilimumab and Tremelimumab, which may inhibit Treg function or allow an enhancement of T cell activation (Fong and Small, 2008). Building on pre-clinical murine models showing the ability of CTLA-4 blockade to allow the rejection of poorly-immunogenic tumours following vaccination (van Elsas et al., 1999) Hodi et al. (2003) administered Ipilimumab to seven melanoma and two ovarian cancer patients who had previously been vaccinated with either TAA peptides, TAA expressing DC or autologous tumour cells modified to express GM-CSF. Extensive tumour necrosis was seen in three melanoma patients, while the two ovarian cancer patients showed reduced or stabilised serum CA-125 levels. Further phase I trials with Tremelimumab predominantly for the treatment of melanoma have also been conducted and showed clinical responses (Ribas et al., 2005; Reuben et al., 2006). Interestingly successful treatment has been associated with the manifestation of autoimmune reactions, suggesting CTLA-4 blockade is able break tolerance (Reuben et al., 2006; Weber et al., 2009; Hodi et al., 2010; Wolchok et al., 2010). Subsequent phase II trials have confirmed these early results showing durable objective responses in response to CTLA-4 blockade alone or in combination with dacarbazine or peptide vaccine (Phan et al., 2003; Fischkoff et al., 2005). Recently phase II (Wolchok et al., 2010) and phase III (Hodi et al., 2010) studies have been completed, with 217 and 676 (pre-treated) advanced melanoma patients respectively. In a dose-ranging study

Wolchok et al. (2010) showed a response rate of 4.6% with 3mg/kg Ipilimumab every three weeks for four cycles (72 patients total) rising to 11.1% at a dose of 10mg/kg (72 patients). This is similar to the response rate of 10.9% following 3mg/kg Ipilimumab every three weeks for four cycles (137 patients) which is oddly significantly higher than the 5.7% response rate reported with Ipilimumab plus a gp100 peptide vaccine (403 patients) reported by Hodi et al. (2010). Interestingly, a number of trials have shown a delay in the initiation of response, while perhaps more surprisingly the initial objective response is not a prerequisite for the achievement of increased survival. These studies suggest that transient CTLA-4 blockade is able to have long lasting effects on the immune system (Hodi et al., 2003; Bulanhagui et al., 2006).

1.2.6.9 CD80 and IL-12 clinical trials

There is a vast body of evidence from animal models and *in vitro* data for the use of many costimulatory molecules and cytokines, alone and in various combinations for the treatment of cancer. There have been many clinical trials using various approaches to utilise costimulation including the use of recombinant cytokines for cancer therapy. Some of the most utilised molecules include CD80 and IL-12. As show in Table 1-2, there have been a number of good clinical responses to therapy with CD80 and IL-12; however these responses are relatively rare. Interpretation of these data is also hampered by the relatively small size of these trials, with assessment of safety and maximum tolerated dose (MTD) being the primary aim of many studies. Equally the use of highly sensitive techniques such as ELISPOT may be able to identify responses in peripheral blood however these may not provide a representative indication of

immune effects within the tumour (Bamias et al., 2007), nor correlate with actual clinical responses (Rosenburg et al., 2004).

The systemic delivery of recombinant IL-12 results in significant toxicity, likewise with IL-2 (Siegel and Puri, 1991; Atkins et al., 1997). Intratumoural delivery of IL-12 DNA or expression vectors offer significant advantages over systemic cytokine delivery as this does not increase circulating levels of cytokine significantly, and good responses are seen with very limited toxicity (Rakhmilevich et al., 1999).

In general these therapies have been applied as a monotherapy to patients with significant tumour burden. While there have been many animal models demonstrating the efficacy of monotherapy, later models using less immunogenic tumours or more representative tumour burdens have shown a requirement for combinations of costimulatory molecules. Studies such as those undertaken by Xu et al. (2004) in a murine melanoma model using intratumoural IL-12 gene transfer and systemic activating anti-4-1BB antibody, may suggest that a combined approach utilising multiple treatment options or a combination of costimulations may be required for efficacy *in vivo*.

It may also be important to choose the correct costimulatory molecules. CD80/CD86 have been show to activate and enhance Tregs (Lohr et al., 2004; Zhou et al., 2009), perhaps explaining relatively transient responses to CD80 therapies. Likewise different costimulatory molecules may be better suited for differing roles in the immune response such as enabling long term expansion and survival or following chronic antigen exposure (Bansal-Pakala et al., 2001; Lathrop et al., 2004; Melero et

al., 2007). Therefore with identification of novel costimulatory molecules and a better understanding of the interactions of various costimulatory molecules response rates and clinical outcomes may improve.

Table 1-2. Examples of previous clinical trials of CD80 or IL-12

Therapy	Patients	Delivery	Regime	Dose	Responses	Toxicity	Ref
rhIL-12	40 Patients (including 20 RCC, 12 MM, 5 Colon)	Systemic (IV)	One dose followed after 2 weeks by once daily for 5 days every 3 weeks	3-1000 ng/kg/day	1 PR (RCC), 1 transient CR (MM), 4 SD. Increased IFNγ, attenuated with further doses	Grade 3/4	Atkins et al., 1997
rhIL-12	28 Patients (20 RCC and 8 MM)	Systemic (IV)	Twice weekly for 6 weeks	30–700 ng/kg	1PR, 2SD (RCC). IFN-γ, IL-15, and IL-18 induced, loss of induction following further cycles associated with progression	Grade 4 in one patient at MTD, no other DLT	Gollob et al., 2000
Autologous IL-12 gene modified cancer cells	6 MM Patients	S/C	4 times in 6weeks	n/a	1 minor clinical response, 2 DTH	Mild	Sun et al., 1998
IL-12 Plasmid DNA	9 MM Patients	Intratumoural		2mg, 4mg, 10-20mg DNA	1CR, 2SD, 8 patients transient response of injected lesions	Mild	Heinzerling et al., 2005
IL-12 cDNA	12 Patients (1 RCC, 11 MM)	Intratumoural	3 times per week for 1 or 2 weeks, retreated at day 28 with same regime if responding	50μg cDNA	3/6 1 week course, 3/6 2 week course had SD after first round, 3/5 of further treated patients SD. No systemic effect was seen.	Mild	Mahvi et al., 2007
IL-12 DNA	24 MM patients	Electroporation of Lesion	Day 1, 5, 8 of 39 day cycle	3.8-5.8mg divided between 3-4 lesions	2/19 CR, 8/19 SD/PR of systemic metastasis	Mild	Daud et al., 2008
CEA and CD80 in ALVAC	18, CEA^{+} 1 Pancreas, 3 Lung, 3 Rectal, 10 Colon, 1 gastric	Intramuscular	1/month for 3 months	Dose escalation, 4.5x10^6,^7,^8	3 SD, Increased ELISPOT to CEA	Mild	Horig et al., 2000

PSA or CD80 in Vaccinia, boost with PSA in Fowlpox	28 Metastatic Androgen insensitive prostate cancer patients	Vaccine S/C With 100ug GMCSF S/C at site of Vaccine for 4 days	Boost after 2 weeks, Vaccine administered 1 day after doc, Doc for 3/4 weeks continued until progression	Vaccine +/- Docetaxel (cross over to Doc following progression)	3.33 fold increase PSA T cells in both arms by ELISPOT, cross over to dox after vaccine alone icreased survival 6.1 months vs 3.7 Dox only (historical), 3.2 Vaccine+Dox, 1.8 Vaccine only, superior decrease in PSA velocity in Post vaccine dox<Vaccine+Dox<Vaccine alone.	Mild associated with Vaccine	Arlen et al., 2006
CEA and TRICOM in Fowlpox and Vacinnia	58 advanced CEA^+ Carcinomas	S/C	28 days for six doses then once every 3 months. Reverting to treatments every 28 days if patients progressed	n/a	23 SD, 14 Prolonged SD, 1 CR, Increased CEA Specific T cells in the majority of Patients	Mild	Marshall et al., 2005
Autologous tumour expressing CD80 vaccine	15 metastatic RCC	S/C	Systemic IL-2	Dose Escalation	2PR 2SD	Mild Associated with Vaccine	Antonia et al., 2002
ALVAC CD80+IL-12	14 Metastatic Melanoma	Intratumoural	1, 4, 8, and 11	9 given escalating does up to $25x10^8$, 5 $25x10^8$+ALVAC-IL-12 ($2x10^6$)	2 SD	Mild	Triozzi et al., 2005
Vaccinia CD80	12 Melanoma	Intratumoural	4.26x10^7 or 4.26x10^8	dose escalation	1PR, 2SD, Increased gp100 and MART-1 T cells by ELISPOT	Grade 1	Kaufman et al., 2005

MM: Malignant Melanoma, NSCLC: Non-small cell lung cancer, RCC: Renal Cell Carcinoma, CR: Complete Response, PR: Partial Response, SD: Stable Disease, MTD: Maximum Tolerated Dose, DLT: Dose Limiting Toxicity, DTH: Delayed-type Hyper sensitivity reaction, ALVAC: Non-replicating canary-pox vector, TRICOM: TRIad of COstimulatory Molecules (B7-1, ICAM-1, and LFA-3), IFA: Incomplete Freuds Adjuvant

1.2.6.10 Trial of agonist CD137 antibodies

As mentioned in sections 1.2.6.5 and 1.2.6.8 there has been marked success using recombinant antibodies as novel cancer therapies. Alongside the use of recombinant cytokines and gene transfer techniques there are a number of costimulatory agonistic antibodies in early stage clinical development. Including the exciting development of BMS-663513 an agonist CD137 antibody developed by Bristol-Myers Squibb, which has been reported to show initial promising results in the early stages of phase I trial (Sznol et al., 2008). The initial results from the BMS-663513 trial are in stark contrast to those shown from the initial testing of TGN1412, a CD28 super-agonist, in humans resulting in all six test subject being hospitalised with four developing severe multi-organ failure (Melero et al., 2007).

1.3 Gene therapy

Since the first in man gene therapy trial for the treatment of adenosine deaminase (ADA) deficient severe combined immunodefiency (SCID) began on 14th September 1990 (Culver et al., 1991) there have been a further 1536 gene therapy trials, utilising a variety of approaches for the treatment of a variety of diseases (www.wiley.co.uk/genmed/clinical).

The targeted indication for the vast majority (64.6%) of the gene therapy trials has been the treatment of cancer, with the delivery of antigenic, cytokine or genes being among the most prevalent approaches of the many trialled (

Figure 1-5A). As
Figure 1-5B shows the choice of vector is also varied, with viral vectors predominating over other delivery methods, and adenoviral vectors being the most

commonly utilized. The use of adenoviral vectors in man for gene therapy also includes the first commercial gene therapy, licensed for the treatment of head and neck squamous cell carcinoma in china in 2003 (Pearson et al., 2004).

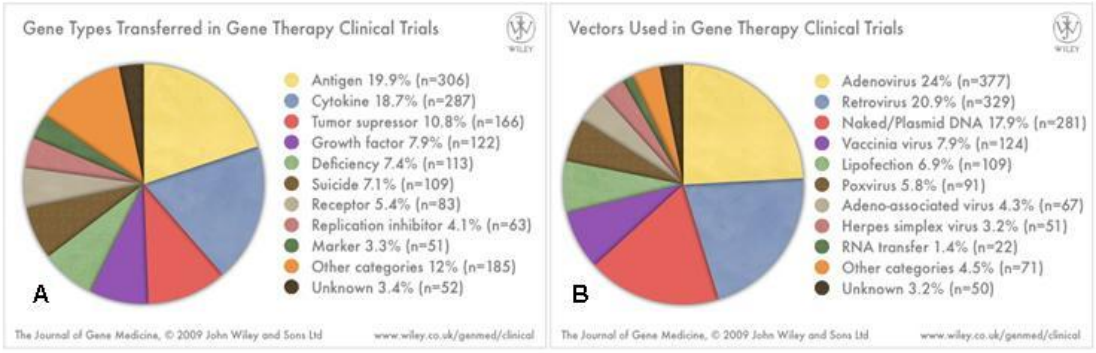

Figure 1-5 Gene and Vector types used in gene therapy trials.
Taken from www.wiley.co.uk/genmed/clinical.

1.3.1 Adenovirus

Adenovirus is a linear, non-enveloped double stranded DNA virus that was first isolated and cultured by Rowe et al, in 1953 from tonsils and adenoid tissue. There have now been 51 adenoviral serotypes identified. Wild type adenoviral infection is generally mild. Associating symptoms are mainly of the upper respiratory and gastrointestinal tracts and the eye, dependent on virus serotype (Horwitz, 2001).

1.3.1.1 Replication defective adenoviral vectors

There is a packaging limit of ~2kb to the size of transgene insert that can be accommodated by addition to the adenovirus genome, therefore in order to allow larger transgene inserts portions of the adenoviral genome have been removed. Primarily the E1 region has been deleted in the majority of vectors used in clinical trial, with the additional removal of the E3 region (which is not required for replication) allowing the insertion of a ~7.5kb fragment to the adenoviral genome.

The deletion of the E1 region of adenovirus also has the advantage of rendering the virus replication defective, necessitating the use of helper cell lines stably expressing complimentary fragments of the adenoviral genome to allow propagation of the virus *in vitro*.

A major attraction for the use of adenoviral vectors is its ability to transduce a wide variety of dividing and non-dividing cell types *in vivo*. Allowing with the use of an appropriate promoter/enhancer sequence very high levels of transgene expression, however in comparison to other viral vectors e.g. retrovirus, expression of the transgene is relatively short lived (5-10 days), making adenoviral vectors a suitable choice for treatments which require only short lived, but high level expression of the transgene, e.g. suicide gene therapy.

1.3.1.2 Immunity to adenovirus

As would be expected for all viruses adenovirus and adenoviral vectors are able to have a profound stimulatory effect on the immune system following infection or administration. There have been a number of HLA class I and class II T cell epitopes identified, predominantly from capsid proteins, and neutralizing antibodies predominantly against hexon have also been defined. Seroprevalence of adenovirus is high (Cheng et al., 2010; Onion et al., 2007).

Following administration of adenoviral vectors for VDEPT (virus directed enzyme prodrug therapy) of prostate cancer it has been shown previously that there is a dramatic increase in adenoviral specific responses, both humoral and cellular. However, no correlation was seen between pre-treatment anti-adenovirus responses

and patient outcome (Onion et al., 2009), suggesting while there is a existing immune response to adenovirus this does not impact significantly on the efficacy of gene delivery, possibly due to the extremely high titre of virus administered.

The administration of virus is likely to cause a number of immune responses to be triggered by both immune and non immune cells. Infection of epithelial and endothelial cells or cancer cell lines with Ad has been shown *in vitro* to induce the production of a number of important chemokines, including IP-10, RANTES, and MIP-2 and may also induce the expression of type I IFN. Likewise triggering of Toll like receptors by Ad has been show to promote DC activation and maturation (Hartman et al., 2008; Sakurai et al., 2008). It could also be expected that adenovirus would activate NK cells via Toll like receptors or other pattern recognition receptors, resulting in production of proinflammatory cytokines. Likewise NK cells may recognise Ad infected cells (Tomasec et al., 2007). T cells are also likely to be able to recognise Ad infected cells, which may be contributed to by the 'leaky' expression of viral proteins by the vector (Christ et al., 1997; Sakurai et al., 2008).

Therefore while individuals may have preformed immune response against adenovirus this need not impact on the efficacy of gene delivery, while activation of innate immune responses may assist as an adjuvant for the activation of anti-tumour immune response.

1.4 Aims of this project

Costimulation may hold significant potential for cancer immunotherapy, the intratumoural delivery of costimulatory molecules for the activation or generation of anti-tumour immune response is a potentially important strategy. It was therefore the aim of this thesis to investigate the effects of the expression of CD80, 4-1BBL and IL-12 for the activation of an anti-tumour immune response.

The initial aim was to revalidate the superiority of the combination of CD80+4-1BBL compared to each costimulatory molecule alone and investigate the effects of the addition of IL-12 to the combination in a model system utilising antigen mimetic OKT-3 stimulation.

It was then aimed to move onto assess the effects of these costimulatory molecules alone and in combination to stimulate viral antigen specific T cell responses from healthy lab donors as a prelude to assessing the response of T cells from ovarian cancer patients to viral antigens, and ultimately to TAAs.

During the course of this project the presentation of 4-1BBL and IL-12 was found to stimulate significant proliferation of an NK cells. As these data were novel and potentially important to cancer immunotherapy it was also decided to further investigate and characterise the response of NK cells to 4-1BBL and IL-12.

2 Materials and Methods

2.1 Suppliers of Materials

Unless otherwise stated all materials were purchased from Sigma-Aldrich (Poole, UK). De-ionised water was obtained from a Maxima Ultrapure Water (ELGA, High Wycombe, UK).

2.2 Molecular biology

2.2.1 DNA restriction digestion

Restriction endonuclease enzymes were supplied by Boehringer Mannheim or New England Biolabs. These enzymes were used in accordance with the reaction conditions described in the manufacturers instructions using the appropriate enzyme buffers.

Typically for diagnostic DNA digestions, 0.5 μg of DNA was cut in a total volume of 20 μl, with 2 μl of appropriate 10x enzyme buffer and 1 U of restriction endonuclease. This was incubated at the optimum temperature for a minimum of two hour unless otherwise instructed. In situations where the use of multiple enzymes was required, an appropriate buffer was selected in accordance with the manufacturer advice which allowed activity for all enzymes being used. Digestion of larger quantities of DNA, up to 20 μg (e.g. for fragment purification) was carried out in a similar way except for the use of 1.5 U of enzyme per μg DNA in a total reaction volume of 100-200 μl.

2.2.2 DNA extraction using phenol and chloroform

Phenol and chloroform were routinely used to remove contaminating proteins from DNA preparations. The DNA solution to be purified was made up to a volume of at least 500µl with 10mM Tris 1 mM EDTA ($T_{10}E_1$) buffer and an equal volume of aqueous phenol was added. The tube was vortexed for 15 seconds to mix, the phases were then separated again by centrifugation in a microfuge for one minute at 13,000 rpm. The lower organic phase was removed into a designated waste container and an equal volume of a 1:1 mixture of phenol and chloroform was added to the remaining supernatant. Vortexing, centrifugation and organic phase removal were repeated and then chloroform alone was added to the aqueous phase. The processing was repeated a third time but on this occasion, the aqueous phase was removed to a fresh tube, ensuring no transfer of organic solvent. In order to allow the DNA to be used in downstream applications, or if the DNA needed to be concentrated, ethanol precipitation was subsequently carried out.

2.2.3 DNA precipitation using ethanol or isopropanol

DNA precipitation in the presence of salt and alcohol was carried out by the addition of either 0.1 volumes of 3 M sodium acetate, or 1/50 volume of 5 M NaCl followed by the addition of 2 volumes of absolute ethanol or 1 volume of propan-2-ol. If small mounts of DNA were to be precipitated linear polyacrylamide (LPA) was also added as a carrier. The solution was then mixed and incubated at -20°C for 20-30 minutes. The DNA was recovered by centrifugation in a microfuge at 16000g for 15 minutes. The supernatant was discarded and the pellet was then air dried before re-suspension in an appropriate volume of $T_{10}E_1$ buffer.

2.2.4 Agarose gel electrophoresis

DNA fragments of between 0.1 kb and 20 kb were separated and visualised by use of submerged horizontal gel electrophoresis. DNA size markers (1kb ladder) were supplied by Invitrogen. Agarose (Invitrogen) was dissolved by heating in a microwave oven, having been prepared in 1 x TAE running buffer (0.04 M Tris acetate, 0.001 M EDTA pH8), at 0.7% - 1.0% for most applications. DNA samples (5-20μl) were made up in 1:6 volume of 6 x Ficoll loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll Type 400; Pharmacia) before loading onto the submerged gel. The DNA electrophoresis was then performed by application of 100 volts for 1-3 hours. The gel was then stained for approximately 10 minutes in ethidium bromide diluted to 1 μg/ml in water or 1 x TAE. The DNA was then visualised by use of a Spectroline TVC-312A variable intensity UV transilluminator. Photographs of gels were taken using a Kodak EDAS290 digital camera.

2.2.5 DNA fragment purification

DNA (20-40μg) was endonuclease restriction digested and run on a Seakem agarose gel following the addition of 6x Ficoll loading buffer (1:6 v/v) and 1/10,000 dilution of stock SYBR® Gold nucleic acid gel stain (Molecular Probes, Eugene, Oregon USA). Visualisation of DNA bands was carried out under blue light (in order to avoid UV damage to the DNA that would occur with EtBr visualisation) with a Dark Reader™ (Clare Chemical Research), and the required band was excised with a fresh scalpel blade. The DNA was subsequently recovered from the gel slice using a QIAquick Gel Extraction kit (Qiagen) following the manufacturers protocol.

2.2.6 Measurment of DNA concentration: Picogreen assay

DNA standards were made in duplicate from serial two fold dilutions of a standard DNA stock solution (New England Biolabs) in a 96-well Falcon Microtest assay plate. Concentrations ranged from 0-200 ng per well in a final volume of 100 µl per well. Subsequently, 100 µl of 1/200 diluted Picogreen (Invitrogen) solution was added to each well. Fluorescence was determined using an absorption wavelength of 485 nm and emission wavelength of 595nm (fluorescein settings) on a Wallac Victor2 1420 Multilabel Counter and the DNA concentration calculated from a standard graph.

2.2.7 Ligation of DNA

Fragments of DNA were joined using T4 DNA ligase (Roche Diagnostics) in order to construct recombinant DNA plasmids and viral vectors. In some cases the 5' ends of the linear DNA fragments were dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (New England Biolabs), dephosphorylation prevented re-circularisation of plasmid vectors without insert, thereby increasing the chance of obtaining the desired product. Dephosphorylation was achieved prior to ligation by incubation with CIAP at 37°C for one hour with 1 x concentration of supplied buffer. The enzyme was then heat inactivated at 75 °C for 10 minutes and the DNA recovered by phenol/chloroform extraction and ethanol precipitation.

In general, 3:1 molar excess of insert to vector was used in sticky end ligations and a 10:1 excess for blunt ended ligations. Reactions were incubated overnight at 14 °C. Following overnight incubation, the DNA was cleaned up by phenol/chloroform

extraction and ethanol precipitation. The pellet was then resuspended in 10µl $T_{10}E_1$ and a small volume was checked on an agarose gel.

2.2.8 Preperation of competent cells

Transformation competent *E. coli* cells were prepared using the calcium chloride method. 1ml of overnight culture was used to inoculate 100ml of LB medium and incubated at 37^{o}C until the $O.D._{600}$ was 0.45-0.55 but for no longer than 4 hours. The culture was equally divided into two 50ml tubes and cooled on ice for 15 to 20 minutes. Cells were pelleted by centrifugation at 4^{o}C (500g for 10 minutes in a Beckman GS-6R benchtop centrifuge), pellets resuspended in 10ml of ice-cold 0.1M calcium chloride and incubated on ice for 30 minutes. Cells were repelleted, resuspended in 2ml of ice-cold 0.1M calcium chloride, incubated on ice for 2-3 hours, repelleted and finally resuspended in 2ml of 0.1M calcium chloride / 15% glycerol. 200µl aliquots were transferred into 1.5ml microcentrifuge tubes, frozen in liquid nitrogen and stored at -80^{o}C until required.

2.2.9 Bacterial growth media

Media for bacterial cultures was prepared as detailed in Table 2-1, prior to autoclaving.

Table 2-1 Bacterial growth media

Culture Medium	Ingredients
LB medium	6g LB broth base (Invitrogen) in 300ml ddH_20
SOB medium	8.4g SOB mixture (Difco) in 300ml ddH_2O
LB agar	6g LB broth base (Invitrogen), 4.5g Bactoagar (Difco) in 300ml ddH_20
SOC medium	SOB medium supplemented with 20mM filter sterilised glucose

2.2.10 Transformation of competent cells with plasmid

Frozen competent bacteria were thawed and kept on ice for 10 min. Approximately 1ng of the control plasmid or the product of a ligation reaction containing ~ 100 – 300ng DNA was added to 100µl of XL-10 competent cells, mixed, and kept on ice for 45 minutes. The cells were subject to a 90-120 second heat shock in 42 °C water bath and then transferred to 900µl of room temperature SOC media. Bacterial mixture was incubated at 37°C for one hour. Bacteria were then plated on SOB agar contained 100µg/ml ampicillin to select transformed cells.

2.2.11 Small scale preparation of plasmid DNA: mini-prep

For small scale preparation of plasmid DNA a single bacterial colony was picked with a sterile loop and used to inoculate 20ml of LB medium containing 100µg/ml of ampicillin. Cells were cultured at 37^oC overnight in a shaking incubator. The next day 10ml of the culture was centrifuged for 5 minutes at 5000 rpm in a Sorvall GSA rotor. The resultant cell pellet was kept on ice and DNA extracted using a QIAprep spin miniprep kit (Qiagen). If restriction digests confirmed the desired clone large scale production of the plasmid DNA could be carried out.

2.2.12 Large scale preparation of plasmid DNA: bulk-prep

For bulk preparation of plasmid DNA bacteria (containing the desired plasmid) in the exponential growth phase were inoculated into 200ml of LB medium containing 100μg/ml of ampicillin. Cells were cultured at 37^{o}C overnight in a shaking incubator. The next day cells were pelleted in a centrifuge pot by centrifugation for 5 minutes at 5000 rpm in Sorvall SM24 rotor. The resultant cell pellet was kept on ice throughout the procedure. First the pellet was resuspended in 4ml of ice cold solution 1 (50 mM glucose, 10 mM EDTA pH 7.5, 25 mM Tris. Cl pH 8.0), followed by the addition of 1ml of lysozyme (10 mg/ml in solution 1) the solution was left on ice for 10 minutes. 10ml of solution 2 (0.2 M NaOH, 1% w / v SDS) was then added and the tube inverted five times and left for 5 minutes on ice. Following this 7.5ml of solution 3 (5 M acetate and 3 M potassium, pH 4.8-5.0) was added and vortexed followed by incubation on ice for 10 minutes. Cellular debris was pelleted by centrifugation for 15 minutes at 8000 rpm at 4^{o}C in GSA rotor and the supernatant was transferred to a new centrifuge pot. The DNA was precipitated with 22.5ml of isopropanol for 30 minutes at -20^{o}C and pelleted by centrifugation at 8000 rpm in GSA rotor. The supernatant was discarded and the crude plasmid DNA pellet was resuspended, usually overnight, in 2.5ml of $T_{50}E_{10}$. The resultant solution was ready for purification by caesium chloride density gradient centrifugation.

2.2.13 Purification of plasmid DNA by caesium chloride density gradient centrifugation

Crude plasmid DNA from bulk prep was purified by caesium chloride (CsCl) density gradient centrifugation. In a 15-ml tube, 3.03 g of Ultrapure™ caesium chloride

(CsCl) (Invitrogen) and 275µl of EtBr solution (10mg/ml) were then added to the plasmid solution. The liquid weight of the plasmid solution was made upto 3.025 g by the addition of $T_{50}E_{10}$. The solution was stored on ice for 10 minutes before centrifugation at 10000 rpm for 10 minutes in a Sorvall SM24 rotor to precipitate contaminating RNA. Cleared plasmid preparation was transferred into 3.9ml Beckman Quick-Seal™ centrifuge tubes, with any gap between the liquid and neck of tube filled with isopycnic CsCl solution (1 g CsCl:1ml $T_{50}E_{10}$). The tube was then spun overnight in a Beckman Optima TLX bench top ultracentrifuge TLN 100 rotor for 4 hours at 100000 rpm followed by 4 hours at 95000 rpm and one hour at 55000 rpm.

After centrifugation the DNA band was harvested by piercing the side of the tube with a 21-gauge needle and 2-ml syringe. The recovered solution was diluted with three volumes of $T_{10}E_1N_{100}$ solution in a 15-ml tube. The DNA was extracted with phenol/chloroform to remove the ethidium bromide, precipitated with two volumes of ethanol, and pelleted by centrifugation. The DNA pellet was resuspended in 500µl of $T_{10}E_1N_{100}$, transferred to an Eppendorf tube and precipitated again with 1ml of ethanol. Finally the DNA pellet was resuspended in 0.2 to 1ml of $T_{10}E_1$ solution, ready for further experiments.

2.3 Virus production

2.3.1 Replication defective Adenoviral vectors

Seed stocks of the replication defective adenovirus vectors expressing CD80, 4-1BBL and GFP which had previously been produced by the Gene Therapy group (Institute for Cancer Studies, University of Birmingham) (Habib-Agahi et al., 2007), were obtained from Dr. P.Searle (Institute for Cancer Studies, University of Birmingham).

2.3.2 IL-12 adenoviral vector resurrection

2.3.2.1 Virus production

Previously a plasmid containing the p35 and p40 subunits of IL-12 driven from the CMV promoter and inserted into the left end of an E1 deleted adenovirus genome had been created within the Gene Therapy group (Institute for Cancer Studies, University of Birmingham). In order to generate a functional Ad-IL-12 vector the following steps were performed. Following verification of the plasmid by restriction digest the plasmid containing the Ad-IL-12 left end was linearised by restriction digest with SwaI and phenol-chloroform extraction and ethanol precipitation performed. A second plasmid containing an overlapping right end of adenovirus was linearised with ClaI and purified as for the left end Ad-IL-12 plasmid (as shown in Figure 2-1). The concentration of the linearised DNA was determined and the two plasmids mixed at a 2.7:1 ratio to give equal molar amounts of each plasmid. This mixture was transfected into 293 cells in a $25cm^3$ flask using Fugene 6 (Roche) following the manufacturers instructions, to allow homologous recombination, (culture of 293 and 911 cells is

described in chapter 2.4). Cells were harvested, and pelleted by centrifugation when cytopathic effect was observed and resuspended in 500µl of infection media and stored at -80^{o}C.

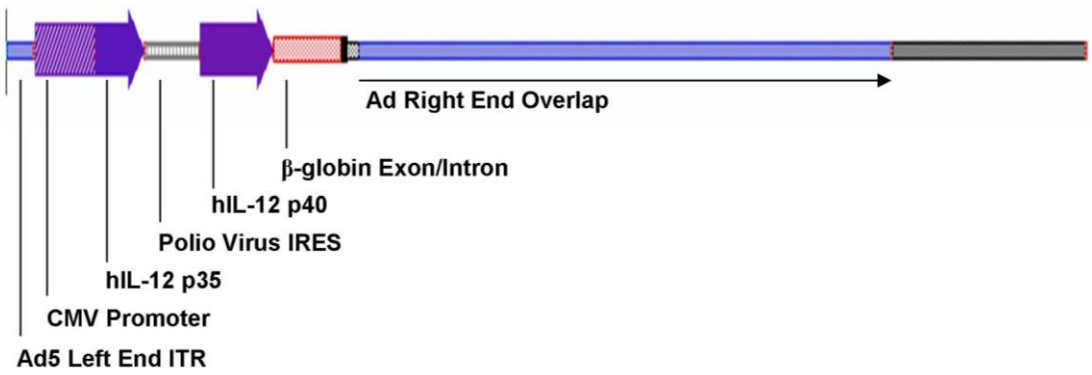

Figure 2-1 SwaI linearised Ad-IL-12 left end plasmid. Shown is a schematic of the Ad-IL-12 left end plasmid linearised following restriction digest with SwaI. Inserted regions and the overlapping region of the Ad right end ClaI linearised plasmid are highlighted.

2.3.2.2 Plaque Purification

In order to obtain a homogeneous stock of virus plaque purification was performed. 1x10^{6} 911 cells were seeded onto 60mm diameter plates (Nunc), 24 h prior to infection. The virus stock was serially diluted 10-fold in infection media (DMEM+2%FCS, see section 2.4.2). Medium was removed from the plates and 500µl of the virus dilution (10^{-1}-10^{-8}) was added to each plate in triplicate. Plates were returned to the incubator and virus allowed to infect for 90 min, with frequent rocking of the plates. Infection media was then removed and 4mls agar-medium overlay (1:1 mixture of 1.4% noble agar, and 2 x DME medium (supplemented with 4% FCS, 2mM glutamine, 5mM $NaHCO_3$, 10mM $MgCl_2$ and 100iu/ml Penicillin and 100mg/ml Streptomycin) was applied to each plate. Once solidified, plates were returned to the incubator. At 4, 7 and 10 days post-infection, cells were re-fed by the addition of 2ml agar-medium overlay. Once plaques were well developed, well isolated plaques were picked using the tip of a transfer pipette into infection media, this was then freeze

thawed three times and used to infect 25cm^3 flasks of 911. When cytopathic effect was evident cells were harvested and plaque purification repeated and used again to infect 911 cells. The harvested virus at the end of this process was used as a seed stock for large scale virus production. The presence of the IL-12 transgene downstream of the CMV promoter was confirmed by PCR using primers specific for regions of the CMV promoter and IL-12 p40 subunit.

2.3.3 Large scale virus production

Large scale crude virus production from stocks of virus were prepared by inoculating sub-confluent monolayers of 293 or 911 cells in 10-20 175cm^2 culture flasks (Nunc) for each virus at a multiplicity of infection (moi) of 10 in 5 ml infection media. Cells were incubated at 37°C for 90 min to allow virus adsorption. A further 20 ml of media was added. Incubation was continued at 37°C until an extensive viral cytopathic effect was observed (bunch of grape like appearance), usually between 4-5 days post infection.

The cell suspension of infected cells were harvested into 50 ml tubes (Falcon) and centrifuged at 840g for 10 min in a refrigerated centrifuge. The supernatant was discarded and the cell pellet re-suspended in 0.5 ml medium per original flask. Cells were subject to three freeze and thaw cycles in liquid nitrogen and 37°C water bath. This crude extract was stored at -80°C prior to purification.

2.3.4 Density gradient virus preparation

Following three freeze thaw cycles n-butanol was added to a final concentration of 1% (V/V), mixed, and incubated on ice for 1 hr. The lysate was then centrifuged at 840g, 10min at 4^{o}C, and the supernatant loaded onto a preformed glycerol/caesium chloride gradient. The gradient consisted of 2ml caesium chloride density ρ=1.45g/ml in 10mM Tris pH 7.9 at the bottom, followed by 3 ml caesium chloride density ρ=1.32g/ml in 10mM Tris pH 7.9, with 2ml 40% glycerol in 10mM Tris pH7.9 on top. Viral supernatant was layered on top of the glycerol, and the tubes centrifuged at 25000 rpm, overnight at 4°C, in a Beckman SW40Ti rotor. Purified virus was visible as an opalescent band at ρ=1.345. The purified virus was collected by puncturing the tube with a 21 gauge needle with 5 ml syringe attached. The collected virus was dialysed to remove contaminating CsCl by inserting the collected virus solution into a 5ml Slidealyser dialysis cassettes (Pierce) and dialyzed against PBS/10% glycerol overnight at 4^{o}C, changing the PBS/glycerol three times. Virus was finally removed from the cassette and aliquoted into Sarstedt O ring sealed 1 ml tubes and stored at -80°C.

2.3.5 Determination of virus particle number by Picogreen assay

0.1% (W/V) SDS solution was mixed in a 1:1 ratio with a sample of virus and heated at 56°C for 30 min to inactivate the virus and release the viral DNA. The titre of viral particles was determined based on DNA concentration using Picogreen reagent (Molecular Probes) as above.

Adenoviruses have one double stranded DNA chromosome therefore every single DNA molecule represent one virus particle. Virus particle number was determined based on the calculated DNA concentration, number of bases in viral DNA, and the average weight of bases by using the following formula:

$$\text{Virus particles} = \text{Measured DNA Conc.} \left(\frac{\text{Avagadro's number}}{\text{Number of base pairs}} \right) \text{X650}$$

2.3.6 Determination of viral titre by plaque assay

Plaque assay were used to determine the titre of infectious particles. $1x10^6$ 911 or 293 cells were seeded per 60mm diameter plate (Nunc), 24 h prior to infection. Viruses were serially diluted 10-fold in media. Medium was removed from the plates and 500µl of the virus dilution (10^{-6}-10^{-10}) was added to each plate in triplicate. Plates were returned to the incubator and virus allowed to infect for 90 min, with frequent rocking of the plates. Subsequently 4mls agar-medium overlay was applied to each plate, once solidified plates were returned to the incubator. At 4, 7 and 10 days post-infection cells were re-fed by the addition of 2ml agar-medium overlay. Plaques were counted as they appeared up until 14 days post infection. The virus titre in plaque forming units (pfu)/ml was then calculated.

2.4 Tissue culture

2.4.1 Maintenance of primary human fibroblasts

Previously primary human fibroblasts had been established from skin punch biopsies from donors in the Institute of Cancer Studies. Cells were fed by half medium change until cells were nearly confluent; each well was then trypsinised and seeded 1:3 in wells of a 6 well plate.

2.4.2 Culture Media

Dulbecco's modified Eagle's medium with HEPES (DMEM-HEPES, Sigma) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine and 100iu/ml Penicillin and 100μg/ml Streptomycin was used to grow A549, HEK-293 and 911 cell lines (see Table 2-2). Complete Roswel Park Memorial Institute medium with L-glutamine (RPMI, Sigma), supplemented with 10% FCS in addition to antibiotics at the above concentrations was used to culture PBMCs (as detailed in section 2.4.7) and for the culture of OVCAR-3 (see Table 2-2). RPMI supplemented with 8% human (h)-AB serum (HD supplies) in addition to antibiotics at the above concentrations was used to culture PBMCs for autologous culture models. Media supplemented as above will be referred to as complete media.

2.4.3 Maintenance and passage of mammalian cell lines in culture

Table 2-2 shows cell lines used in this study. All cell lines were obtained from colleagues (as indicated) in the CRUK Institute for Cancer Studies, University of Birmingham.

Table 2-2 Cell lines.

Cell line	Cell type	Source	ATCC No.
HEK293	**Primary human embryonic kidney transformed by sheared human Ad5**	**Dr. V. Mautner**	**CRL-1573**
911	**Human embryonic retinoblasts (HER) transformed by a plasmid containing base pairs 79-5789 of Ad5**	**Dr. V. Mautner**	**n/a**
OVCAR-3	**Human ovarian adenocarcinoma**	**Dr. P.Searle**	**HTB-161**
A549	**Human lung carcinoma**	**Dr. P.Searle**	**CCL-185**
K562	**Human chronic mylogenous leukaemia**	**Dr. O. Goodyear**	**CCL-243**

Cultures were kept at 37°C in humidified incubator with 5% CO_2. At approximately 80% confluence cultured cells were passaged by removing the media, washing with Phosphate Buffered Saline (PBS) and incubating in trypsin (0.05%) until cells were detached. Trypsin was inactivated by the addition of complete culture media. Harvested cells were either grown in cell culture flasks typically at a 1:5 or 1:10 dilution or were collected by centrifugation at 680g for five min. and diluted to a known concentration for use in experiments.

2.4.4 Cryopreservation/Recovery

Cell lines were kept as frozen stocks in liquid nitrogen. To prepare cells for cryo-storage, trypsinised cells were centrifuged as before at 4^oC and re-suspended in a

appropriate volume of freezing media (10% DMSO in FCS) 1ml aliquots in Cryo-tubes (NUNC) were placed into a NALGENE Mr Frosty pre-cooled to 4^{o}C and transferred immediately to a -80°C freezer overnight. Cells were transferred to vapour phase of liquid nitrogen storage tanks for long term storage. To revive frozen stocks, cells were warmed to 37°C rapidly and washed in 10mls of appropriate pre-warmed complete media by centrifugation at 300g for 10 min. before transfer to flasks in the appropriate medium for growth.

2.4.5 Microscopy

A Zeiss Axiovert 25 inverted microscope was used for all phase-contrast and fluorescence microscopy using 5, 10, 20 and 40x objectives.

2.4.6 Subjects and ethics

Local ethical approval (LREC) was obtained; Healthy volunteers 06/Q2604/156, Ovarian cancer patients 04/Q2707/190, Renal cell carcinoma patients 06/Q2706/82.

Blood and tissue samples were obtained following an informed written consent from healthy consenting members of the Institute for Cancer Studies, Birmingham or renal cell carcinoma patients undergoing partial or complete nephrectomy at Queen Elizabeth Hospital, Birmingham. Ascitic fluid was obtained from ovarian cancer patients undergoing paracentesis at the Cancer Centre, Queen Elizabeth Hospital. The HLA types of donors used for HLA restricted peptide specific responses had previously been determined within the Institute for Cancer Studies, Birmingham by

molecular tissue typing of the HLA class I locus, carried out by the Blood Transfusion Service (BTS).

2.4.7 Isolation of peripheral blood mononuclear cells, PBMCs and depletion of plastic adherent cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised peripheral blood samples by Ficoll density-gradient centrifugation. Whole blood was diluted 1:1 with Complete RPMI, 30mls was then layered over 15mls Ficoll (Lymphoprep, Norway) and centrifuged for 30 min at 680g at 20^{o}C with the brake off. The layer of PBMC was transferred with a transfer pipette to a fresh sterile tube and re-suspended in complete RPMI. Following two washes by re-suspension in RPMI and centrifugation at 540g for 10min the cell pellet re-suspended in complete RPMI. The lymphocyte yield was determined by haemocytometer (ISL) count.

The isolated PBMCs ($5x10^{6}$ cells/ml) were incubated in tissue culture flasks in complete RPMI at 37°C for a further 2 hr. The non-adherent cells were washed off with pre-warmed media and transferred to a fresh container. Isolated cells were used immediately or cryo-preserved in freezing media in liquid nitrogen.

2.4.8 Isolation of tumor infiltrating lymphocytes from ascitic fluid

Ascitic fluid was transferred to 250ml centrifuge tubes (BD Falcon) and spun at 840g for 15 min, the pellet was then resuspended (volume dependent on pellet size and volume of initial ascitic fluid sample) with complete RPMI and passed through a

100μm cell strainer (BD Falcon). The sample could then be treated as for PBMC isolation (2.4.7).

2.4.9 Isolation of 'normal' and tumour cells from RCC patient tissue

Samples of tumour tissue or normal margin were collected. Tissue was finely minced with sterile scalpel blades in a 60cm tissue culture dish (Nunc). The tissue was then either centrifuged (680g, 10 min) and cryopreserved or immediately processed for culture.

Minced tissue to be used to culture primary cell lines was suspended in complete RPMI and passed through a 100 μm cell strainer (BD Falcon); further tissue dissociation was carried out using a cell scraper to break tissue apart through the cell strainer. Cells were then centrifuged (680g, 10 min), re-suspended in complete RPMI and transferred to a 75cm^2 tissue culture flask. Following overnight culture at 37^{o}C non adherent cells were removed. Adherent cells were then cultured in complete RPMI and passaged as required.

2.4.10 Infection with recombinant adenovirus

Adenoviral vectors were used at the following MOI unless otherwise stated; Ad-CD80 was used at 300 virus particles/cell, Ad-4-1BBL and Ad-GFP were used at 3000 virus particles/cell and Ad-IL-12 was used at 600 virus particles per cell, (For clarity standard infections will use the following notation; Costimulatory

combination$_{\text{Cell type}}$ i.e. A549 infected with 300 particles/cell Ad-CD80 and 3000 particles/cell of Ad-4-1BBL will be defined as CD80+4-1BBL$_{\text{A549}}$).

These doses of Ad-CD80 and Ad4-1BBl were initially determined by flow cytometric analysis of expression by A549 cells 48hrs post infection, Ad-GFP was used in a dose equivalent to Ad-4-1BBL, i.e. the highest level of single virus used.

A549, OVCAR-3, cultured fibroblast and adherent PBMC (monocytes) were used to express CD80, 4-1BBL, IL-12 or GFP after infection with the corresponding adenoviral vectors. 24 or 48 hours in advance, cells were harvested from tissue culture flasks or purified from peripheral blood. The cells were re-suspended in DMEM supplemented with 2% FCS in addition to antibiotics for infection of cell lines or complete RPMI media for primary cell infection at $1x10^6$ cell/ml. The required number of virus particles for each infection were calculated and added to the cell suspensions. The virus-cell mixture was incubated at 37°C for 90 min. with frequent rocking to allow virus adsorption. Cells were then distributed to appropriate wells of tissue culture plates and additional media added to form a uniform monolayer growth.

Expression of the transgenes was examined by flow cytometry using specific monoclonal antibodies or fluorescence in the case of GFP expression.

2.5 T cell and Natural Killer cell assays

2.5.1 Anti-CD3 T cell activation

A schematic of the OKT-3 response model system is shown in Figure 2-2. A549 cells were infected with the appropriate adenovirus vectors as described in section 2.4.10 and cultured at $1x10^5$ cells/well of a 24 well plate 48 hrs in advance. Infection media was removed and $1x10^6$ PBMCs then added to each well in a total volume of 2 mls of complete RPMI supplemented with 100ng/ml OKT-3 (Orthoclone™, Janssen-Cilag). Lymphocyte proliferation was studied by cell counting (in the presence of trypan blue to exclude dead cells), cell surface marker staining and flow cytometry or CFSE dilution at appropriate time points.

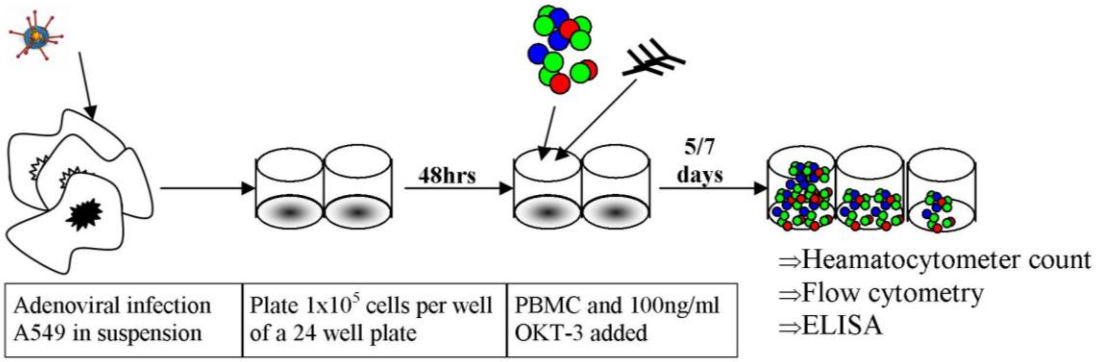

Figure 2-2 OKT-3 response model system. A549 were infected with adenoviral vectors for 90min in suspension at $1x10^6$ cells/ml prior to plating into wells of a 24 well plate. 48hrs after infection media was removed and $1x10^6$ non adherent PBMC added. PBMC responses were assessed by differing methods after 5 or 7 days.

2.5.2 Allogenic stimulation of Natural Killer cells

A schematic of the NK cell response model system is shown in Figure 2-3. OVCAR-3 cells were infected with the appropriate adenovirus vector as described in section 2.4.10 and cultured at $1x10^5$ cells/well of a 48 well plate 48 hrs in advance. Infection

media was removed and $0.5x10^6$ PBMCs then added to each well in a total volume of 1 ml of complete RPMI. Lymphocyte proliferation was studied by cell counting, cell surface marker staining and flow cytometry or CFSE dilution at appropriate time points.

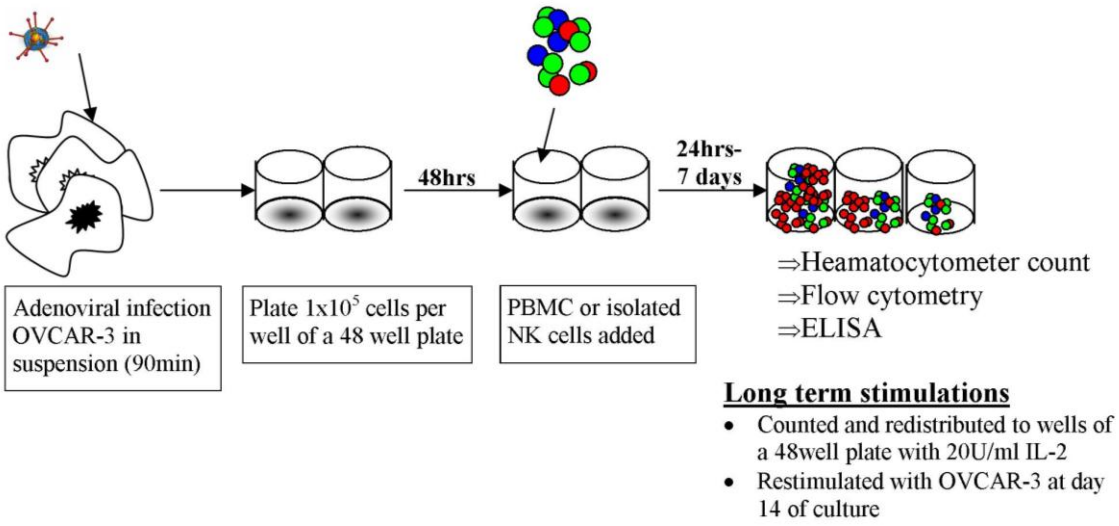

Figure 2-3 Allogeneic NK cell culture model. OVCAR-3 cells were infected with adenoviral vectors for 90min in suspension at $1x10^6$/ml then plated at $1x10^5$ cells/well of a 48 well plate. 48hrs after infection media was removed and a non adherent PBMC population or an isolated NK cell population added. Responses of the PBMC or NK cell population was assessed between 24hrs to 7 day of culture, dependent on experiment. Long term stimulations were also performed by the addition of 20U/ml IL-2 at day 7 and re-stimulation with OVCAR-3 pre-infected as for day 0 at day 14.

2.5.3 Autologous viral peptide stimulation of T cells

A schematic of the OKT-3 response model system is shown in Figure 2-4. 24 hrs prior to the addition of non-adherent PBMC or purified T cells autologous adherent PBMC were allowed to adhere in $75cm^2$ tissue culture flasks for two hours, non adherent PBMC were removed by washing with media. Adherent cells were removed by scraping and re-suspended at $1x10^6$ cells/ml for infection. Cells were distributed and infected with the appropriate adenovirus vectors at $2x10^5$ cells/well of a 48 well plate and the volume adjusted to 0.5mls with complete RPMI after a 90min infection. Four

hours before the addition of non-adherent PBMC or purified T cells, the stimulating cells were peptide-pulsed by the addition of 5μg/well of peptide (Table 2-3); unbound peptide was removed after four hours by three washes by the gentle addition and removal of media. $0.5x10^6$ non-adherent PBMC or purified T cells were then added and cultured for 7 days.

48 hours prior to the addition non-adherent PBMC or purified T cells fibroblasts were trypsinised and re-suspended at $1x10^6$ for infection with the appropriate adenovirus vectors cells, then plated out in 48 well plates at $1x10^5$ cells/well and the volume adjusted to 0.5mls with complete RPMI after a 90min infection. Fibroblasts were peptide pulsed in the same manner as for adherent cells.

Table 2-3 Virus specific peptide epitopes.

Peptide Sequence	Origin	HLA Restriction	Manufacturer
*CLG*GLLTMV	LMP-2 (EBV)	HLA-A2	Mimotopes
L*TDL*GQNLLY	Hexon (Adenovirus)	HLA-A1	JPT Peptide Technologies
*NLV*PMVATV	pp65 (CMV)	HLA-A2	JPT Peptide Technologies

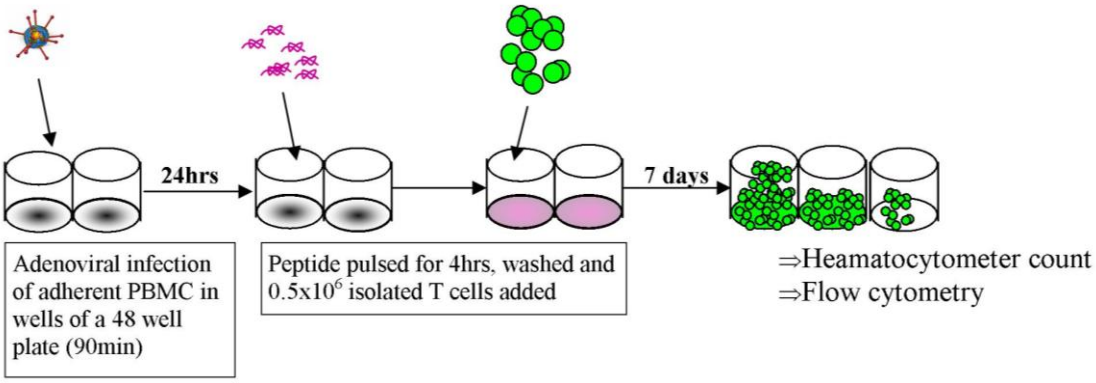

Figure 2-4 Viral peptide specific response model. Adherent PBMC or autologous fibroblasts were infected with adenoviral vectors for 90min in suspension at $1x10^6$ cells/ml in wells of a 48 well plate ($2.5x10^5$ cells/well). 24-48hrs after infection cells were peptide pulsed with 5µg of peptide for 4hrs. Media was then removed and unbound peptide removed by gentle washing with media. $0.5x10^6$ isolated T cells were then added. T cell responses were assessed after 7 days of culture.

2.5.4 Isolation of lymphocyte subsets

2.5.4.1 Negative isolation of T cells

A population of untouched T cells was obtained using the Miltenyi Biotec Pan T Cell Isolation Kit II following the manufactures protocol. Non-T cells were removed by a two step process. Firstly a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123, and Glycophorin A was added to bind Non-T cells followed by the addition of anti-biotin microbeads to allow the capture and magnetic removal of these cells.

2.5.4.2 Isolation of Natural Killer cells

A population of purified NK cells was obtained using the Miltenyi Biotec $CD56^{+}CD16^{+}$ NK Cell Isolation Kit. This kit firstly allowed the purification of an untouched pan NK cell population by negative isolation. As with the negative isolation of T cells, Non-NK cells were removed by two steps, involving first binding with a cocktail of biotin-conjugated antibodies against lineage specific antigens followed by the addition of anti-biotin microbeads to allow the capture and magnetic removal of these cells. This kit also allowed the subsequent separation of $CD16^{+/-}$ cells, by the positive selection of $CD16^{+}$ cell by anti-CD16 microbeads. Following the initial negative isolation NK cells, this allowed the isolation of a highly purified population of NK cells of the $CD56^{dim}$ subset. These procedures were carried out following the manufacturers' protocol.

2.5.5 Cell staining for flow cytometry analysis

Cultured or fresh PBMC or cell lines were washed with PBS/FCS (2%) and incubated with appropriate fluorochrome-conjugated antibodies at 4^{o}C in the dark for 30 min. Cells were washed with PBS/FCS (2%) and cells were re-suspended in 500μl buffer for flow cytometry or fixed with 2% paraformaldehyde for later analysis. Cells were analysed using a four-colour Beckman Coulter XL flow cytometer using Coulter System II software for data acquisition or a 16 colour Beckman Coulter LSR II. Data was analysed using FlowJo or WinMDI software.

Unstained or isotype control stained samples were used first to set forward scatter and side scatter properties. Voltages were set so the peak fluorescence generated from the negative control in each channel was between 10 and 1. If multicolour staining was being used then appropriate colour controls were analysed and compensation performed with reference to every other colour being analysed. Once this was completed voltages, gates and compensation were kept constant for all cells analysed. Table 2-4 lists the anti-human monoclonal antibodies and the flurochrome labels used in the course of this project.

Data was analysed using two parameters: percentage positive and mean fluorescent intensity (MFI). Percentage positive refers to the percentage of gated cells fluorescing above the level of the negative control (isotype control) (Serotec or Beckman Dickinson) this was measured using a marker set to include less than 1% of the negative control cells. Mean fluorescence was taken as the arithmetic mean fluorescence in each channel for all the cells gated.

Table 2-4 Main antibodies used in this project.

Antibody	Label	Supplier
Anti-CD3	FITC/Pacific Blue	BD Pharmingen
Anti-CD4	FITC	DAKO
Anti-CD4	PE	Beckman Coulter
Anti-CD8	FITC/PE-Cy5	Beckman Coulter
Anti-CD16	FITC	BD Pharmingen
Anti-CD25	PE	Serotec
Anti-CD56	APC/PE	Becton Dickinson
Anti-CD62L	APC	BD Pharmingen
Anti-CD80	FITC	BD Pharmingen
Anti-CD137	PE	BD Pharmingen
Anti-CD137L	PE	BD Pharmingen
CCR5	PE-Cy7	BD Pharmingen
CCR7	PE-Cy7	Beckman Dickinson
CXCR3	Unconjugated	Beckman Dickinson
IFNγ	PE	BD Pharmingen
FoxP3 (PCH101)	PE	eBioscience

2.5.6 Measurement of lymphocyte division using CFSE (Carboxy Fluorescein diacetate, Succinimidyl Ester) dilution

2.5.6.1 CFSE labelling of lymphocytes

PBMCs were washed three times with PBS, centrifuged at 540g, 10 min and re-suspended at 2×10^7cells/ml in PBS. 10 mM stock CFSE (Molecular Probes) in DMSO was diluted in PBS to achieve a 5 µM stock. Diluted CFSE was added to the cells at a ratio of 1:1 (vol:vol) and vortexed gently. The cell suspension was then incubated for 15 minutes at 37°C agitating periodically. An equal volume of complete RPMI culture medium was added to the cells and left for one minute at room temperature to stop the labelling process. The cells were washed 3 times with media and re-suspended in culture media.

2.5.6.2 Analysis of cell proliferation by CFSE dilution

CFSE is a fluorescein related dye consisting of a fluorescent molecule containing a succinimydyl ester functional group and two acetate moieties. CFSE diffuses freely into cells where intracellular esterases cleave the acetate groups converting it to a fluorescent, membrane impermeable dye which binds to intracellular proteins. As cells divide the dye is partitioned equally between mother and daughter cells, the number of cell divisions can therefore be determined according to the number of equally spaced peaks of CFSE fluorescence, typically up to six divisions can be discerned from CFSE dilution. The area under each peak was determined to calculate the percentages of dividing lymphocytes in each round of cell division or simply as a measure of dividing and undivided populations.

2.5.7 Production of CLG Tetramer

2.5.7.1 Induction of protein expression

Glycerol stocks of E.coli transformed with expression plasmids for HLA-A2 or β_2M were a kind gift from Geothy Chakupurakal, (Institute for Cancer Studies, Birmingham). All bacterial culture media were supplemented with 100μg/ml ampacillin. Bacteria were streaked out onto LB Agar plates, and cultured overnight at 37^{o}C. Plates were removed from the incubator and stored at 4^{o}C, colonies were picked using a sterile loop into 10mls LB medium which was cultured overnight at 37^{o}C. The following day 4L of low salt LB medium and 2L of LB were prepared for HLA-A2 and β_2M growth respectively, 10mls of the overnight culture was added per litre of media and cultured at 37^{o}C. The OD_{600} was measured using a spectrophotometer periodically until OD_{600} reached between 0.3-0.4 at which point protein expression

was induced by the addition of Isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5mM for 4 hours, cultures were then stored at 4^{o}C overnight. Cultures were centrifuged at 4000 rpm in a Sorvall GSA rotor at 4^{o}C for 20 mins. The pellet was then resuspended in ice cold PBS ice cold and transferred to a 50ml tube, and made up to 25ml and stored at -80˚C.

2.5.7.2 Purification of protein

In order to release the inclusion bodies containing the recombinant HLA-A2 and β_2M molecules it was necessary to sonicate the bacterial cells. Bacteria were thawed at room temperature and subsequently kept on ice. A flat horn was used in bursts of 45 secs, followed by 1 minute between bursts on ice to allow the sample to cool. In total this was repeated eight times until the sample was completely lysed. The sample was then stored on ice for about 30 mins.

The sonicated sample was spun at 4^{o}C at 15000 rpm in a Sorvall SS-34 rotor for 10 mins, the supernatant was discarded, and the pellet was resuspended in 4ml/l of Triton Wash (0.5% Triton X-100, 50mM Tris pH 8.0, 100mM NaCl, 0.1% Sodium Azide, 1mM EDTA, 1mM DTT) and transferred into a glass homogeniser using a transfer pipette and homogenised until completely re-suspended. Following homogenisation the solution was once again spun, this procedure was repeated twice. After the final wash the supernatant was discarded and 2ml per litre of re-suspension buffer (50mM Tris pH 8.0 100mM NaCl, 1mM EDTA, 1mM DTT) was added, the solution was again transferred into a clean glass homogeniser and completely re-suspended. The sample was then spun at 15000 rpm in a Sorvall SS-34 rotor for 10 mins at 4^{o}C. 15mls of freshly prepared Urea solubilisation solution (8M Urea, 50mM Mes pH 6.5, 0.1mM

EDTA, 0.1mM DTT) was then added to the pellet, which was and re-suspended by vortexing then incubated rotating overnight at 4^{o}C. The solubilised sample was transferred to clean centrifuge pots and centrifuged at 15000 rpm in a Sorvall SS-34 rotor for 20 min in a precooled centrifuge, the supernatant was aliqoted and stored at -80^{o}C. The purity and concentration of the isolated protein was determined by SDS-PAGE and Bradford assay.

2.5.7.3 Refolding Class I complexes

The HLA-A2 and β_2M was now ready to be refolded with the CLG peptide to form HLA monomers. The refolding buffer was made, firstly 400ml of initial buffer (125mM Tris pH 8, 0.4M L-Arginine HCL 2.5mM EDTA) was made and left overnight stirring at 4^{o}C. Continuing to stir at 4^{o}C the solution was then made up to contain 0.6μM Reduced Glutathione, 0.06μM Oxidised Glutathione, and 0.25mM PMSF and 5mg of CLG peptide. A 12.5mg aliquot of β_2M was then thawed and diluted to a concentration of 1mg/ml by drop wise addition of the refold buffer (containing peptide), this mixture was then added back drop wise to the bulk refold buffer, this mixture was left stirring at 4^{o}C for 1 hr. A total of 33.68mg of HLA-A2 was then required to form tetramer monomer. This was added as above in eight separate stages over a 48hr period. The HLA-A2 was thawed and the required amount diluted to 1mg/ml in refold buffer (containing peptide and β_2M) as for β_2M and transferred back to the main refold. Following the final addition of HLA-A2 the refold was left stirring for a further 2 days.

2.5.7.4 Purification and biotinylation of refolded CLG monomer

The refolded complex was then concentrated to allow purification using a Millipore Pellicon XL (10,000 MW) plate to approximately 7 ml. In order to allow biotinylation of the monomer buffer exchange was performed using a PD10 column (Pierce). The column was washed three times with Bir A reaction buffer (100mM Tris pH 8, 20mM NaCl, 5mM MgCl, 0.1mM PMSF), the sample was then run onto the column and then eluted using Bir A reaction buffer. The monomer was then biotinylated overnight at room temperature by the addition of a final concentration of 1.5nM Biotin, 15mM ATP, Protease inhibitor cocktail and Bir A enzyme (Roche). The biotinylated CLG monomer was then purified on a G75 Superdex column (GE healthcare) and concentrated using a Millipore Centricon filter spun at 840g at 4^{o}C. The concentration of the monomer was determined by spectrophotometry and diluted to 1mg/ml using FPLC buffer (20mM Tris pH8.0, 50mM NaCl) and stored as 100µg aliquots at -80^{o}C.

Successful biotinylation of the monomer was subsequently confirmed by ELISA, briefly monomer was bound to a well of a maxisorp plate (Nunc) at room temperature for 2hr, washed three times with PBS+0.1% Tween 20, and Extravidin-peroxidase added for 30min, washed five times and 100µl tetramethylbenzidine (TMB) substrate (Pierce) added, colour generation indicated successful biotinylation.

2.5.8 Tetramer staining

2.5.8.1 Tetramerisation with streptavidin-PE

Tetramers were stored as frozen monomers, in order to produce tetramers the monomer was mixed at a ratio of 0.312mg streptavidin-PE:1mg of monomer. A

aliquot of frozen monomer was thawed and the required volume of streptavidin-PE (Invitrogen) added in 10 aliquots over a 48hr period at 4^{o}C. Tetramer was generally considered to be stable at 4^{o}C for upto four weeks.

2.5.8.2 Cell staining with tetramers and antibodies

0.5-1×10^{6} cultured or fresh PBMCs were incubated with appropriate PE-labelled MHC tetramer reagent at 37°C for 15 min. After washing with cold PBS/FCS (2%) buffer, cells were re-suspended and incubated with the appropriate fluorescently labelled antibodies for 20 min at 4°C. The stained cells were washed with cold PBS/FCS (2%) buffer and re-suspended in 300-500µl of buffer for analysis by flow cytometry.

2.5.9 Quantitative enzyme linked immunosorbent assay (Elisa)

Media was collected at appropriate time points from stimulated cultures and stored at -20^{o}C until required.

2.5.9.1 Interferon gamma ELISA

Maxisorp 96-well plates (Nunc) were coated with 50µl/well of anti-human-IFNγ antibody (0.75µg/ml) (clone B133.5, Thermo scientific) in coating buffer (0.1M Na_2HPO_4, pH9) by overnight incubation at 4°C. The unbound antibody was washed off by three washes with PBS+0.1% Tween 20, the plate was then blocked with 200µl/well 1% BSA, PBS+0.1% Tween 20 for1 h incubation at RT. The plate was washed 5 times and 50µl sample added to each well in triplicate. Culture supernatants were serially diluted 1:10 to 1:1000 and IFNγ standard solutions were serially diluted

1:2 (range 2000-31.5pg/ml). Plates were incubated at RT for 3hrs and subsequently washed with PBS+0.1% Tween 20 five times. Biotinylated anti-IFNγ (clone B133.5, Thermo scientific) was diluted in the blocking buffer at 1/300 and 50µl added to each well. Plates were incubated at RT for 1 h and again washed five times. Extravidin-peroxidase was diluted in blocking buffer (1:1000) and 50µl added to each well. After a 30 min incubation at RT and nine final washes, 100µl TMB chromogen (Pierce) was added to each well. The reaction was stopped with 100µl of 1M hydrochloric acid and the absorbance was measured at 450nm by a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer, Monza, Italy (Formally Wallac)).

2.5.9.2 IL-10 ELISA

IL-10 concentrations were determined using a matched antibody set and recombinant IL-10 standard purchased from ebioscience following the manufactures guidelines. Briefly a Maxisorp 96 well plate (Nunc) was coated with anti-IL-10 (clone JES3-9D7) diluted in supplied coating buffer, overnight at 4^{o}C. Following blocking with 1%BSA in 0.1%PBS-Tween samples were incubated at room temperature for 5hr, washed with 0.1%PBS-Tween and anti-IL-10-biotin (clone JES3-12G8) added in blocking buffer for a further 1hr. Detection was then achieved following washing by addition 50µl/well of Extravidin-peroxidase diluted in blocking buffer (1:1000). After a 30 min incubation at RT and nine final washes, 100µl TMB chromogen was added to each well. The reaction was stopped with 100µl of 1M hydrochloric acid and the absorbance was measured at 450nm by a Wallac Victor2 1420 Multilabel Counter (PerkinElmer, Monza, Italy (Formally Wallac))

2.5.9.3 Granulocyte-macrophage colony-stimulating factor ELISA

GM-CSF concentrations were determined using a GM-CSF Elisa Max kit (Bioledged, Cambridge Bioscience) following the manufacturers guidelines. Briefly anti-GM-CSF antibody was coated onto Maxisorp plates overnight at 4^{o}C, followed by blocking, incubation with sample and labelling with detection antibody and Steptavidin-HRP. Analysis was performed as for detection of IFNγ ELISA; timings followed the manufacturers' instructions.

2.5.10 Interferon gamma capture

To allow the detection of specific populations of cells producing IFNγ within a cultured population a Miltenyi Biotec IFNγ Secretion Assay - Cell Enrichment and Detection Kit was used following the manufacturers protocol. Cells were initially labelled with a bi-specific antibody (anti-lymphocyte/anti-IFNγ) following a period of culture with constant rotation (45min at 37^{o}C) to allow secretion and capture of IFNγ. IFNγ production was detected by the addition of an anti IFNγ PE conjugated antibody. Additional phenotype markers were also stained at this point by addition of the appropriate fluorescently labelled antibodies for 20 min at 4°C. The stained cells were washed with cold MACS buffer and re-suspended in 300-500µl of buffer for analysis by flow cytometry.

2.5.11 Intracellular interferon gamma staining

Intracellular staining was performed using ADG (Caltag) Fix & Perm reagents following the manufacturers' instructions. Briefly cells were cultured in the presence of 10µg/ml brefeldin A for 4hr. Cells were then washed with cold PBS+2%FCS and

stained with surface antibodies as described in 2.5.5. Following washing cells were fixed with Reagent A (formaldehyde). Following fixing, cells were washed and permeabilised with Reagent B (Saponin/Formaldehyde), to which 20µl anti-IFNγ-PE antibody was added or the appropriate isotype control. Following 30 min incubation at 4^{o}C, cells were washed and analysed by flow cytometry.

2.5.12 Intracellular FoxP3 staining

Intracellular staining was performed using a FoxP3 staining buffer set (eBioscience) following the manufacturers' instructions. Briefly cells were stained with surface antibodies as described in 2.5.5, following washing cells were fixed and permeabilised (Saponin/Formaldehyde). Following blocking with 2% rat serum for 15 min at 4^{o}C 20µl anti-FoxP3-PE antibody was added or an appropriate isotype control. Following 30 min incubation at 4^{o}C cells were washed and analysed by flow cytometry.

2.5.13 Chromium release cytotoxicity assay

2.5.13.1 K562 cells as targets

Typically K562 cells were counted and 1x10^{6} were spun down, 540g 10min. The media was removed with a transfer pipette, taking care to ensure all media was removed, the cells were then resuspended in 0.25 mCi ^{51}Cr (Sodium Chromate) (Amersham) and incubated at 37^{o}C for 90 min.

Cells were washed twice with 8 ml of complete RPMI, target cells were then resuspended at 5x10^{4}/ml in complete RPMI. 100µl of the target cell suspension was

added to appropriate wells of a 96 well V bottom plate (Nunc). Effector cells were also counted and resuspened and added to targets to give Effector:Target ratios of 33:1, 10:1, 3:1 and 1:1 or 0.3:1. Targets were also plated out with the addition of media only (to determine spontaneous release) and with RPMI+0.1% SDS (to give maximal release). Controls were typically plated out in four separate triplicate sets. The plates were the centrifuged at 355g for 3min then incubated at 37^{o}C for 5hrs.

100µl of the supernatant was then removed from each well and placed into individual LP2 tubes. These were then loaded on a Packard Cobra II D5010 Ten Detector Scintillation Gamma Counter. Results are expressed as percentage specific lysis which was calculated using the following formula;

$$\% \text{ Specific Lysis} = \left(\frac{\text{Exp. Release} - \text{Spontaneous}}{\text{Max. Release} - \text{Spontaneous}} \right) \text{x100}$$

2.5.13.2 Autologous tumour and 'normal' cells as targets

In the case of frozen tissue, tissue was defrosted as 2.4.3. Dissociated tissue was resuspended and cells isolated as for fresh tissue (2.4.9). In the case of cultured cell lines, cells were trypsinised (2.4.2).

Target cells from tissue isolated by either method were now treated identically. Cells were counted by hemocytometer and typically 1-5x10^{5} cells spun down (540g 10 min) and media removed with a transfer pipette. ^{51}Cr labelling was as for K562 cells (2.5.13.1).

Target cells were mixed with effector cells at appropriate ratios or plated as minimal and maximal lysis controls. Following 5 hour incubation 100 µl supernatant was removed from each well and specific lysis determined as above.

2.5.14 Syto 16 cell death assay

SYTO dyes are cell permeable dyes that are non-fluorescent in aqueous medium but become highly fluorescent when bound to nucleic acids. Previously SYTO dyes have been used to distinguish live, apoptotic and necrotic cells. The green fluorescent SYTO 16 especially has been used to detect early apoptotic changes in lymphocytes. As SYTO 16 fluoresces when bound to nucleic acids live cells fluoresce brightly, while apoptosis can be discerned by a reduction in florescence (Sparrow and Tippett, 2005). With the addition of propidium iodide (P.I.) late stage apoptotic/necrotic cells could be discerned as $P.I.^{+}$ having lost membrane integrity while early apoptotic cells appeared $P.I.^{-}$ retaining membrane integrity.

Cells were removed from culture and washed with cold saline 540g 10 min at $4^{o}C$. The cell pellet was then resuspended in 500µl Saline+10nM Syto 16 (Invitrogen) supplemented with 0.5%BSA+ 30 µM verapamil (a gift from Yi-Jun Qi, Institute for Cancer Studies, Birmingham) and incubated at $37^{o}C$ for 45min. Cells were then washed in MACS buffer (PBS(pH7.2)+0.5%BSA+2mM EDTA), subsequently antibody staining was performed with the required antibodies, and cells were then analysed by flow cytometery on a BD LSR II.

2.6 Graph plotting and statistical analysis

All graphs were plotted and statistical analysis performed using Graphpad Prism version 4 software (Graphpad software Inc., La Jolla, CA, USA).

3 Results: T cell Stimulation

3.1 Costimulation of T cell Response to OKT-3

3.1.1 Introduction

Costimulation is a prerequisite for the generation of an adequate T cell response. In order to study the ability of costimulation delivered by adenoviral vectors to provide costimulation for the activation and expansion of a T cell response a model system was used. A549 cells where used to express costimulatory molecules of interest, while the anti-CD3 antibody OKT-3 was used to provide a generic antigenic stimulation to the T cell population. While this system is highly artificial it allows a large scale response to be easily monitored without the added complexities of small peptide specific responses.

Previously within this laboratory the cDNA sequence for human CD80 and 4-1BBL or green fluorescent protein (GFP) have been isolated and inserted into replication defective adenoviral vectors, where the E1 region was replaced with one of the aforementioned genes, under the control of the CMV immediate–early promoter, to drive high level expression. The sequence for the p35 and p40 subunits of IL-12 had also been isolated and linked with an IRES, expression was also driven by the CMV immediate–early promoter, this was used to create a replication defective adenovirus vector to express IL-12 within the course of this project.

3.1.2 Expression of CD80 and 4-1BBL by adenoviral vector

In order to validate the ability of these vectors to express the required costimulatory molecules a range of virus particles per cell were used to infect the cell line A549. 48

hours following infection expression of the relevant molecules were assayed by flow cytometry. It was determined that a virus particle/cell ratio of 300 was required for Ad-CD80 to provide good infection and expression, while Ad-4-1BBL required a ten fold higher level of virus. This level of virus was shown to also be consistently effective for OVCAR-3, adherent PBMC and fibroblasts (Figure 3-1).

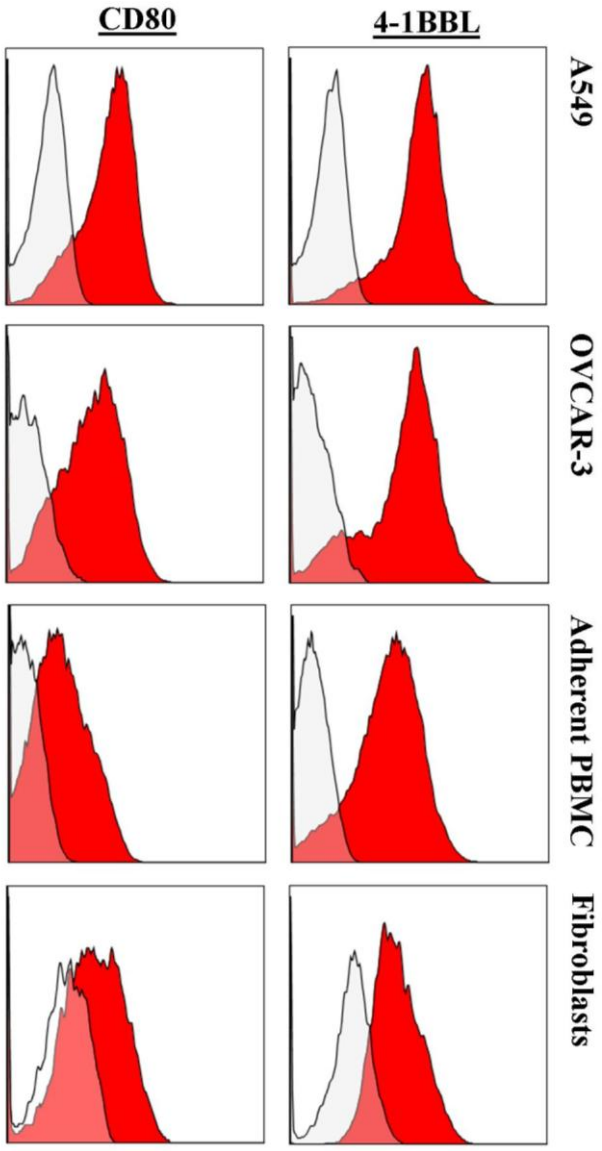

Figure 3-1 Expression of CD80 and 4-1BBL following infection with Ad-CD80 and Ad-4-1BBL. The cell types shown were infected in suspension with 300vp/cell and 3000vp/cell of Ad-CD80 and Ad-4-1BBL respectively. Following culture for 48hrs expression of CD80 and 4-1BBL were assessed by flow cytometry. Shaded histogram show mock infected cells stained with antibody. Results shown are typical of at least three A549 and OVCAR-3 experiments, four donor adherent PBMC samples, and results from fibroblasts of one donor used for long term stimulation.

3.1.3 Costimulation of short term OKT-3 stimulated T cells

Using a similar model system Habib-Agahi et al. (2007) previously showed that a combination of CD80/CD86+4-1BBL was superior to either costimulatory molecule alone in the ability to promote the expansion of T cells during short term (7 day) culture. In order to revalidate the ability of CD80+4-1BBL to allow the expansion of T cells, and also to investigate the effect of the addition of IL-12 to the combination; experiments were set up using A549 infected to express our chosen combinations of costimulation.

Assessing the total number of PBMC (rather than actual expansion of the T cell population) we were able to show that after short term culture within our system there is little expansion in CD80 stimulated cultures (1.3 fold), while 4-1BBL alone is able to stimulate a modest increase in cell number (2.3 fold). As expected the combination of CD80+4-1BBL is superior (3 fold) to either single costimulation. Interestingly the addition of IL-12 to the combination further enhances the proliferation of these cultures (3.9 fold), while IL-12 alone has no effect, likewise there is no expansion in GFP controls (Figure 3-2A).

Having reconfirmed the superiority of the dual combination of CD80+4-1BBL costimulation to costimulation with either ligand individually, we wished to further assess the ability of the addition of IL-12 to the dual combination to allow enhanced proliferation of cultures. As shown in Figure 3-2B, the triple combination of CD80+4-1BBL+IL-12 is able to significantly enhance the proliferation of cultures in response to OKT-3 compared to the dual stimulation.

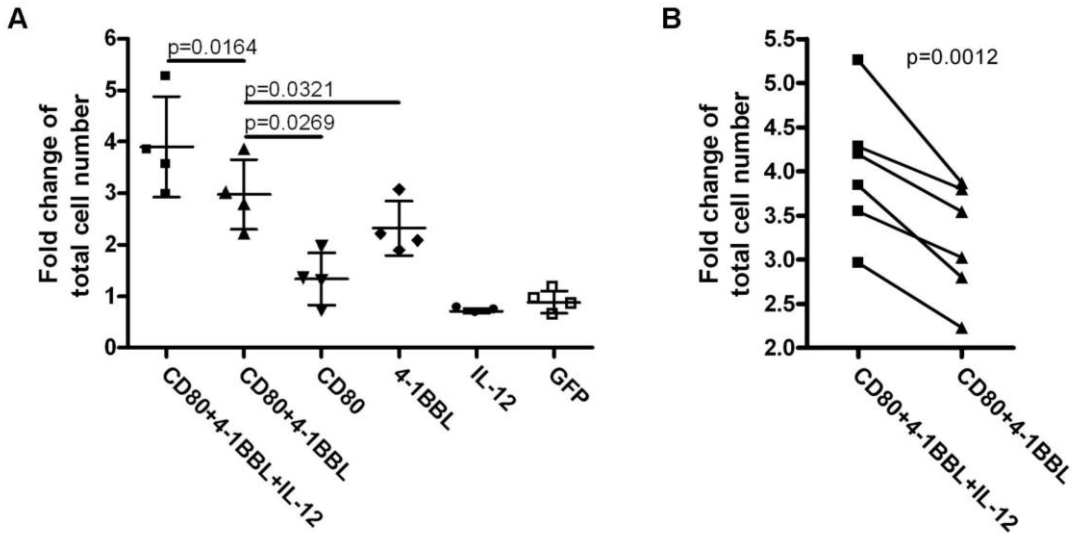

Figure 3-2 Expansion of whole PBMC populations in response to OKT-3 and adenoviral vector directed costimulation. $1x10^6$ adherent cell depleted PBMC were cultured in wells of a 48 well plate with $1x10^5$ pre-infected A549 cells/well (expressing costimulation as detailed in the figure) for 7 days in the presence of 100ng/ml OKT-3. A. shows fold increase of the total PBMC number from four healthy lab donors (N=4) compared to day 0 cell numbers, determined by haemocytometer count. Standard deviation (SD) and mean are shown, paired t-test. B. Shows fold increase in total PBMC number for six healthy lab donors following culture for 7 days with A549 expressing the combination of CD80+4-1BBL±IL-12, paired t-test.

3.1.4 Inclusion of IL-12 increases CD8 T cell expansion

In order to validate the expansion of a T cell population using our combinations of costimulation I examined the expansion of $CD4^+$ and $CD8^+$ T cells in culture. The populations following 7 day culture were predominantly T cells (Figure 3-3A), looking at the type of cell which were expanding we found that the $CD4^+$ T cell population was exhibiting minimal proliferation in response to either the dual or triple costimulation combinations. The majority of the expansion in response to CD80+4-1BBL appeared to be coming from the $CD8^+$ T cell population, the addition of IL-12 further enhanced proliferation of the $CD8^+$ T cell subset (see Figure 3-3).

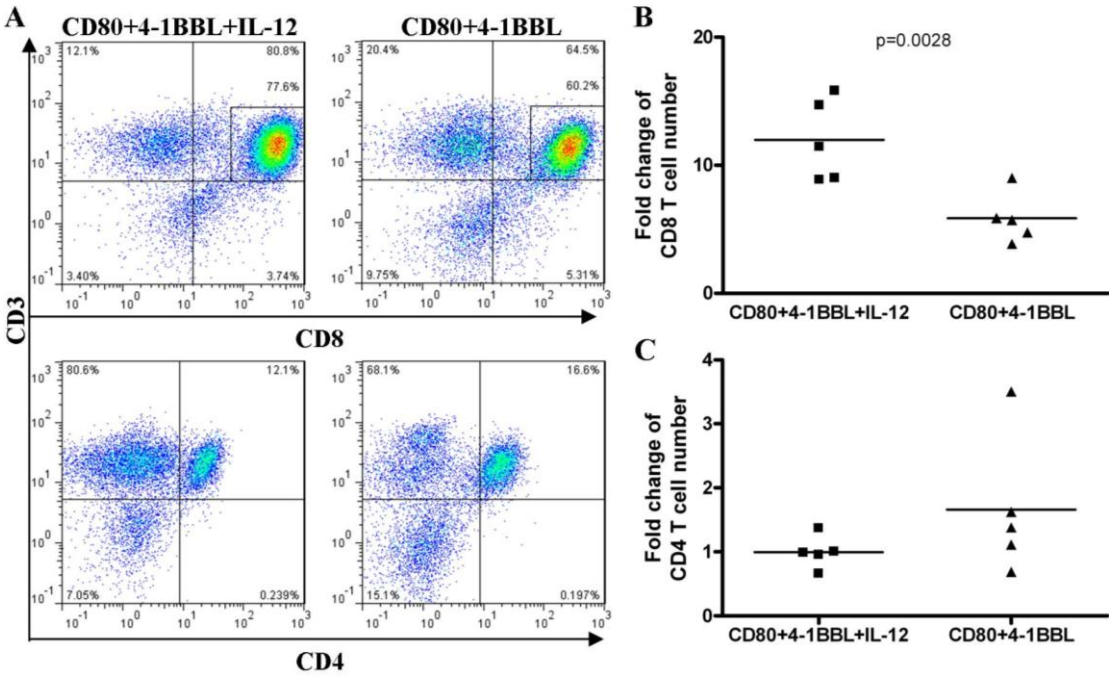

Figure 3-3 Expansion of $CD8^+$ and $CD4^+$ T cells. $1x10^6$ adherent cell depleted PBMC were cultured in wells of a 48 well plate with $1x10^5$ pre-infected A549 cells/well, (expressing costimulation as detailed in the figure following infection with adenoviral vectors) and 100ng/ml OKT-3 for 7 days. $CD3^+CD4^+$ and $CD3^+CD8^+$ populations were then determined by flow cytometry (A). Total PBMC number was also assessed by haemocytometer and total CD4 and CD8 T cell numbers obtained, the expansion of these cells was then determined as fold change in comparison to the number present at day 0. B. shows expansion of $CD8^+$ T cells, C. shows expansion of $CD4^+$ T cells. Paired t-test.

These data therefore reconfirm the superior ability of CD80+4-1BBL compared to either alone to enhance the expansion of a PBMC population in response to OKT-3 stimulation. Interestingly the addition of IL-12 to the combination further enhances expansion, although IL-12 alone was unable to induce expansion of the PBMC population. This suggests additional stimulation is able to enhance expansion further. The CD8 T cell population was shown to be the main subset proliferating in this model system which was enhanced by the addition of IL-12 which is in contrast to previous data using this model system.

3.1.5 Cytokine production following costimulation

Having shown the ability of our system to promote the expansion of T cells in response to antigen-mimetic OKT-3 stimulation we were also keen to infer functionality of these cells. In order to do this we measured production of the cytokines IFNγ and IL-10 by the various cultures. As shown in Figure 3-4, cultures stimulated with CD80+4-1BBL+IL-12 show a higher level of IFNγ compared to other cultures, however cultures stimulated with CD80+4-1BBL also show good levels of IFNγ albeit dwarfed by the effects of addition of IL-12 to the combination. IL-12 alone induced a slightly higher levels of IFNγ production compared to CD80+4-1BBL, mean 499.8ng compared to 254.7ng respectively, but was significantly lower than the combination of CD80+4-1BBL+IL-12 (mean 2115ng) (data not shown). In order to ensure that we were promoting a Th1 type response and to rule out the production of regulatory $CD8^+$ T cells we also assessed the production of IL-10 within the cultures. In comparison to IFNγ production there is very little IL-10 produced (Figure 3-4), suggesting that these cultures contained cells predominantly of the Th1 subset.

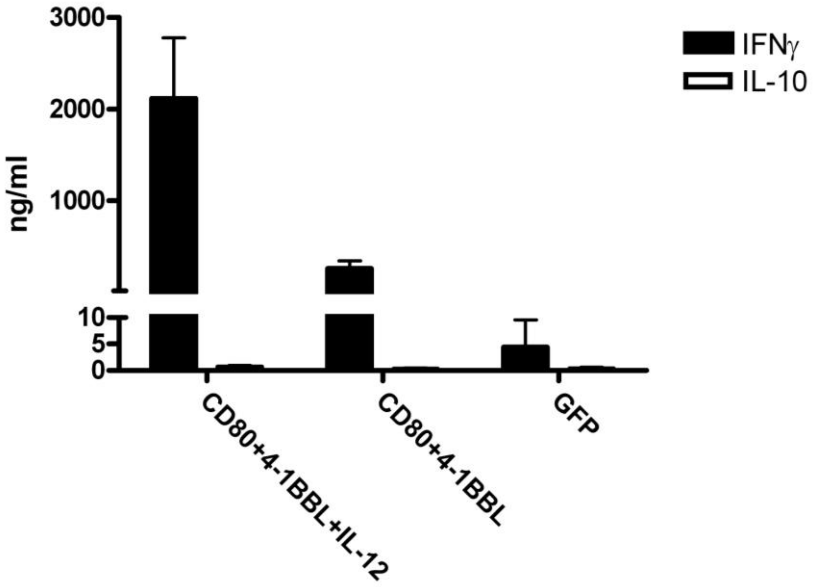

Figure 3-4 Cytokine production by cultured PBMC. $1x10^6$ adherent cell depleted PBMC were cultured in wells of a 48 well plate with $1x10^5$ pre-infected A549 cells/well (expressing costimulation as detailed in the figure following infection with adenoviral vectors) and 100ng/ml OKT-3 for 5 days. Supernatant was collected and concentrations of IFNγ and IL-10 determined by ELISA. N=3 SD.

3.1.6 TGF-β reduces IFNγ production but not proliferation

As discussed in section 1.2.3 tumours and associated regulatory T cells are capable of producing TGF-β, which is a major immunosuppressive cytokine. To test the effect TGF-β may have on IFNγ production and proliferation of PBMC in response to costimulation, PBMC were cultured on adenovirus infected A549 with OKT-3 as previously described with or without the addition of 2ng/ml recombinant human TGF-β, which had previously been shown to induce Treg (Chen et al., 2003; Zheng et al., 2007). TGF-β has also previously been reported to inhibit cytokine production (Schröder et al., 2003). The effect of TGF-β on the production of IFNγ by cultured cells was determined by ELISA, as shown in Figure 3-5A TGF-β was able to reduce the production of IFNγ in response to OKT-3 and CD80+4-1BBL or CD80+4-1BBL+IL-12 costimulation, therefore showing this concentration of TGF-β is sufficient to suppress function in this model system. However, as Figure 3-5B shows the addition of TGF-β did not inhibit expansion of the PBMC population. This suggests in a tumour micro environment the ability of the combination of CD80+4-1BBL+IL-12 to stimulate cytokine production may be impaired by the presence of TGF-β, however overall expansion may be unaffected. Further studies would be required to determine further functional consequences of TGF-β or other inhibitory factors during T cell stimulation in the presence the active costimulatory combinations. As this is a highly artificial model further studies were intended to be carried out during assessment of viral antigen specific responses in section 3.3.

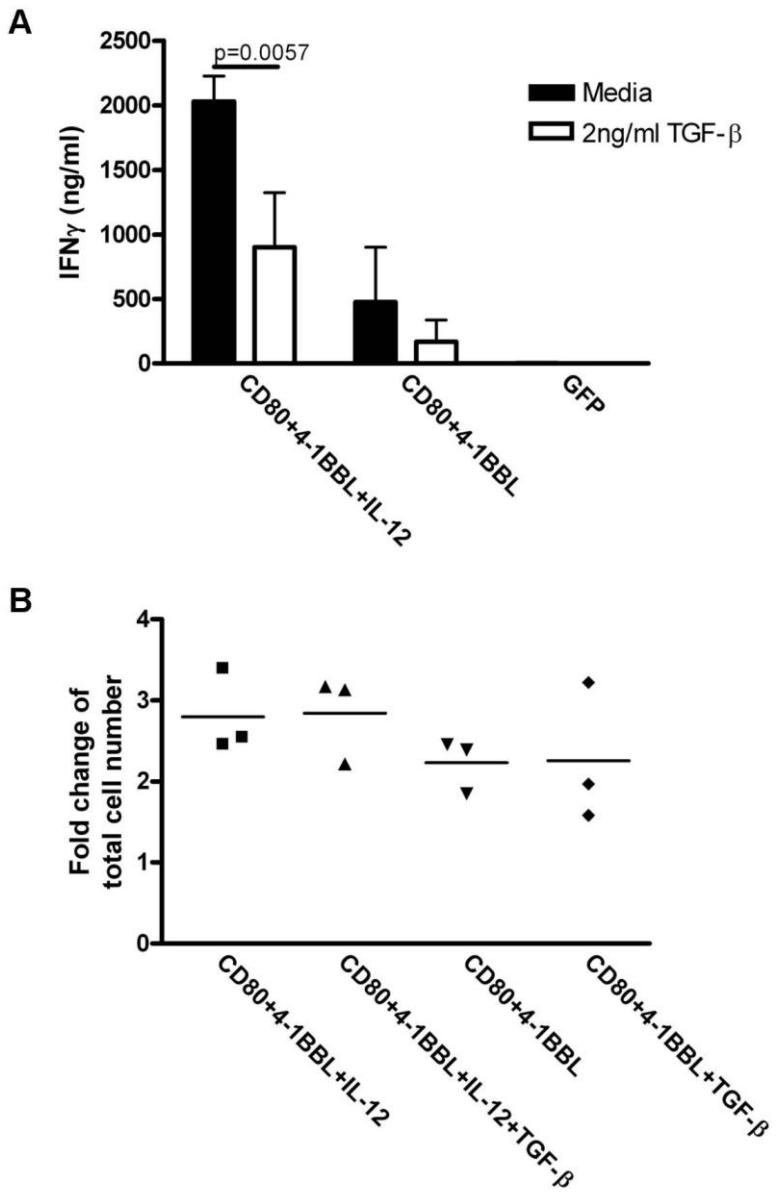

Figure 3-5 Effects of TGF-β on proliferation and IFNγ production by cultured PBMC. $1x10^6$ adherent cell depleted PBMC were cultured in wells of a 48 well plate with $1x10^5$ pre-infected A549 cells/well (expressing costimulation as detailed in the figure following infection with adenoviral vectors) and 100ng/ml OKT-3, in the presence or absence of 2ng/ml TGF-β. A. At 5 days of culture supernatant was collected and concentrations of IFNγ determined by ELISA. N=4, SD, paired t-test. B. Shows fold expansion of the total PBMC number following 7 days of culture from three healthy lab donors relative to total starting PBMC number, determined by haemocytometer count.

3.1.7 FoxP3 expression by expanded T cells

As there have been reports of costimulatory molecules' promoting the proliferation of Tregs under appropriate conditions it was of interest to determine if there was an effect on the Treg population. Figure 3-6A shows an example of FoxP3 staining from one donor and Figure 3-6B shows four out of five donors with no difference in the overall proportion of $CD4^{+}FoxP3^{+}$ T cells following costimulation with CD80+4-1BBL+IL-12 or CD80+4-1BBL.

As discussed, tumours tend to develop many immunosuppressive mechanisms in order to evade the immune system. This includes the production of TGF-β, which has been show to attenuate IFNγ production in our culture system. In order to further test the effect the costimulatory combinations of CD80+4-1BBL with or without IL-12 may be having on a Treg population, cultures were supplemented with 2ng/ml TGF-β; conditions know to promote Treg generation. Following 7 day culture under these conditions there is a significant increase ($p=0.0308$) in the proportion of $CD4^{+}FoxP3^{+}$ T cells following culture with CD80+4-$1BBL_{A549}$ supplemented with TGF-β compared with culture without TGF-β. The addition of IL-12 to the combination partially ablates the TGF-β induced increase in $CD4^{+}FoxP3^{+}$ T cells. However there is not a statistically significant difference between the $CD4^{+}FoxP3^{+}$ population following culture in the presence or absence of IL-12 in cultures supplemented with TGF-β (Figure 3-6A and Figure 3-6C).

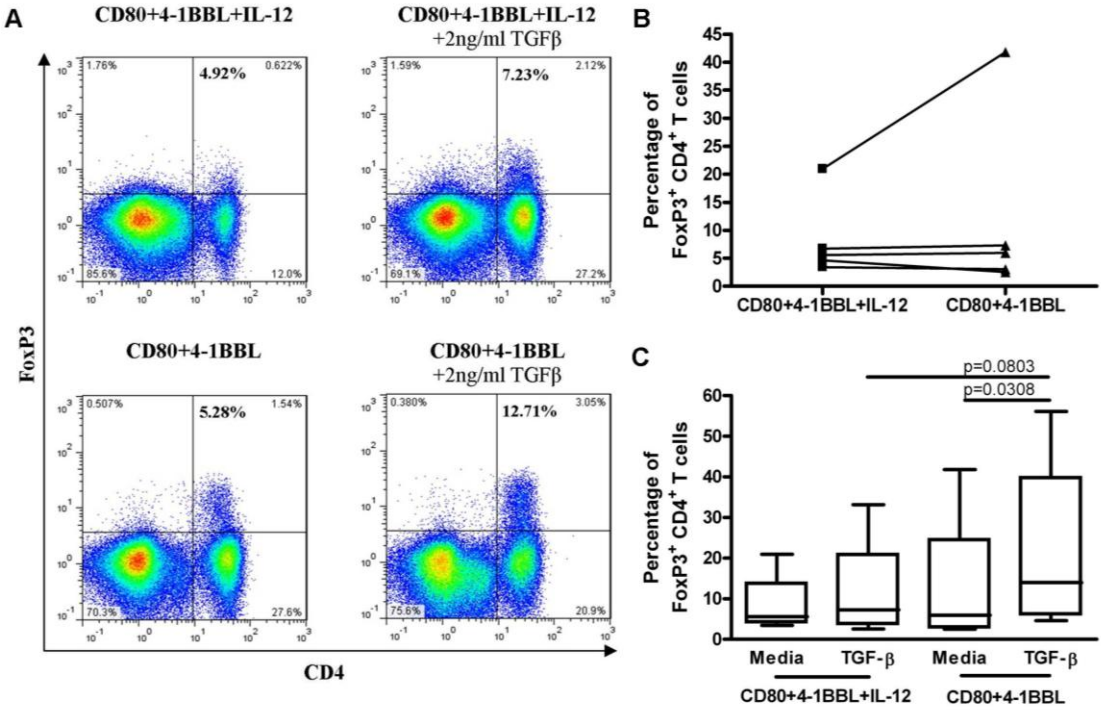

Figure 3-6 Foxp3 expression by CD4$^+$ T cells in response to TGF-β. 1x10^6 adherent cell depleted PBMC were cultured in wells of a 48 well plate with 1x10^5 pre-infected A549 cells/well (expressing costimulation as detailed in the figure following infection with adenoviral vectors) and 100ng/ml OKT-3 for 7 days in the presence or absence of 2ng/ml TGF-β. FoxP3$^+$ expression and associated markers were then determined by flow cytometry as shown for one donor in A. Percentage of the total CD4$^+$ population which are FoxP3$^+$ are shown in A (bold numbers) and B. Percentage of total CD4$^+$ population which are FoxP3$^+$ in the presence or absence of 2ng/ml TGF-β are shown in A and C. N=5, paired t-test.

The population of CD4$^+$ T cells is typically reduced in cultures with CD80+4-1BBL+IL-12$_{A549}$ compared with CD80+4-1BBL$_{A549}$ (p=0.0186) most likely due to increased proliferation of CD8$^+$ T cells (3.1.4). Interestingly, in the presence of there is a marked increase in the proportion of CD4$^+$ T cells present in cultures (p=>0.0001) abolishing the dominance of CD8$^+$ T cells induced by the addition of 12 (Figure 3-6A and

Figure 3-7).

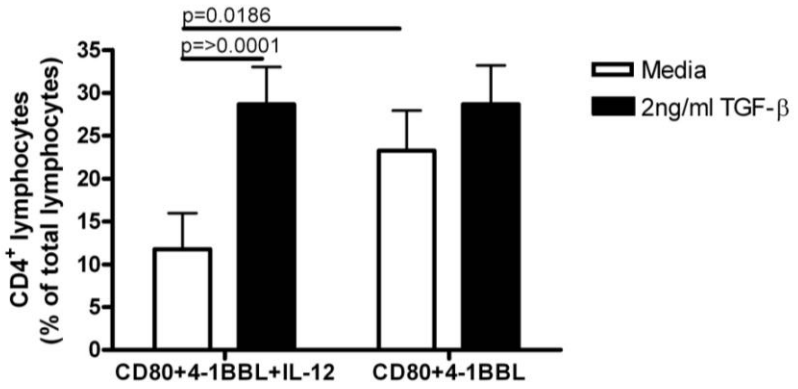

Figure 3-7 $CD4^+$ population is increased by the addition of TGF-β. $1x10^6$ adherent cell depleted PBMC were cultured in wells of a 48 well plate with $1x10^5$ pre-infected A549 cells/well (expressing costimulation as detailed in the figure following infection with adenoviral vectors) and 100ng/ml OKT-3 for 7 days in the presence or absence of 2ng/ml TGF-β. The percentage of $CD4^+$ cells was determined by flow cytometry. N=5, SD, Paired t-test.

These data suggest that the combination of CD80+4-1BBL±IL-12 does not induce or expand a significant $CD4^+FoxP3^+$ population in this model system. However in the presence of the Treg inducing cytokine TGF-β, the addition of IL-12 to the combination reduces the proportion of $FoxP3^+$ $CD4^+$ cells, suggesting the addition of IL-12 to a costimulatory combination maybe be important if used as an immunotherapy, and therefore warrants further investigation. Interestingly the addition of TGF-β increased the proportion of $CD4^+$ cells in cultures stimulated with CD80+4-1BBL+IL-12 to levels similar to CD80+4-1BBL stimulated cultures, without an associated decrease in expansion of these cultures (Figure 3-5), this would suggest that there is a positive effect on the expansion or survival of $CD4^+$ T cells. As IFNγ production is reduced in these cultures (as shown in Figure 3-5) to levels similar to CD80+4-1BBL stimulated cultures, this may suggest that the production of high levels of IFNγ is suppressing or having a negative effect on the $CD4^+$ T cell population, contributing to the predominant expansion of $CD8^+$ T cells.

3.2 Ovarian cancer patient responses to OKT-3 with co-stimulation

3.2.1 Introduction

Ovarian cancer is highly prevalent with a lifetime incidence of 1/50 in the UK and is the leading cause of death from gynaecological cancers. Patient five year survival is low and recurrence is common. However the infiltration of lymphocytes to tumour has been shown to correlate with better prognosis and survival. There is therefore a requirement for new and novel treatment for ovarian cancer and a rational for the investigation of immunotherapy strategies.

I was therefore keen to investigate the ability of our system to allow the expansion of T cells from ovarian cancer patients. Tumour associated lymphocytes were isolated from a sample of ascitic fluid and stimulated using our model system. Unfortunately these data are preliminary only, due to a lack of further samples.

3.2.2 PBMC expansion

Figure 3-8A shows the expansion of a total TAL population from one ovarian cancer patient in two independent experiments performed in duplicate. As can be seen CD80+4-1BBL$_{A549}$ is able to allow the expansion of TAL in response to OKT-3 stimulation, albeit at a lower level than was generally seen in healthy donors, while the addition of IL-12 to the combination seems to have little impact on expansion.

3.2.3 Cytokine production

The general immunosuppression associated with ovarian cancer, the immunosuppressive environment which may exist within the tumour, and the active recruitment of regulatory T cells to the tumour environment could potentially lead to a lack of effector function, also as the cells isolated from ascetic fluid could be regulatory it was important to ensure that the cells which were being expanded were functional. Again we assayed the production of IFNγ and IL-10. As shown in Figure 3-8B the dual costimulation produced a notable level of IFNγ whereas IL-10 remains virtually undetectable. The addition of IL-12 to the combination enhanced the production of IFNγ as was previously seen, and the levels of IL-10 remain low.

Therefore we can conclude that the combination of CD80+4-1BBL is also capable of expanding a TAL population. As could be expected there seems to be a reduction in the level of expansion of TAL in response to OKT-3 compared with PBMC from healthy donors. This could be for a number of reasons including the age of the donor (57 years old), the general immunosuppression of ovarian cancer patients, or the health of lymphocytes isolated from ascitic fluid, or this may be due to donor variation. Likewise the lack of increase in expansion associated with the addition of IL-12 was reproducible for this donor however it is impossible to draw general conclusions from these data without investigation of TAL from further ovarian cancer patient.

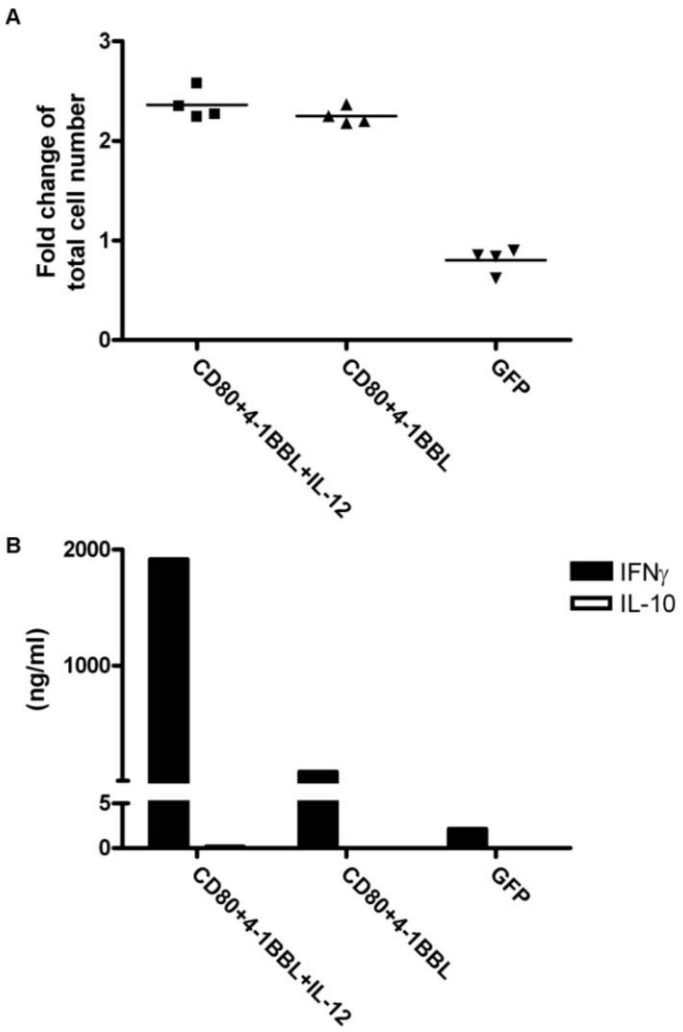

Figure 3-8 Expansion and cytokine production of TAL isolated from an ovarian cancer patient. TAL were isolated from the ascitic fluid of an ovarian cancer patient $1x10^6$ TAL were cultured in wells of a 48 well plate with $1x10^5$ pre-infected A549 cells/well (expressing costimulation as detailed in the figure following infection with adenoviral vectors) and 100ng/ml OKT-3. A. Total TAL number was determined by haemocytometer cell count and fold change compared to the starting population determined, from two independent experiments in duplicate. B. IFNγ and IL-10 concentrations in supernatant of cultures after 5 days of culture were determined by ELISA.

3.3 Costimulation of T cell Response to Viral Peptide

3.3.1 Introduction

Having reconfirmed the superiority of combination of CD80+4-1BBL over individual stimulations to allow the expansion of T cells in response to OKT-3 and shown that the addition of IL-12 to the combination could further enhance the proliferation of T cells and more specifically enhance the proliferation of $CD8^+$ T cells we were keen to move away from the strong generic antigenic stimulus of OKT-3 to a more physiologically relevant antigenic response, with the ultimate goal of assessing TAA specific responses. In order to assess the ability of our chosen costimulatory molecules to induce antigen specific responses we chose to begin by examining viral peptide specific responses. Primarily by using autologous adherent cells which were infected with combinations of adenoviral vectors 24 hours prior to loading with peptide and establishment of co-culture with PBMC for 7 days. Expansion of peptide specific T cells was tracked with tetramer. Primary human fibroblasts were also used in some experiments in order to allow the repeated stimulation of T cells. Fibroblasts were infected with combinations of adenoviral vectors 48 hours prior to loading with the appropriate peptide and use for stimulation of T cells.

3.3.2 Stimulation with viral peptides results in expansion of non-T cells

Initial experiments using adenoviral vector infected, peptide pulsed autologous adherent PBMC to stimulate a mixed population of non adherent PBMC showed that

in cultures stimulated with either 4-1BBL or IL-12, or to a greater degree those cultures combining 4-1BBL+IL-12 showed a large population and presumable expansion of a non-T cell ($CD3^-$) population, which will be discussed in the next chapter. In order to remove any effect the stimulation or proliferation of a non-T cell population may be having on the response of peptide specific T cells, it was decided that a pure T cell population would be used. An untouched T cell population (above 95% T cells) was isolated using a Miltenyi Pan T Cell Isolation Kit II; these data are based on a purified T cell population. As shown in Figure 2-4 adherent PBMC or autologous fibroblasts were infected with adenoviral vectors for 90min in suspension at $1x10^6$ cells/ml in wells of a 48 well plate ($2.5x10^5$ cells/well). 24-48hrs after infection cells were peptide pulsed with 5μg of peptide for 4hrs. Media was then removed and unbound peptide removed by gentle washing with media. $0.5x10^6$ isolated T cells were then added. T cell responses were assessed after 7 days of culture.

3.3.3 Costimulation of adenoviral TDL peptide specific T cells

Initial experiments began by examining the effect of adenovirus delivered costimulation on the proliferation of specific T cells in response to the adenovirus hexon derived TDL peptide.

Autologous adherent cells infected with combinations of adenoviral vectors were peptide pulsed and used to stimulate isolated autologous T cells in a 7 day culture without the use of exogenous cytokines. The results for one donor are shown in Figure 3-9. It can be seen in the case of TDL specific $CD8^+$ T cells that the combination of CD80+4-1BBL_{Mono} with or without IL-12 is able to induce a

significant population of TDL specific T cells compared with mock cultures. Surprisingly stimulation with 4-1BBL$_{Mono}$ alone or in combination with IL-12 is able to produce similar levels of tetramer specific T cells as the combinations with the addition of CD80. In contrast the combination of CD80+IL-12$_{Mono}$ appears to produce the greatest population of TDL specific CD8^{+} T cells. Whereas the population in the Mock$_{mono}$ (peptide pulsed monocytes) stimulated culture had a negligible tetramer specific T cells population. Puzzlingly however the GFP$_{Mono}$ control cultures show a marked population of TDL specific CD8^{+} T cells, only surpassed by the combination of CD80+IL-12$_{Mono}$. The dominance of CD80+IL-12$_{Mono}$ and the significant population of tetramer specific T cells in response to GFP$_{Mono}$ culture were seen in two donors in independent experiments.

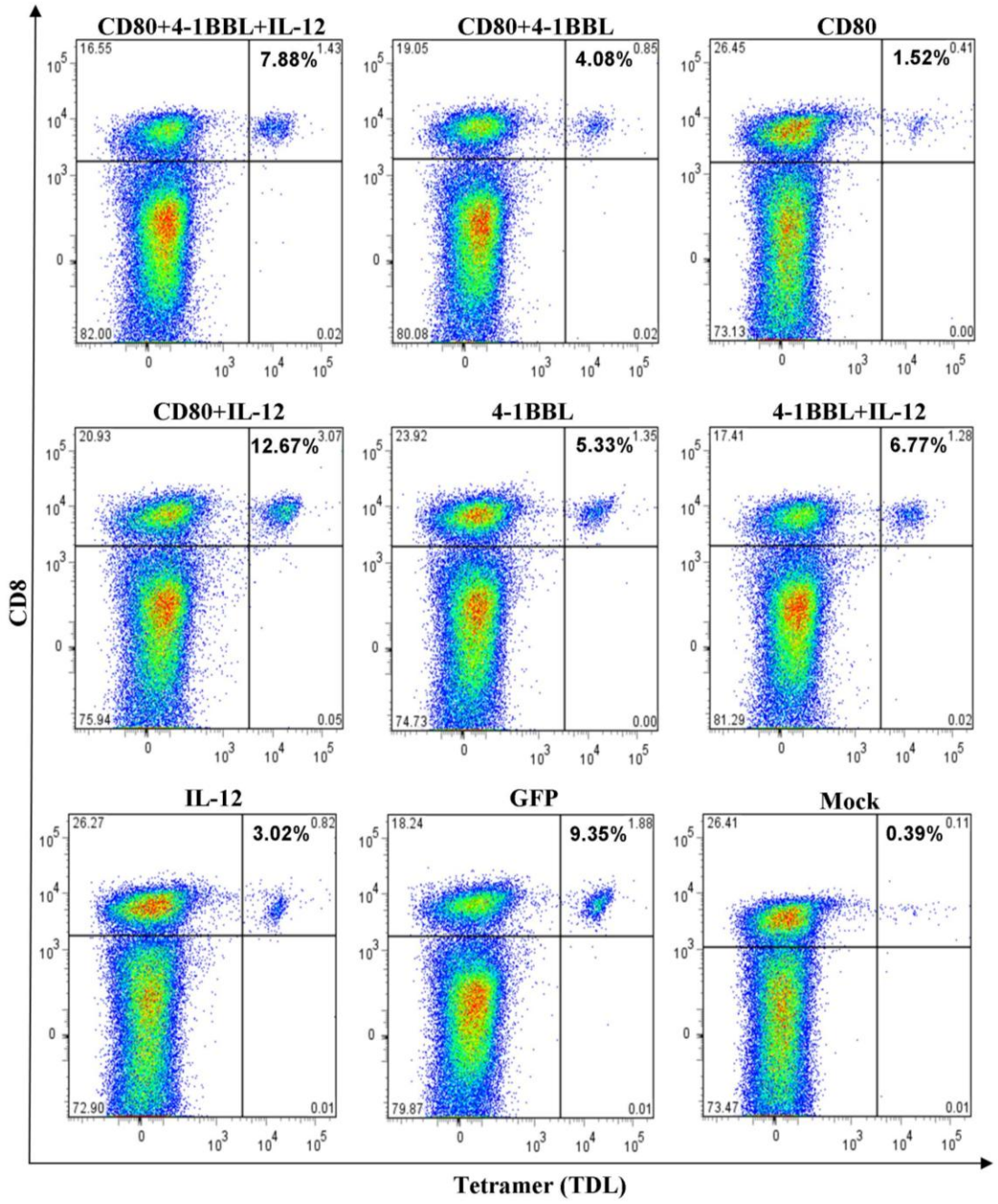

Figure 3-9 Expansion of TDL specific T cells in response to peptide stimulation. $0.5x10^6$ isolated T cells were cultured for 7 days with $2x10^5$ autologous adherent cells which had been pre-infected with appropriate adenoviral vectors 24hrs earlier and pulsed with TDL peptide for 4hrs prior to addition of T cells. Tetramer staining was then performed following 7 days of culture and analysed by flow cytometry, data shown are gated on the T cell lymphocyte population ($CD3^+$). Bold numbers indicate the percentage of $CD8^+$ T cells which are $tetramer^+$.

3.3.4 Costimulation of CMV NLV peptide specific T cells

The presence of a seemingly stimulatory effect of the GFP control virus was startling and inexplicable. In order to examine whether this was possibly due to the adenovirus vector somehow stimulating a better TDL specific recall response than stimulation with combinations of costimulatory molecules I chose to examine an unrelated peptide response.

The chosen epitope was the CMV pp65 derived NLV peptide. As for the TDL stimulated cultures isolated T cells were stimulated with adenovirus vector infected autologous adherent cells for 7 days. In a different pattern to that that seen for TDL specific responses and in contrast to the results of stimulation with OKT-3 it can be seen in Figure 3-10 that stimulation with combinations of costimulatory molecules appear to be resulting in a smaller portion of the population being specific for tetramer. Stimulation with $IL\text{-}12_{Mono}$ or $CD80_{Mono}$ however results in a significant population of tetramer specific $CD8^+$ T cells. As with the results of TDL stimulation a significant population of tetramer specific $CD8^+$ T cells is also present in GFP_{Mono} cultures; in this donors case higher than any of the active costimulatory cultures. A similar pattern of tetramer specific $CD8^+$ T cells was again seen in three donors in independent experiments.

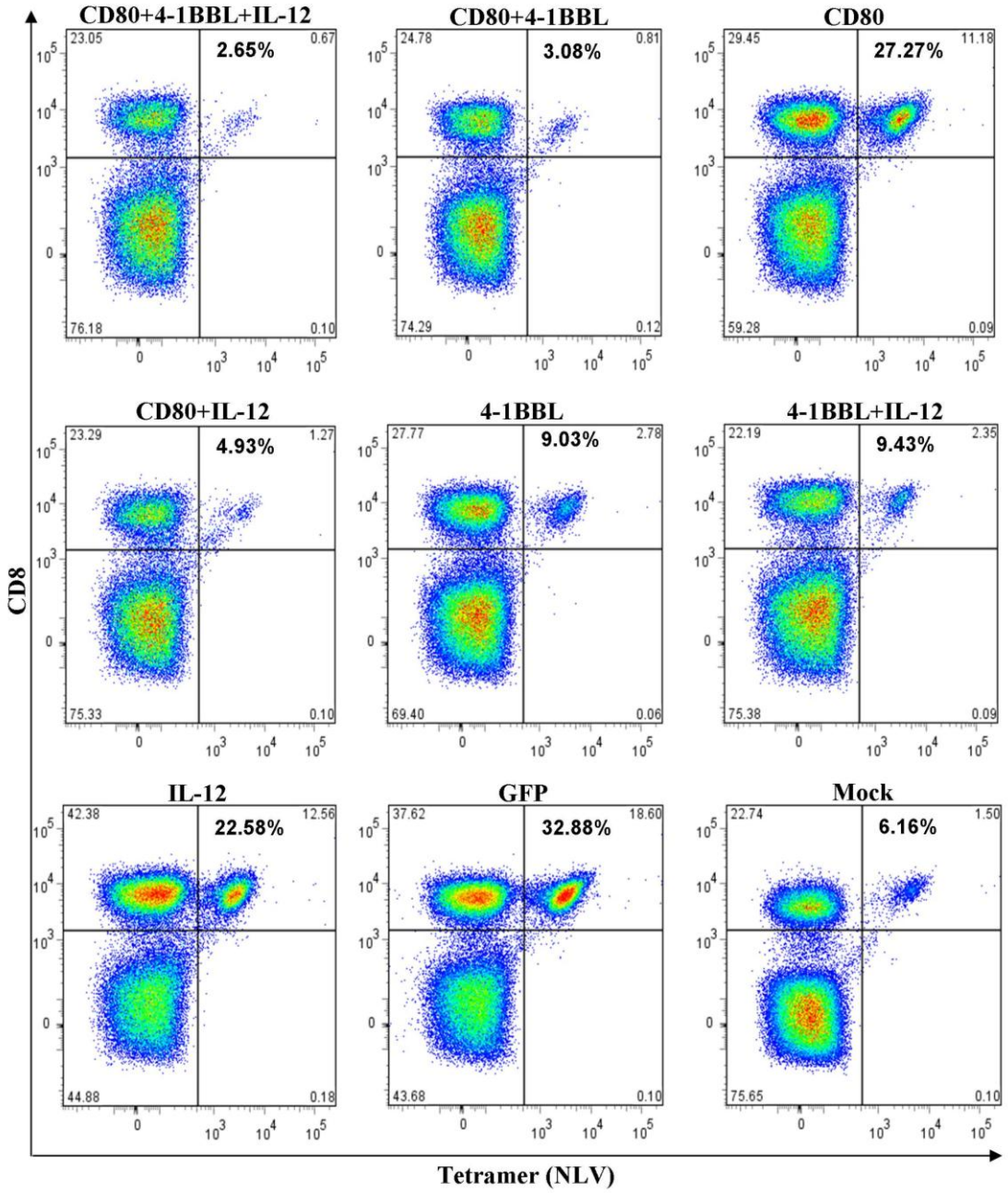

Figure 3-10 Expansion of NLV specific T cells in response to peptide stimulation. $0.5x10^6$ isolated T cells were cultured for 7 days with $2x10^5$ autologous adherent cells which had been pre-infected with appropriate adenoviral vectors 24hrs earlier and pulsed with NLV peptide for 4hrs prior to addition of T cells. Tetramer staining was then performed following 7 days of culture and analysed by flow cytometry, data shown are gated on the T cell lymphocyte population ($CD3^+$). Bold numbers indicate the percentage of $CD8^+$ T cells which are $tetramer^+$.

3.3.5 Costimulation of EBV CLG peptide specific T cells

A third viral response was examined using the EBV LMP2 derived CLG epitope. This was also hoped to provide a starting point for the investigation of the effects of adenovirus directed costimulation on the proliferation of T cells specific for TAA, as EBV is associated with a number of malignancies including Burkitt's lymphoma, other non-Hodgkin's lymphomas, Hodgkin's disease, nasopharyngeal carcinoma and gastric cancer (Hsu and Glaser, 2000).

3.3.5.1 Short term culture

Again isolated T cells were cultured with autologous adherent PBMC infected with combinations of adenoviral vector for 7 days. As is shown in Figure 3-11 stimulation with IL-12_{Mono} resulted in the greatest expansion of tetramer specific $CD8^+$ T cells, while the addition of CD80 does not seem to affect proliferation in response to IL-12 other combinations of costimulatory molecules show reduced levels of tetramer specific $CD8^+$ T cells compared with IL-12_{Mono} or CD80+IL-12_{Mono} stimulation. Again however there is a significant tetramer positive population in GFP_{Mono} stimulated cultures.

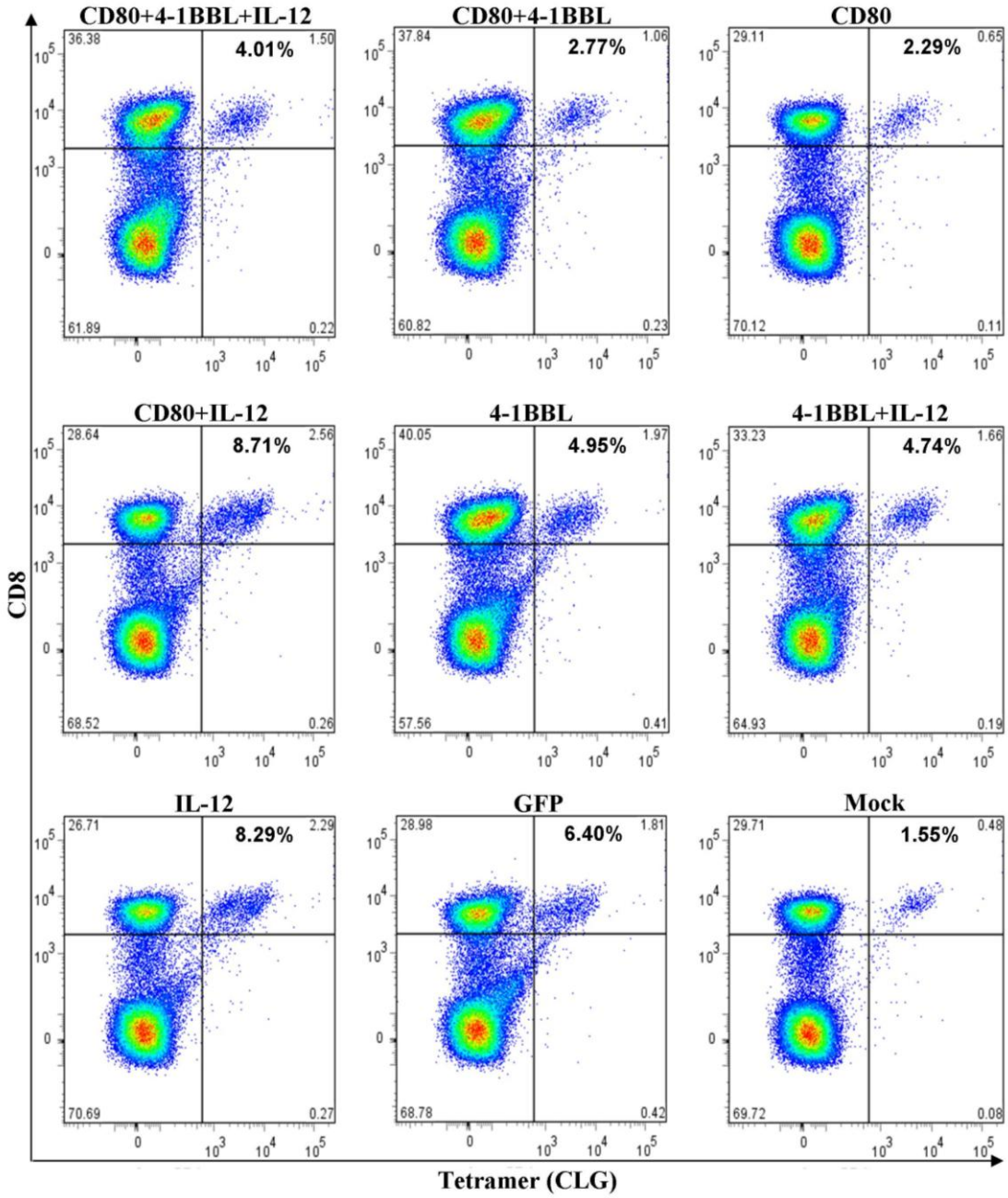

Figure 3-11 Expansion of CLG specific T cells in response to peptide stimulation. $0.5x10^6$ isolated T cells were cultured for 7 days with $2x10^5$ autologous adherent cells which had been pre-infected with appropriate adenoviral vectors 24hrs earlier and pulsed with CLG peptide for 4hrs prior to addition of T cells. Tetramer staining was then performed following 7 days of culture and analysed by flow cytometry, data shown are gated on the T cell lymphocyte population ($CD3^+$). Bold numbers indicate the percentage of $CD8^+$ T cells which are $tetramer^+$.

3.3.5.2 Long term expansion

In order to test whether the benefit of stimulation with IL-12 alone was transient, only allowing short term proliferation, while a combination of costimulatory molecules may become more important in longer term cultures, cultures were set up using autologous fibroblasts in place of adherent PBMC. The use of fibroblasts allowed repeated restimulation. Isolated T cells were stimulated with virus infected peptide pulsed fibroblasts on day 0 and day 14 of culture with 20U/ml of IL-2 added to cultures after 7 days. This method of stimulation was in accordance with the method used for long term culture of NK cells in section 4.2. The actual number of tetramer specific T cells rather than the proportion of the population which was tetramer specific was determined in these experiments, to allow for any changes in the non-tetramer specific population.

As can be seen in Figure 3-12 there is a benefit of 4-1BBL$_{Fibro}$ culture between day 7 and 14 of stimulation, with the addition of CD80 to 4-1BBL reducing the increase in CLG specific $CD8^+$ T cells, with 4-1BBL$_{Fibro}$ alone or in combination with IL-12 allowing the greatest expansion. Following re-stimulation at day 14 however a significant decrease in the population of 4-1BBL+IL-12 $_{Fibro}$ or 4-1BBL $_{Fibro}$ stimulated T cells can be seen with the exclusion of CD80+4-1BBL $_{Fibro}$ stimulation which shows a marginal increase in the tetramer specific population. However again even in long term culture there is a baffling increase in tetramer specific T cells in the GFP$_{Fibro}$ stimulated cultures which continues until day 21.

Alongside the perplexing increase in tetramer specific T cells in response to Fibro$_{GFP}$ stimulation it was also noted that despite initial isolation of T cells prior to culture

there was a significant population of non T cells becoming apparent in longer term proliferation (Figure 3-13).

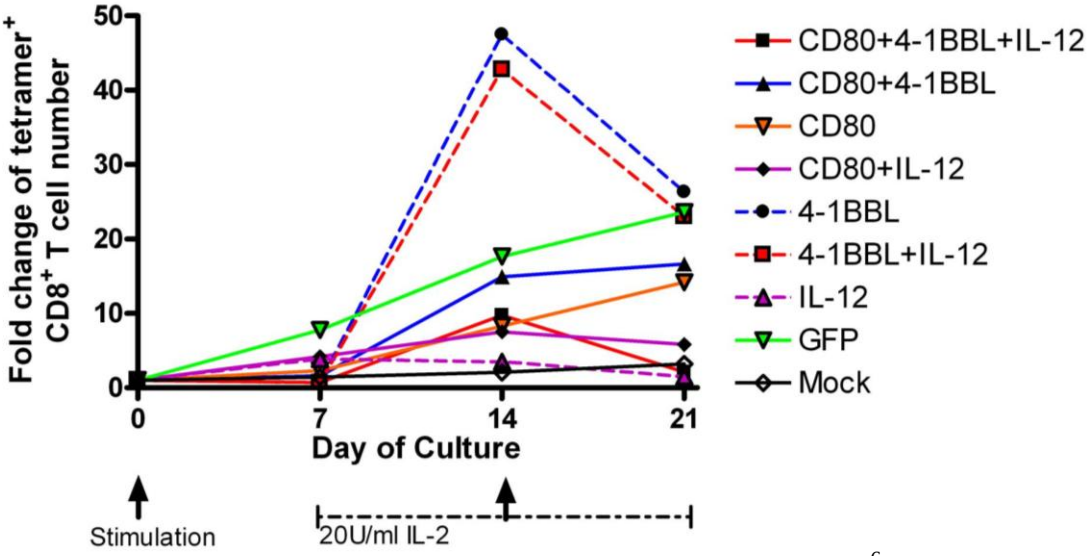

Figure 3-12 Long term expansion of CLG specific T cells. $0.5x10^6$ isolated T cells were stimulated with $2x10^5$ autologous fibroblasts which had been infected with appropriate adenoviral vectors 48hrs earlier and pulsed with CLG peptide for 4hrs prior to stimulation of T cells at days 0 and day 14. Cultures were supplemented with 20U/ml IL-2 from day 7. Tetramer staining and haemocytometer counts were performed at days 0, 7, 14 and 21 and the total CLG tetramer specific $CD8^+$ T cell population calculated, and expansion determined relative to the starting population.

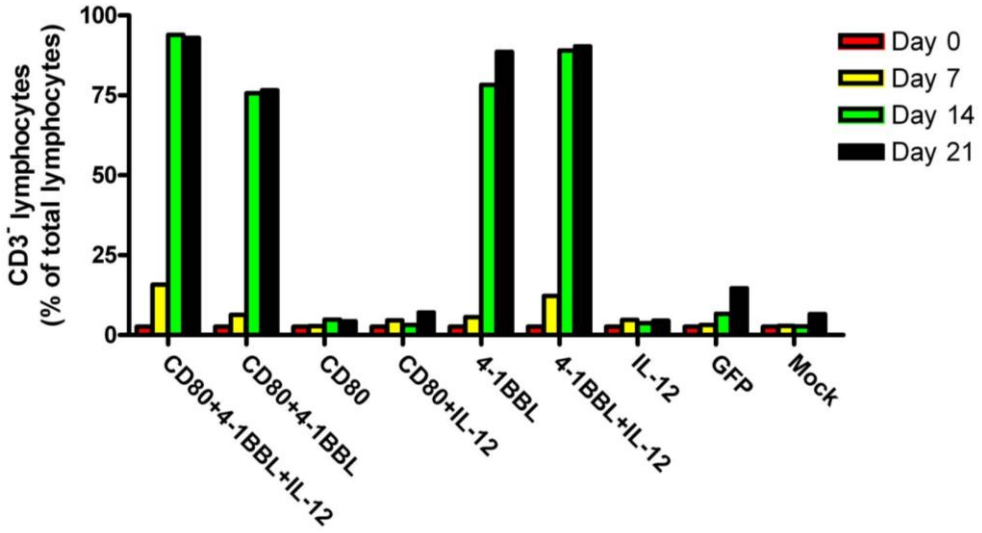

Figure 3-13 Non T cell population in long term peptide cultures. $0.5x10^6$ isolated T cells were stimulated with $2x10^5$ autologous fibroblasts which had been pre-infected with appropriate adenoviral vectors 48hrs earlier and pulsed with CLG peptide for 4hrs prior to stimulation of T cells at days 0 and day 14. Cultures were supplemented with 20U/ml IL-2 from day 7. The percentages of cells staining $CD3^-$ in cultures was determined by flow cytometry at days 0, 7, 14 and 21 of culture.

These results show that in contrast to initial results using OKT-3 the stimulation of peptide specific responses requires differing costimulatory requirements and may vary on the peptide antigen which is used. There is however a stimulatory effect seen in response to Ad-GFP suggesting adenovirus is able to stimulate T cell expansion. Additionally the presences of small numbers of non T cells in cultures and their expansion during long term culture makes interpretation of long term T cell cultures problematic.

3.3.6 Ad-GFP background

To determine whether the expansion of peptide specific T cells in response to Ad-GFP infected cells was due directly to an effect Ad-GFP was having on the T cells or whether there was a possibility of Ad-GFP having an effect to stimulate the antigen presenting functions of the infected adherent cells, we tested the HLA-A2 restricted peptide NLV response comparing autologous adherent PBMC and OVCAR-3 (which are HLA-$A2^{+}$) for peptide presentation. As can be seen in Figure 3-14 there is expansion in the NLV specific population in both cultures, thus suggesting Ad-GFP is having an effect on the T cell population.

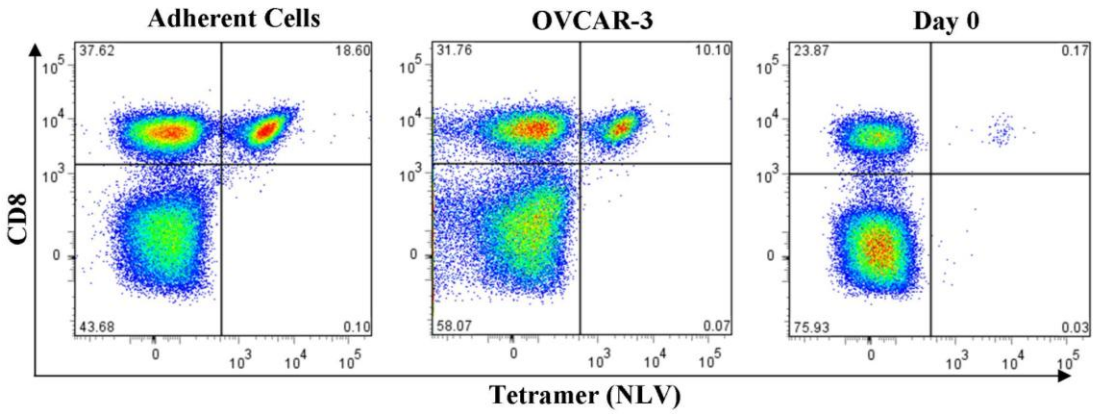

Figure 3-14 Ad-GFP stimulate antigen specific T cells directly. $1x10^5$ OVCAR-3 or $2x10^5$ autologous adherent cells infected with Ad-GFP 48hrs or 24hrs prior to T cell stimulation respectively and pulsed with NLV peptide for 4hrs were used to stimulate $0.5x10^6$ isolated T cells in wells of a 48 well plate. Following culture for 7 days cells were tetramer stained and analysed by flow cytometry. Shown plots are gated on $CD3^+$ lymphocytes.

I also chose to test whether the use of Ad-GFP was causing a non-specific binding of tetramer, to do this I cultured cells with or without peptide. As can be seen in Figure 3-15 there is an increase in the tetramer positive population only in cultures which have received peptide, confirming that this is a true peptide specific response.

Alternative stocks of Ad-GFP and the E1-deleted adenovirus dl312 (lacking exogenous transgene) were tested to rule out any contamination of the Ad-GFP stock with a stimulatory adenoviral vector (Figure 3-15), however all stocks tested resulted in an expansion of tetramer positive cells.

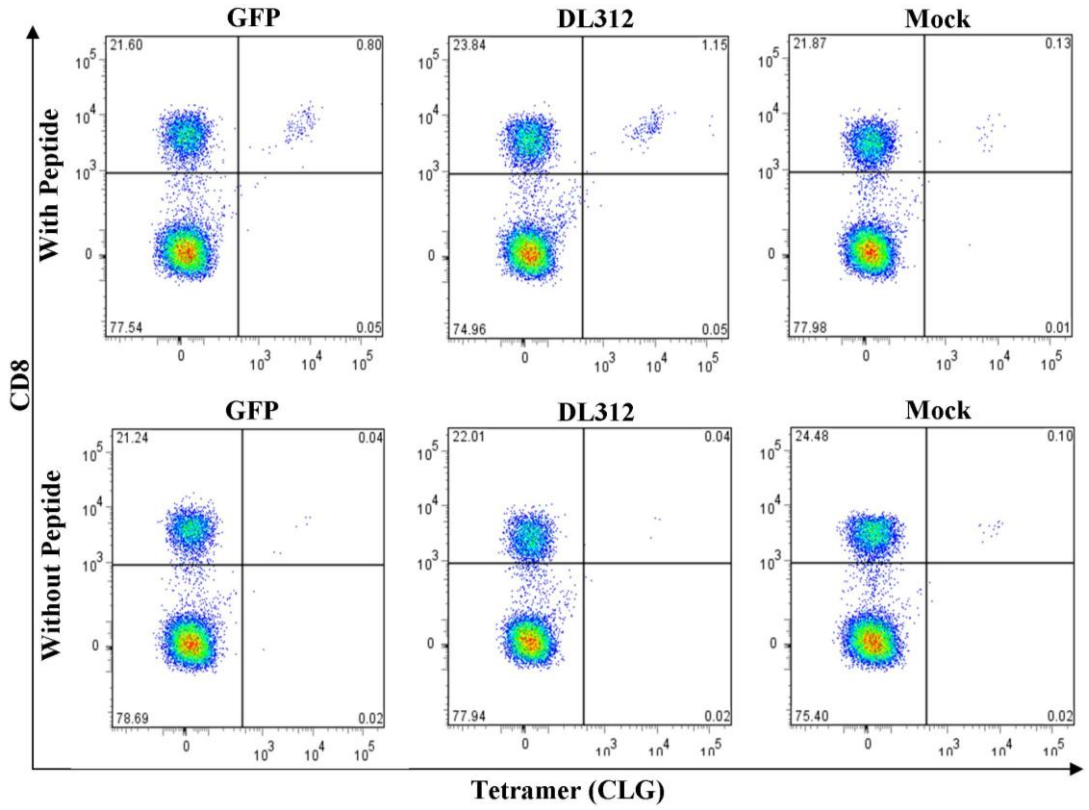

Figure 3-15 Adenoviral vector mediated expansion of T cells is peptide specific. $0.5x10^6$ Isolated T cells were stimulated with $2x10^5$ autologous fibroblasts pre-infected with appropriate adenoviral vectors as detailed in the figure 48hrs prior to stimulation; fibroblasts were pulsed with CLG peptide or vehicle for 4hrs prior to stimulation. Following 7 days of culture the populations of tetramer specific T cells was determined by flow cytometry, shown plots are gated on $CD3^+$ cells.

These data show that the background stimulation of antigen specific T cells is not due to an effect of Ad infection of monocytes stimulating APC maturation, as would also be suggested by fibroblast results in the preceding section. These affects are shown to be peptide specific, ruling out non-specific tetramer binding or effects of GFP. Additionally the effect of control adenoviral vector is not confined to Ad-GFP suggesting this is a general property of Ad infection. The use of dl312 which is effectively E1A deleted (like all Ad vectors used), also provides evidence that this effect is not due to the deletion of E3 in Ad-GFP (and other vectors) as dl312 retains E3 (Jones & Shenk, 1979; Deryckere & Burgert, 1996). Further experiments would be required to determine the mechanisms responsible for the stimulatory effects of adenoviral infection of presenting cells; these may include the use of transwells to determine if this is due to cytokine production by infected cells. These data also suggested if future investigation of T cell stimulation is carried out other vectors such as lentiviral vectors may be required to provide a cleaner model system.

3.3.7 Discussion

I have utilised model systems initially to examine the effects of CD80 and 4-1BBL alone or in combination which was previously shown to be superior to either stimulation alone (Habib-Agahi et al., 2007). I have also added IL-12 to the combination in order to determine whether IL-12 would facilitate an enhancement of effector function. These initial experiments were conducted using OKT-3.

The combination of CD80+4-1BBL was reconfirmed to enhance the proliferation of T cells in short term culture. The addition of IL-12 to the combination further enhanced proliferation of the PBMC population; I have shown that the predominant expansion

occurred in the $CD8^+$ T cell population in response to costimulation with CD80+4-1BBL which is enhanced by the addition of IL-12. Previous data by Habib-Agahi et al. (2007) showed the expansion of $CD4^+$ and $CD8^+$ T cells to be comparable in response to OKT-3; these data are therefore in contrast to the results obtained here. Conversely the data presented here are in accordance with numerous other reports in the literature showing a preference for $CD8^+$ T cell expansion following 4-1BBL stimulation (Dawicki et al., 2004; Shuford et al., 1997).

I have shown substantial IFNγ production in response to CD80+4-1BBL stimulation, which is increased drastically by the addition of IL-12. In a model system also utilising adenoviral vectors to express CD80 and 4-1BBL on autologous cells, Bukczynski et al. (2005) examined HIV peptide responses in uninfected healthy donors and early and late stage HIV infected donors. In this model system IFNγ produced in culture was shown to be inhibitory to the expansion of T cells from healthy donors; the addition of anti-IFNγ in particular augmented expansion of Flu peptide specific $CD8^+$ T cells. Likewise perforin or IFNγ knockout mice have been shown to have increased $CD8^+$ T cell numbers in the contraction phase of the immune response (Badovinac et al., 2000), while there is an increase of 30-40% in the $CD4^+$ T cell population in IFNγ-deficient mice during a model of mycobacterial infection (Dalton et al., 2000). The production of IFNγ by cultured cells therefore could be exerting a negative effect on the proliferation of T cells in this *in vitro* culture system; As I have shown TGF-β reduces the production of IFNγ in response to costimulation, if IFNγ was having a negative effect on the $CD4^+$ T cell population the increased population of $CD4^+$ T cells may be attributed to this. However as overall expansion is not affected an associated negative effect would need to be exerted on the $CD8^+$ T cell

population in this case either by TGF-β directly or through competition with the increased CD4 T cell population. Also as 4-1BBL has been suggested to favour the survival of $CD8^+$ T cells it could be suggested that the predominant expansion of $CD8^+$ T cells is due to stimulation with 4-1BBL overcoming the negative effects exerted by high concentrations of IFNγ in these cultures.

The ability of 4-1BBL to promote the long term proliferation of T cells was documented previously by Habib-Agahi et al. (2007), in a comparable model system utilising OKT-3 or viral peptides (EBV derived RAK, CMV derived ELK and influenza ELR epitopes), however I was unable to show long term expansion of T cells. The inability to expand T cells long term was unfortunate, a number of possibilities have been considered; firstly the presence of replication competent virus within the culture has been suggested, however as I have later shown the long term expansion of NK cells this seems unlikely. Secondly the batch and origin of OKT-3 will have varied, however this is impossible to test. It has been demonstrated that while the initial stimulation of T cells requires to be prolonged to induce activation, a prolonged stimulation of effector T cells results in the induction of apoptosis (Iezzi et al., 1998). Iezzi et al. (1998) also showed costimulation to extend the survival of T cells in response to extended antigenic stimulation. However in these cultures it is likely that costimulation will not be presented at all times due to death of presenting cells, in contrast OKT-3 will continue to be present. Therefore if the OKT-3 I used induced stronger TCR signals possibly this may have a negative effect on the long term expansion and survival of T cells.

In the literature there are also a number of paradoxical or dichotomous results associated with 4-1BBL stimulation of T cells. While 4-1BBL has been documented to enhance T cell proliferation and survival, T cells have also been shown to be hyper-responsive from 4-1BB deficient mouse models in response to protein and peptide antigen stimulation *in vivo* (Lee et al., 2005; Lee et al., 2006; Kwon et al., 2002). There have also been reports in the literature of negative effects of strong or continued 4-1BB stimulation of T cells. Kim et al. (2005) showed in a murine model of GVHD that delivery of agonistic anti-4-1BB antibody reduced T cell numbers with a particularly strong effect on $CD4^+$ T cells through the induction of AICD; Sun et al. (2002) also showed a similar effect in an experimental autoimmune encephalomyelitis model. Interestingly Sun et al. (2002) also showed that the administration of anti-4-1BB did not significantly reduce initial expansion of $CD4^+$ T cells, on the contrary expansion appears to be enhanced. Rather, anti-4-1BB induced AICD of these T cells at later time points.

This raises the question whether 4-1BBL stimulation is having a negative effect on $CD4^+$ T cell expansion or the expansion of peptide specific responses due to the high level ectopic expression provided by adenoviral vectors. Another difference between the model system used by Habib-Agahi et al. (2007) and that used within this thesis is the level of Ad-4-1BBL being higher here. As a higher amount of virus was used this may have an impact on levels of expression which may therefore impact on the outcome of stimulation.

Therefore, while these data conflict in part with previous data by Habib-Agahi et al. (2007), and data in the literature, these data also fit with other data in the literature

which has been published regarding the preferential expansion of $CD8^+$ T cells. In this manner these data and those in the literature show the effects of costimulation to be dynamic and highly complex.

There are numerous reports of the expansion of Tregs in response to costimulation and their generation in response to TGF-β (Chen et al., 2003; Golovina et al., 2008; Perez et al., 2008). It was also therefore of interest to monitor the effect costimulation was having on a putative Treg population. Initial experiments showed very little difference between levels of $CD4^+FoxP3^+$ T cells in the tested culture conditions, the proportion of the $FoxP3^+$ $CD4^+$ T cells observed was also in line with the size of the $CD4^+FoxP3^+$ T cell population expected in the peripheral blood of healthy donors (Liu et al., 2006); therefore further experiments were not carried out. The expression of FoxP3 as a marker of Tregs in the murine system is reliable, however it has become increasingly apparent that the expression of FoxP3 by human T cells is less dependable as a marker of Treg function as activated T cells transiently induce expression of FoxP3 (Allan et al., 2007; Tran et al., 2007) Therefore if further experiment were to be carried out more rigorous Treg phenotyping would need to be employed.

Ultimately it was desired to characterise the response of T cells from ovarian cancer patients toward viral and ideally tumour associated antigens. Initial experiments examined the effect of costimulation on the proliferation of adenovirus TDL specific T cells; these data showed a superior expansion of TDL specific T cells in response to CD80+IL-12 costimulation compared to any other combination. This combination was not tested in initial OKT-3 stimulation experiments, as these initial experiments

were intended to assess the function of IL-12 when added to the combination of CD80+4-1BBL, therefore direct comparisons cannot be draw between these data. The presence of 4-1BBL in the combination of CD80+4-1BBL+IL-12 reduced the TDL specific population. This may be due to an effect on a non-specific population of T cells, or may be directly attributed to a negative effect of strong 4-1BBL stimulation as discussed. More perplexingly there was a significant population of TDL specific T cells seen to arise in the GFP adenovirus control cultures. Given these results it was important to examine the effects of costimulation on further peptide responses. These experiments showed the CMV and EBV derived NLV and CLG epitopes expanded to the greatest extent through costimulation with IL-12 alone, in contrast to TDL specific T cell results. Again cultures stimulated with the control Ad-GFP in place of viruses expressing costimulatory ligands were shown to result in a significant population of peptide specific T cells. Interestingly in the case of NLV the addition of IL-12 to 4-1BBL reduce expansion compared to IL-12 alone. Likewise the addition of either IL-12 or 4-1BBL to CD80 stimulation resulted in a drastically reduced peptide specific T cell population in comparison to CD80 costimulation alone, or other stimulations including Mock cultures. These data are intriguing, and interestingly bear some resemblance to data from Habib-Agahi (unpublished data); RAK and ELK specific T cell responses were shown to be greatest in response to CD80, combining CD80 and 4-1BBL resulted in no advantage or a reduction in specific T cells respectively. While in contrast 4-1BBL alone was able to drive an ELR specific T cell response, while other costimulations were ineffective. Furthermore an effect of stimulation with the Ad-GFP control virus was also shown by Habib-Agahi (unpublished data).

The proliferation of antigen-specific T cell in response to control adenoviral vector infected cultures and differences seen in responses to combination of adenoviral vectors expressed costimulation is puzzling. There is no clear explanation for these effects; however a number of possible effects could be suggested to be mediating in part these results. A non-specific response to viral infection such as the production of type I IFN by the adenoviral infected cells may contribute to the stimulatory effects of Ad-GFP. Type-I interferon have been implicated in the activation, expansion and survival of antigen specific T cells in response to viral infection (Brinkmann et al. 1993; Kolumam et al., 2005; Sikora et al., 2009). In contrast however type-I interferon's have also in some studies been implicated in the suppression of T cell responses, which may be dependent on the activation state of the T cell at the time of IFN stimulation; antigen activated T cells may respond positively to IFN stimulation (Dondi et al., 2003; Gallagher et al., 2009). If T cells were responding to the viral infection of presenting cells this effect should in theory enhance proliferation of all cultures; therefore this alone cannot explain the expansion of antigen specific T cells in response to Ad-GFP. Alongside the stimulatory effects of costimulation negative effects have also been suggested. Besides the negative interaction with CTLA-4, (section 1.1.2.3.2.2) CD80 has been associated with the activation and expansion of regulatory cells (Golovina et al., 2008), which in the case of the NLV response may explain the negative effects of CD80; however this does not explain why these effects are only seen clearly in NLV responses. Zhu et al. (2007) have shown a role for 4-1BBL in the antigen independent homeostatic expansion of T cells. The expansion of T cells in response to 4-1BBL in the absence of antigen may seem counter intuitive given the antigen induced expression of 4-1BB, however Pulle et al. (2006) showed the expression of 4-1BB could be induced by stimulation of memory T cells with IL-

15. Exerting a non-specific activating stimulus driving expansion of a non-specific T cell population would increase competition within the cultures, possibly inhibiting antigen-specific T cell expansion. This is in contrast to type I interferon which would only expand cells stimulated with antigen. IL-12 on the other hand has also been show to act as a growth factor for the proliferation and homeostatic expansion of memory T cells (Kieper et al., 2001). A model of a potential mechanism by which Ad-GFP shows significant T cell stimulatory capacity is shown in Figure 3-16. A definitive explanation of these effects however is not possible.

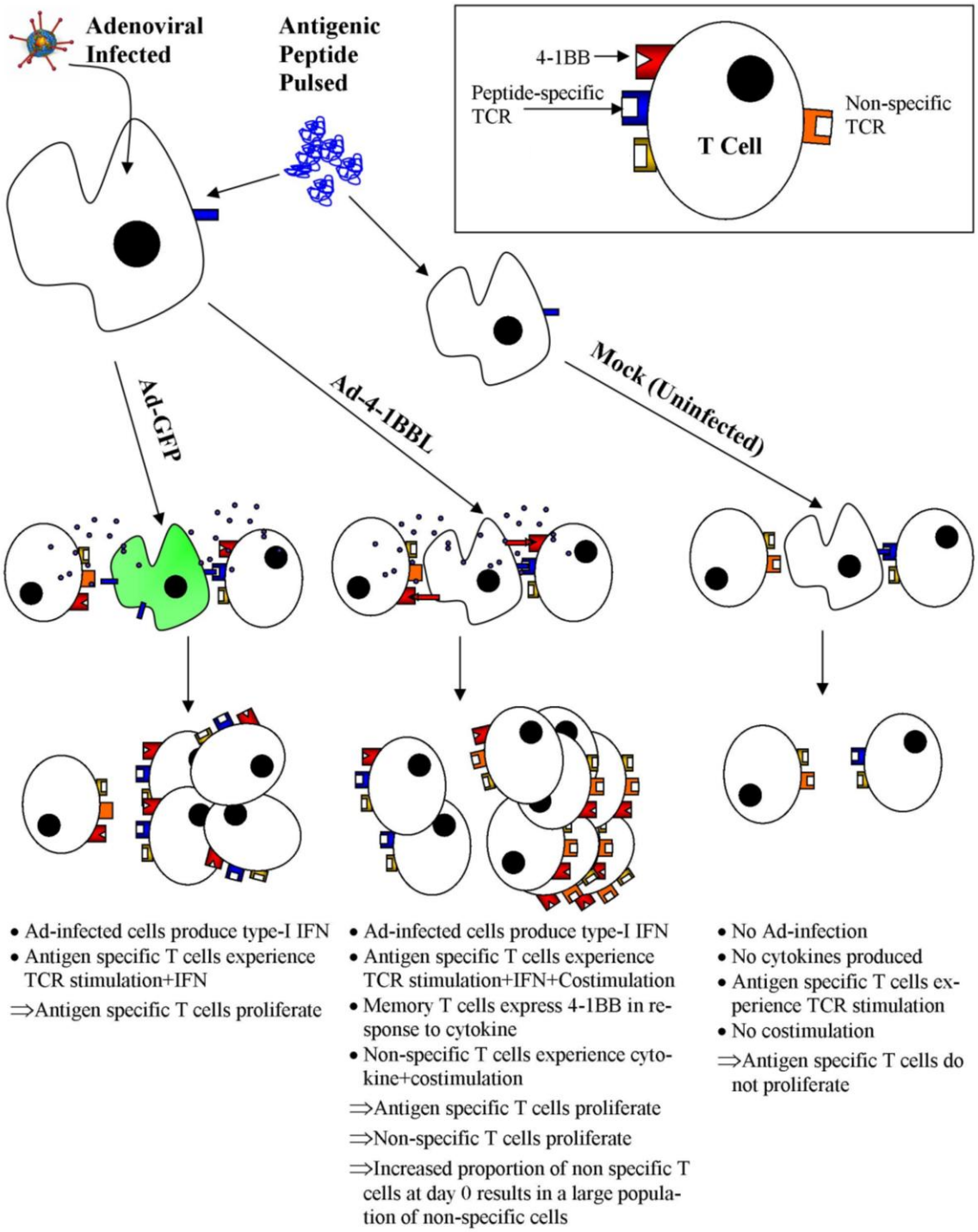

Figure 3-16 Potential mechanisms of the superior Ad-GFP driven expansion of tetramer specific T cells.

Due to the inexplicable but reproducible effects of the GFP control virus, and the presence of a large population of contaminating non-T cells in longer term cultures, interpretation and validity of these experiments was questionable. These uncertainties would also hamper interpretation of further data as such further work would be required to explain these effects. Therefore it was decided that further attempts would not be made with antigen specific T cell culture allowing focus to be moved to the more novel aspect of stimulation of NK cell proliferation.

4 Results: Natural Killer Cell Stimulation

4.1 Short term Natural Killer cell responses to 4-1BBL and IL-12

4.1.1 Introduction

Natural killer cells are a powerful element of the immune response. As effectors, NK cells are known to recognise and kill tumour cells, while they have also been shown to coordinate immune responses. As discussed in chapter 1.2 and below, NK cells have been implicated in the efficacy of numerous *in vivo* animal immunotherapy models and human therapies, therefore understanding the potential effects adenoviral vector delivery of costimulatory molecules may have on the NK cell population is important.

Expression of 4-1BB (CD137) was first documented in murine NK cells by Melero et al. (1998) who went on to show a requirement for NK cells for the anti-tumour efficacy of anti-4-1BB antibody in an *in vivo* murine tumour model. However, while NK cells were required they were not sufficient alone for efficacy. Therefore while 4-1BB is able to induce anti-tumour responses in an NK cell-dependent manner, there was no direct effect on the cytolytic function of stimulated NK cell.

Subsequent experiments combining 4-1BB stimulation with IL-12 stimulation through intratumoural adenoviral delivery, (Martinet et al., 2000) or intratumoural delivery of IL-12 DNA and systemic application of anti-4-1BB (Chen et al., 2000) have shown a synergistic effect for the combination in a colon cancer metastasis model. Through depletion experiments it has been shown that NK cells are also required for the efficacy of this combination in these model systems. These models have also

identified the ability of this combination, not only to allow long term survival of the mice, but also allow the development of an anti-tumour T cell mediated immune response to parental tumour through re-challenge experiments which has a requirement for NK cells. Importantly the combination of intratumoural gene delivery of IL-12 and systemic application of anti-4-1BB has also been shown to be efficacious in poorly immunogenic primary and metastatic melanoma models in which monotherapy was ineffective or showed only marginal therapeutic efficacy (Xu et al., 2004).

In contrast to murine NK cells, little work has focussed on the role of 4-1BBL, or the ability of 4-1BBL to stimulate, human NK cells. Unlike murine NK cells, human NK cells do not express 4-1BB in response to IL-2 stimulation without further activation (e.g. PHA) (Imai et al., 2005; Lin et al., 2008). K562 cells which alone are able to activate NK cells were genetically modified by Imai et al. (2005) to express 4-1BBL and an IL-15R-CD8α (transmembrane domain of CD8) construct. NK cells were cultured in media containing low dose IL-2 (10U/ml) for 7 days. Unmodified K562 were shown to induce a 2.5 fold expansion, while K562 modified to express 4-1BBL or IL-15 resulted in greater expansion of NK cells from some but not all donors. The combination of both resulted in consistently improved expansion, with a median expansion of 1089-fold following 3 weeks of culture. Cultured NK cells were shown to express higher levels of CD56 but similar levels of CD16, KIR and inhibitory receptors compared to the starting population, suggesting that all subsets had expanded, retaining the immunophenotypic diversity of the NK cell pool rather than targeting specific subsets. Lin et al. (2008) were able to show the induction of 4-1BB expression in response to the ligation of CD16 by immobilised Fc fragments (but not

by non-immobilised antibody), expression was transient becoming undetectable after 72hrs. Interestingly expression of 4-1BB was shown to be inversely correlated with CD16 expression.

Despite the knowledge that 4-1BB is expressed by human NK cells in response to activation there is very little work focusing on the role of 4-1BBL or its potential application for the expansion or activation of NK cells for immunotherapy. The work by Imai et al. (2005) and the subsequent work by the same group (Fujisaki et al., 2009b) is the only relevant work which has been performed to investigate the potential of 4-1BBL as a NK cell-directed therapeutic. However, their system is directed toward the *ex vivo* expansion of NK cells. We were therefore keen to investigate the ability of adenovirus delivered 4-1BBL and IL-12 to expand human NK cell, with its potential to allow intratumoural delivery and activation of NK cells.

4.1.2 Natural Killer cells proliferate in response to 4-1BBL and IL-12 in autologous cultures

As reported in the previous chapter (3.3.2) I initially observed an increase in the non-T cell population in culture when using autologous monocytes to deliver 4-1BBL or IL-12 to PBMC. As NK cells are known to express 4-1BB and previous data from mouse and human studies suggested that NK cells are able to proliferate in response to 4-1BBL stimulation, and IL-12 is known to induce proliferation of NK cells (Kobayashi et al., 1989), I examined the presence of NK cells in cultures following 7 day stimulation with autologous monocytes infected with adenovirus vectors.

As can be seen in Figure 4-1 there is a significant population of NK cells ($CD3^-$ $CD56^+$) in cultures stimulated with CD80+4-1BBL+IL-12_{Mono}, or CD80+4-1BBL $_{Mono}$, or 4-1BBL $_{Mono}$ alone. In contrast, stimulation with CD80 $_{Mono}$ alone or GFP $_{Mono}$ results in a very small population of NK cells in these cultures. This pattern is consistent with the pattern of non-T cells seen in culture (data not shown). Note these results are from preliminary experiments following on from the OKT-3 response model, prior to use of an isolated T cell population. Thus only a limited range of costimulatory molecule combinations were used, however these data show that a large population of NK cells were produced in response to some combinations of costimulation which promoted further study.

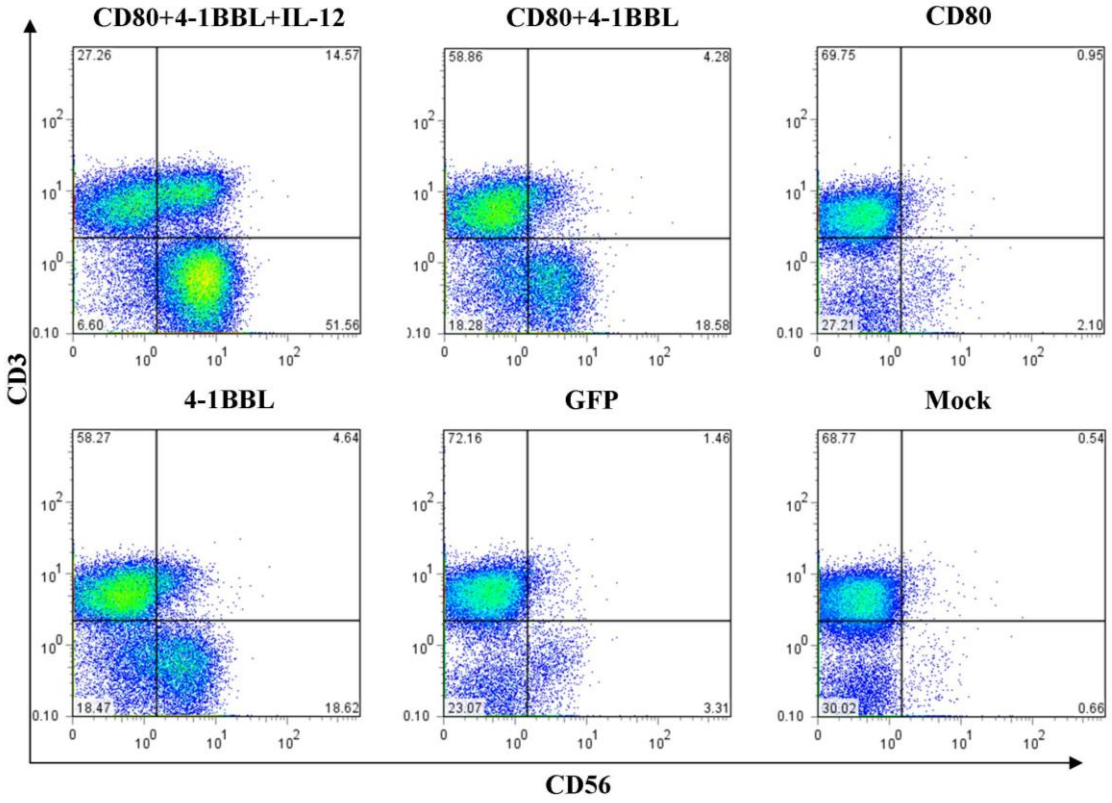

Figure 4-1 NK cell population following culture on autologous adenoviral vector infected adherent PBMC. $2.5x10^5$ adherent PBMC were pre-infected with adenoviral vectors as detailed in the figure 24hrs prior to addition of $0.5x10^6$ autologous adherent cell depleted PBMC. Following culture for 7 days cultures were stained to determine CD3 and CD56 populations and analysed by flow cytometry.

4.1.3 Natural Killer cell proliferation is masked in the presence of OKT-3

Previously we observed the dominance of T cells within cultures stimulated with OKT-3; however in the absence of OKT-3 (i.e. when using peptides) there appeared to be a sizable proliferation of the NK cell population. To assess whether the strong antigenic stimulation of the whole T cell population by the use of OKT-3 was masking the effect of costimulation on the NK cell population, cultures were tested in parallel in the presence or absence of 100ng/ml OKT-3. A549 cells were infected with adenovirus vectors to express CD80+4-1BBL with or without IL-12 and GFP as a negative control, this combination was chosen as NK cells had been shown to proliferate in response to this combination of costimulation while these combinations had also been shown to allow the highest level of T cells proliferation.

As can be seen in Figure 4-2 stimulation with OKT-3 and cells expressing CD80+4-1BBL+IL-12_{A549} and CD80+4-$1BBL_{A549}$ results in T cells dominating the culture as was previously observed. Whereas in the absence of OKT-3 CD80+4-$1BBL_{A549}$ stimulated cultures can be seen to have a sizable non-T cell population, while the addition of IL-12 to the combination markedly skews the proportions of the population towards non-T cells dominating the culture. These cells were also shown to be $CD56^{+}$ (data not shown) and therefore were NK cells. These data show that the presence of OKT-3 in earlier experiments masked or suppressed the proliferation of a non-T cell population, which are NK cells.

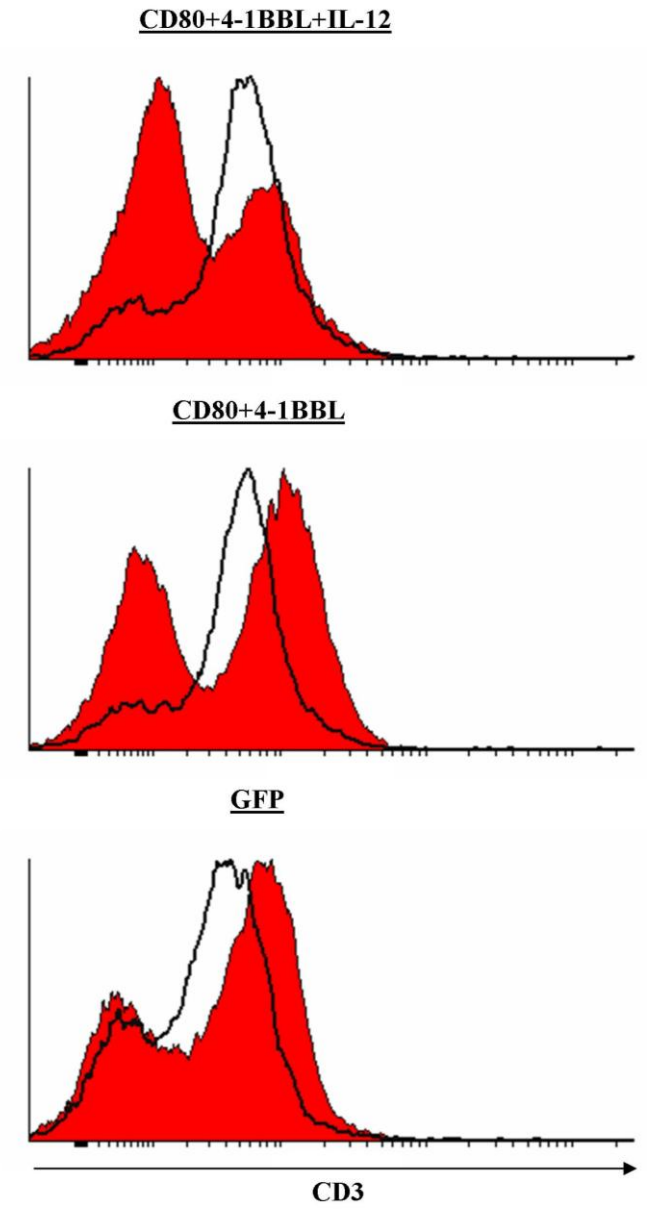

Figure 4-2 NK cell proliferation in the presence and absence of OKT-3. $0.5x10^6$ adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected A549 (with the appropriate adenoviral vectors) in the presence (clear) or absence (red) of 100ng/ml OKT-3. Following 7 day culture CD3 cell populations were analysed by flow cytometry.

4.1.4 Short term proliferation of Natural Killer cells

It now appeared that the proliferation of Natural Killer cells in response to stimulation through Adenoviral vector delivery of 4-1BBL and/or IL-12 was the predominant effect in the absence of the strong antigenic stimulus of OKT-3. In order to further test the ability of our chosen costimulatory molecules to 'costimulate' the proliferation of NK cells, OVCAR-3 were infected, singly or with combinations of Ad-CD80, Ad-4-1BBL and Ad-IL-12, or Ad-GFP (as a control for adenovirus), or OVCAR-3 alone (Mock). After 48h, adherent cell-depleted PBMC were added at a final concentration of $0.5x10^6$/ml, and cultures were assayed after a further seven days.

As shown in Figure 4-3A, following stimulation of adherent cell-depleted PBMC there was little to no proliferation of T cells ($CD3^+CD56^-$) in response to any stimulation. There was however a small increase in the number of cells resembling NKT cells ($CD3^+CD56^+$) in response to costimulatory combinations containing either 4-1BBL or IL-12. The combination of 4-1BBL+IL-12 further enhanced the proliferation of NKT-like cells. Although it can not be ruled out that these cells may be activated T cell, as CD56 may be expressed on T cells when activated (Kelly-Rogers et al., 2006).

While there is a notable proliferation of NKT-like cells in response to co-stimulation with 4-1BBL or IL-12 the predominating effect appears to be the proliferation of Natural Killer cells ($CD3^-CD56^+$), which again is enhanced by the combination of both costimulatory molecules. As there was little stimulation of proliferation of Natural Killer or NKT cells by Ad-CD80 above background subsequent experiments

used only Ad-4-1BBL and Ad-IL-12 alone or in combination, with Ad-GFP (as control for adenovirus), or OVCAR-3 alone (Mock). Following further experiments with further healthy donors, Figure 4-3B shows that the mean expansion of NK cells in response to 4-1BBL+IL-12_{OVCAR} was 27 fold while stimulation with 4-$1BBL_{OVCAR}$ or IL-12_{OVCAR} alone induced a 10 fold and 12 fold expansion respectively. While there is apparent variation between donors in terms of fold change the pattern for all donors tested held true with the proliferation of NK cells in response to the combination of 4-1BBL+IL-12 far exceeding the proliferation of NK cells in response to either alone.

Therefore the stimulation of a PBMC population in the absence of the strong TCR stimuli of OKT-3 results in a significant expansion of NK cells in response to 4-1BBL and/or IL-12. As this combination has shown promise in murine tumour immunotherapy models, in a NK cell dependent manner (Chen et al., 2000; Martinet et al., 2000; Xu et al., 2004), but remains novel for the stimulation of human NK cell responses, and as very little was know about the role or effects of 4-1BBL stimulation of NK cells we were keen to investigate the effects of this combination further.

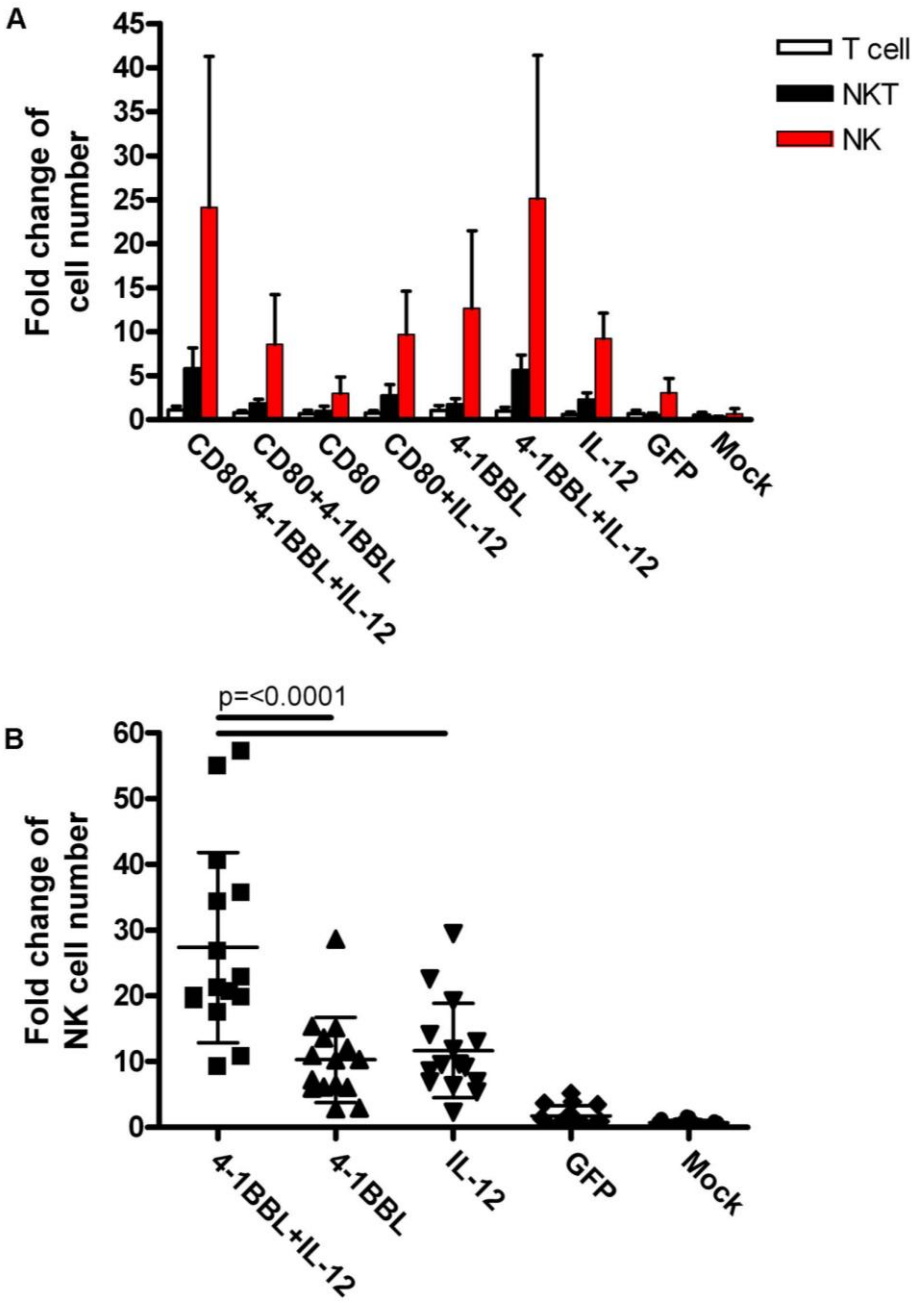

Figure 4-3 Quantification of expansion of T, NKT and NK cells in short term culture with costimulation. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 for 7 days. Cultures were counted by haemocytometer and populations determined by flow cytometry. Lymphocyte populations were defined as T cells $CD3^+CD56^-$, NKT cells $CD3^+CD56^+$ and NK cells $CD3^-CD56^+$. A. Expansion of T, NKT and NK cells from 5 healthy lab donors, expressed as fold change compared to the starting population at day 0. B. Expansion of NK cells expressed as fold change compared to the starting population at day from 14 healthy lab donors. SD, paired t-test.

4.1.5 Functionality of Short term Cultures

While stimulation of NK cells was able to induce their proliferation it was also important to evaluate the functionality of these cells. The two major roles for Natural Killer cells in an immune response centre on their ability to produce cytokines and show cytotoxicity toward sensitive targets. IFNγ is a major cytokine in the host immune response, and is involved in Th-1 polarisation as well as having anti-tumour effects (Bird et al., 1998; Grogan et al., 2001). GM-CSF has a role in the generation of DCs, and is required for the NK cell induced maturation of DCs (Zhang et al., 2007). Both of these cytokines may be produced by NK cells and are of direct functional relevance.

4.1.5.1 Cytokine Production

The ability of the cultures to produce IFNγ and GM-CSF was tested by ELISA; following 7 days of culture, cells were counted and re-suspend at $0.5x10^6$cells/ml in fresh medium. After overnight culture, the medium was tested for the presence of IFNγ and GM-CSF. Figure 4-4A shows that cultures stimulated with either 4-$1BBL_{OVCAR}$ and/or IL-12_{OVCAR} alone produce far higher levels of IFNγ than the GFP_{OVCAR} or $Mock_{OVCAR}$ control, however 4-$1BBL_{OVCAR}$ stimulated cultures produce far lower amounts of IFNγ than those stimulated with IL-12_{OVCAR} alone or in combination with 4-1BBL. Figure 4-4B shows that cultures stimulated with either 4-$1BBL_{OVCAR}$ and/or IL-12_{OVCAR} are superior in their production of GM-CSF, compared to controls. However in contrast to IFNγ production, cultures stimulated with 4-$1BBL_{OVCAR}$ alone or in combination with IL-12 produce higher levels of GM-CSF than IL-12_{OVCAR} stimulated cultures. Therefore while stimulation with either 4-

1BBL or IL-12 alone promotes differential cytokine production by cultures, 4-1BBL+IL-12 in combination is able to promote the most robust overall production of these important cytokines.

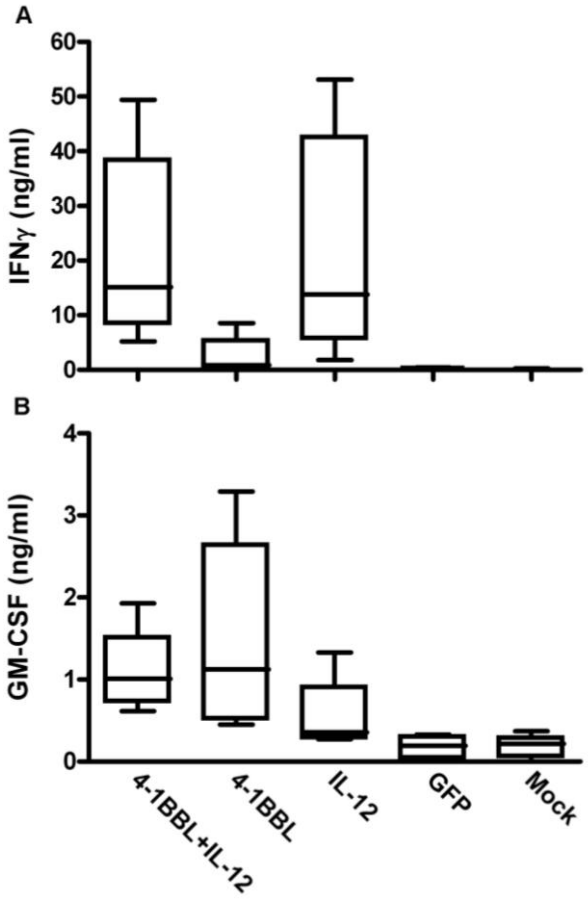

Figure 4-4 Production of IFNγ and GM-CSF by short term cultured cells. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells for 7 days. Cultured cells were then re-suspended at $0.5x10^6$ cells/ml in fresh media and cultured overnight. Concentrations of IFNγ (A) and GMCSF (B) in overnight culture supernatants was then analysed by ELISA. N=6 and N=5 respectively.

While these experiments demonstrate the ability of stimulated cultures as a whole to produce cytokine we could not be certain of the origin of the cytokines produced, therefore in order to assess the producers of IFNγ in culture, intracellular staining was performed. As can be seen in Figure 4-5, IFNγ production is not limited to a single cell type but is induced in the majority of NK cells, NKT-like cells and a subset of T cells following stimulation with either 4-1BBL or IL-12 alone or in combination.

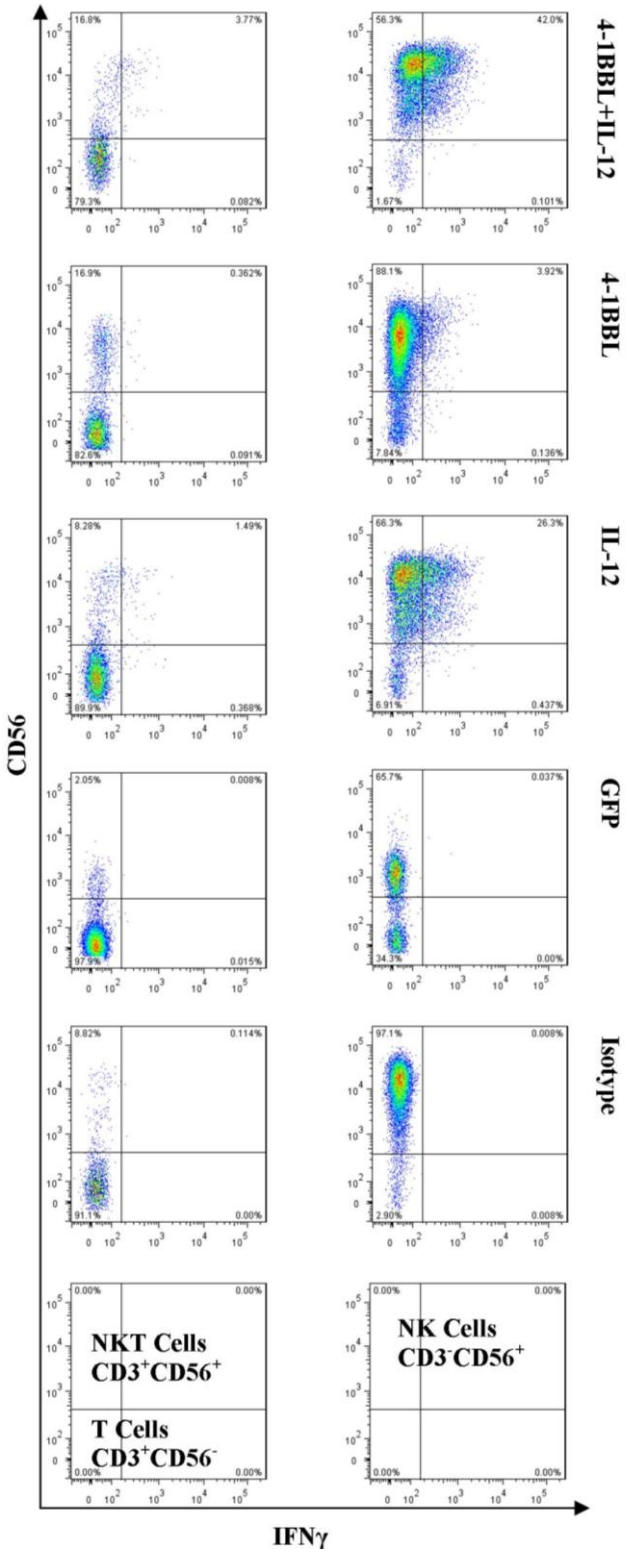

Figure 4-5 Analysis of IFNγ producing populations by intracellular staining. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ OVCAR-3 cells (pre-infected with adenoviral vectors as detailed in right hand legend), for 7 days. Cultured cells were then incubated for 4hr in the presence of 10μg/ml brefeldin A and then assessed by intracellular staining for the production of IFNγ. Left hand panels are gated on $CD3^+$ cells, right hand panel gated on $CD3^-$ cells, staining for CD56 expression defines T cells ($CD3^+CD56^-$), NKT cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$) as detailed in the lower two panels.

4.1.5.2 Cytotoxicity

Cytotoxicity was tested using the NK sensitive cell line K562 in a standard 5 hour chromium release assay. As shown in Figure 4-6 mock stimulated cultures showed virtually no cytotoxicity at any effector:target ratios tested. Interestingly, the GFP_{OVCAR} stimulated cultures showed higher levels of cytotoxicity compared to mock cultures. 4-1BBL stimulated cells however showed clearly increased cytotoxicity at all ratios, in excess of 60% specific lysis at an effector:target ratio of 33:1.

While 4-1BBL+$IL\text{-}12_{OVCAR}$ stimulated cultures show the highest level of cytotoxicity towards K562, IL-12 stimulated cells have a similar level of cytotoxicity to the dual stimulated cultures at the highest ratio tested, however at lower ratios cytotoxicity was slightly reduced.

The testing of NK cell function was of importance to indicate potential efficacy of this combination for tumour immunotherapy. This has become particularly pertinent following the reports of Baessler et al. (2010) of a negative effect of 4-1BBL stimulation of NK cells. Within this model system NK cells are cytotoxic and capable of producing IFNγ.

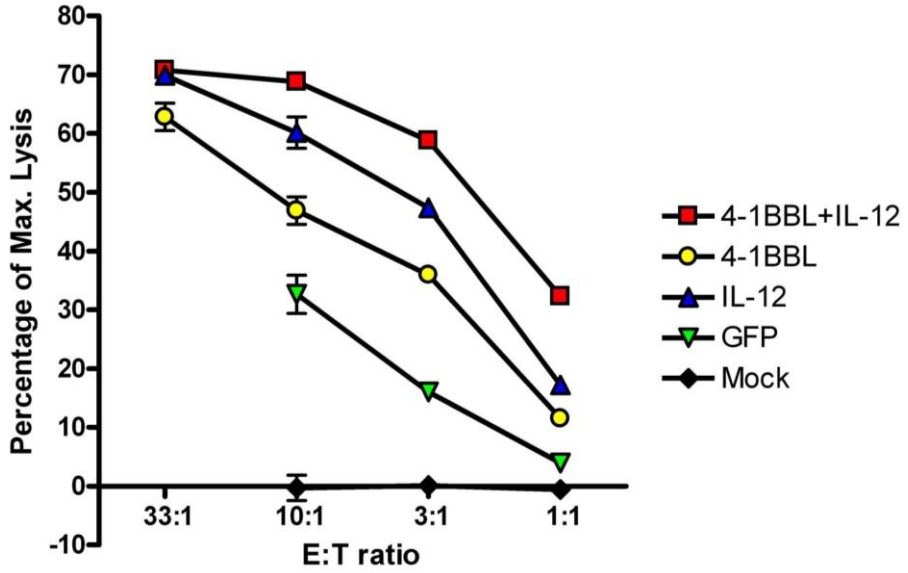

Figure 4-6 Cytotoxicity following short term culture. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells for 7 days. Cultured cells were then assessed for cytotoxicity against K562 targets in a standard 5hr chromium release assay (SD). Data shown are representative of four healthy donors.

4.1.6 Phenotype of Natural Killer cells following short term culture

Previously it had been shown that the expansion of NK cells through the K562-4-1BBL-IL15 expansion system resulted in an expansion of the whole population without a skewing effect in terms of subsets or receptor expression (Imai et al., 2005). Given the functional differences in the subsets of NK cells we wished to elucidate the effect of stimulation with our costimulatory molecules delivered by adenovirus on the subsets of NK cells.

Through staining of CD56 during our initial studies of NK cell proliferation I noted that the majority of the NK population were $CD56^{bright}$ by the seventh day of culture as shown in Figure 4-7A and Figure 4-7C, which is in contrast to proportion of these cells at Day 0. I therefore wished to determine if these cells displayed further phenotypic markers consistent with the $CD56^{bright}$ subset. One of the major hallmarks dividing the $CD56^{dim}$ and $CD56^{bright}$ subsets is high level expression or absence of CD16 respectively. As can be seen in Figure 4-7B and Figure 4-7C the NK cells which have been stimulated with 4-1BBL+IL-12_{OVCAR} are predominantly $CD16^{-}$, which is also the case for cultures stimulated with either 4-1BBL or IL-12 alone, while cells in the control cultures predominantly retain expression of CD16.

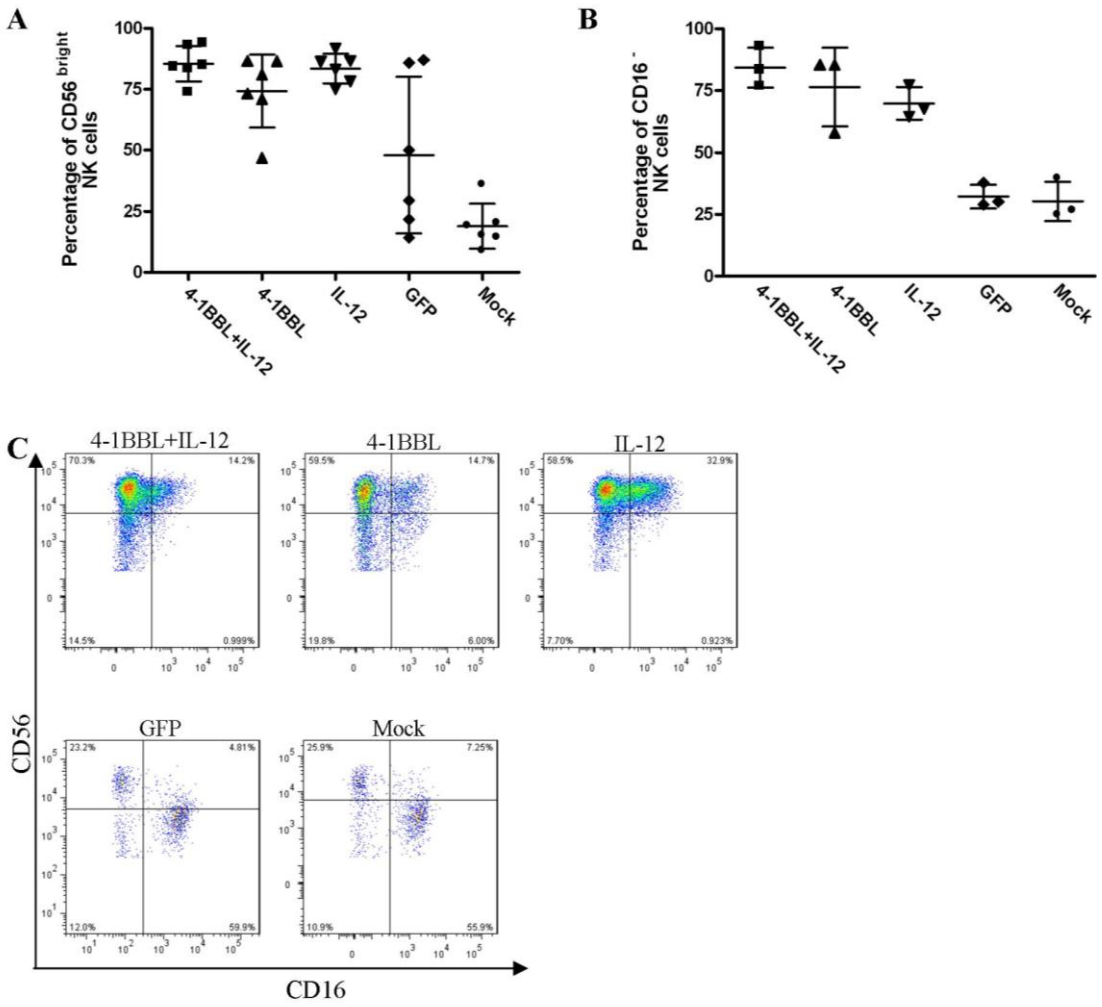

Figure 4-7 Expression of CD56 and CD16 following short term culture. $0.5x10^6$ adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells (infected with adenoviral vectors expressing costimulation as indicated) for 7 days. Following 7 day culture the population of NK cells ($CD3^-CD56^+$) obtaining a $CD56^{bright}$ (A) or CD16 negative (B) phenotype was assessed by flow cytometry. SD and mean are shown. C. shows representative plots of $CD56^{bright}CD16^-$ NK cell populations following culture (gated on NK cells ($CD3^-CD56^+$).

I also assessed the expression of other CD56^{bright} subset markers. As shown in Figure 4-8 there is also a clear acquisition of CD25 expression by stimulated cells consistent with the CD56^{bright} subset, however the majority of NK cells lack the expression of CD27.

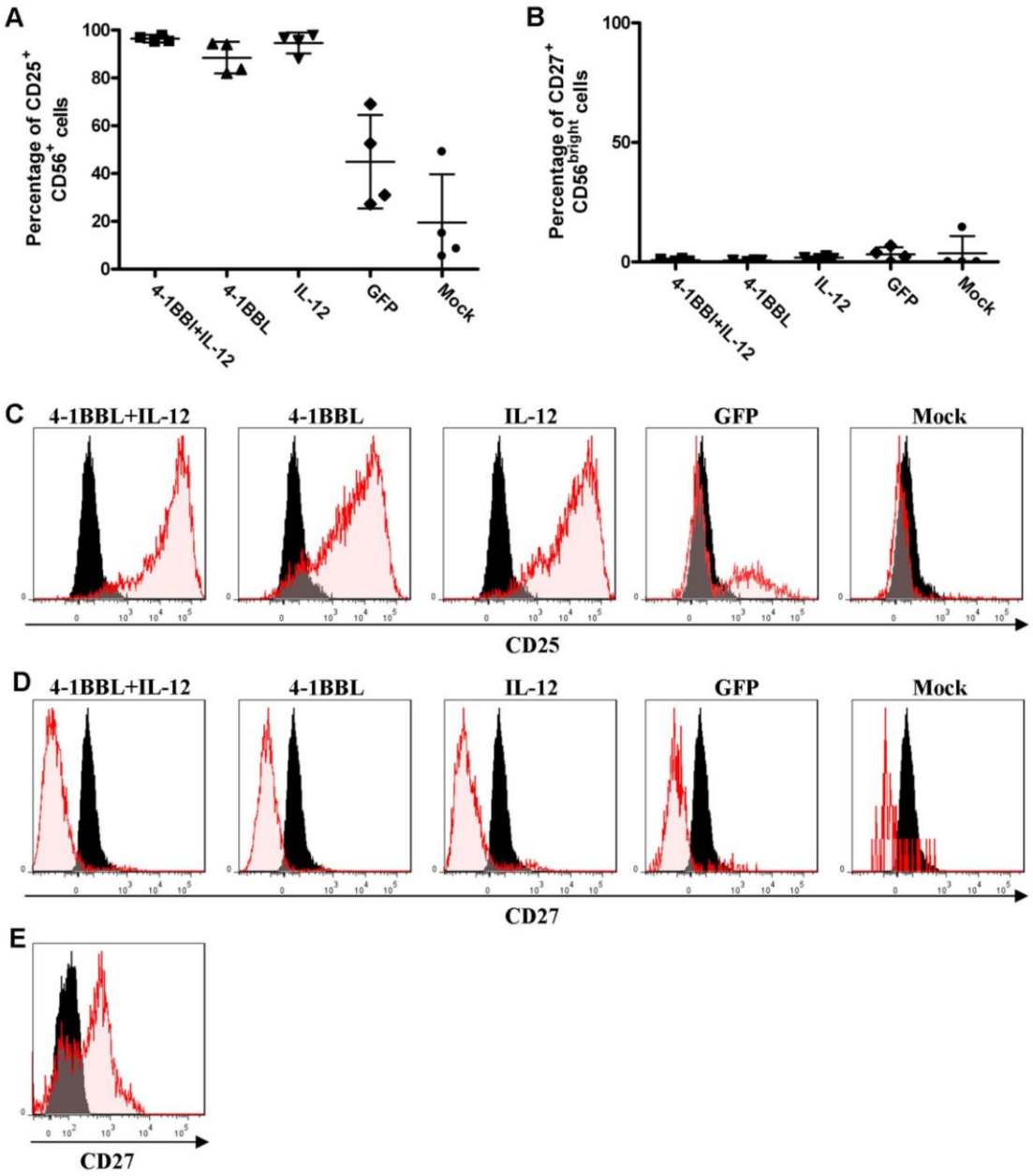

Figure 4-8 Expression of CD25 and CD27 following short term culture. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells (infected with adenoviral vectors expressing costimulation as indicated) for 7 days. Following culture, expression of CD25 by the $CD56^+$ cell population was determined (A); or expression of CD27 analysed on $CD56^{bright}$ lymphocytes (B) by flow cytometry. SD and mean are shown. An example of CD25 (C) and CD27 (D) staining of $CD56^+$ and $CD56^{bright}$ cells respectively are shown. E shows CD27 staining of $CD56^-$ cells. Stained cells are shown shaded red, black fill indicates isotype control staining.

$CD56^{bright}$ and $CD56^{dim}$ NK cells are also reported to have a differential expression of homing markers. I therefore examined the expression of secondary lymphoid homing markers CCR7 and CD62L as shown in Figure 4-9. Although all three of the active stimulatory conditions induced similar percentages of $CCR7^{+}$ and $CD62L^{+}$ cells, marked differences were apparent in the levels of CD62L induced, with the MFI of NK cells stimulated with 4-1BBL_{OVCAR} being substantially lower than induced by IL-12_{OVCAR} alone or in combination with 4-1BBL.

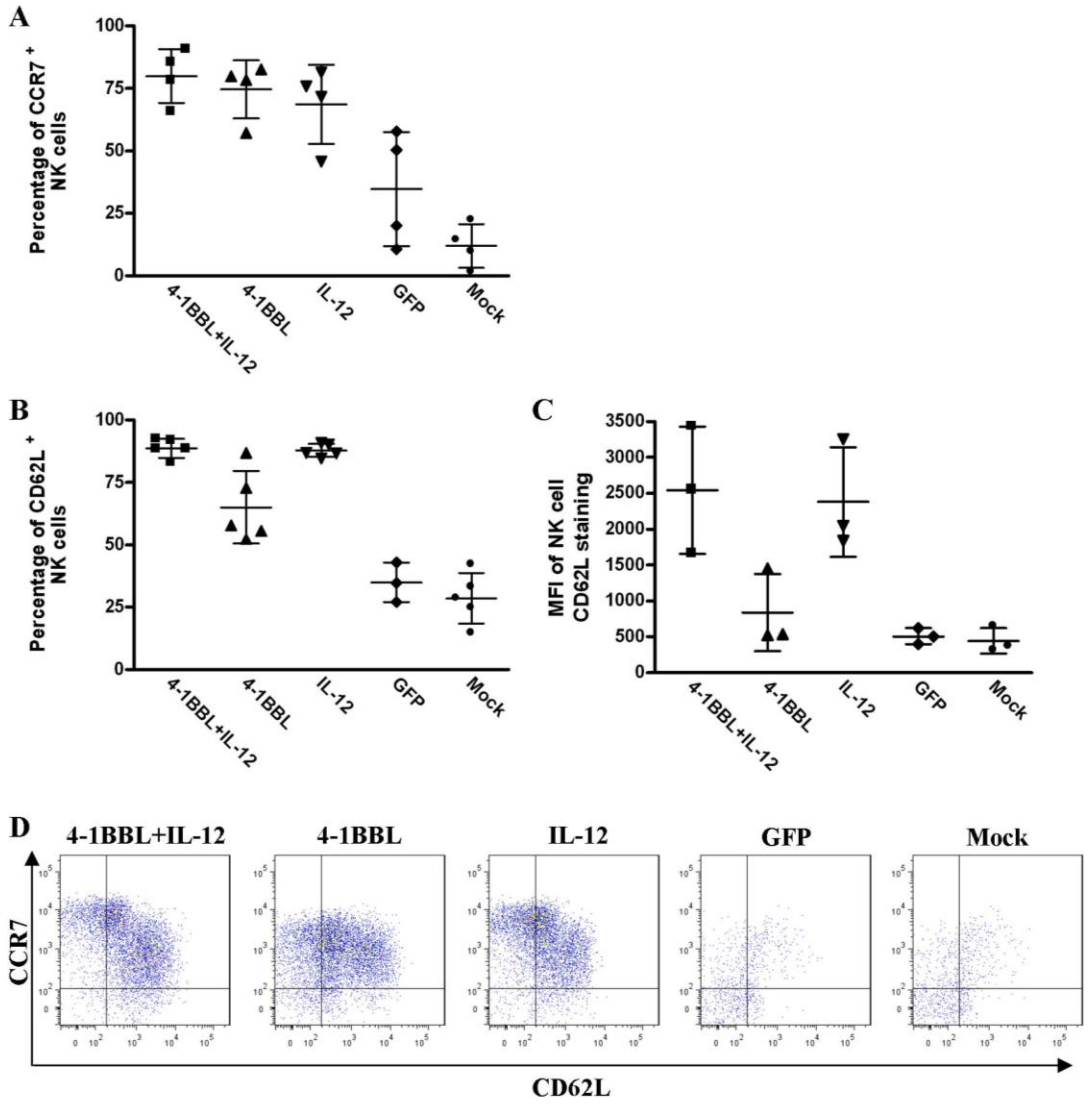

Figure 4-9 Expression of CCR7 and CD62L by short term cultured NK cells. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells (infected with adenoviral vectors expressing costimulation as indicated) for 7 days. Expression of CCR7 (A) and CD62L (B) was determined as a percentage of the NK population ($CD3^-CD56^+$) or MFI of CD62L staining (C). SD and mean are shown. D. shows plots of CCR7 and CD62L staining. Gated on the NK cell population ($CD3^-CD56^+$).

I also stained for the expression of inflammatory site homing markers CCR5 and CXCR3 (Figure 4-10). Again there appears to be a disparity in expression of these molecules between the stimulations. IL-12_{OVCAR} stimulation results in a greater proportion of cells expressing CCR5 than in all other stimulations, while 4-$1BBL_{OVCAR}$ results in no difference compared with controls. Interestingly in 4-1BBL+IL-12_{OVCAR} stimulated cultures 4-1BBL results in an attenuation of the expression of CCR5 induced by IL-12 alone resulting in a significantly lower population of CCR5 expressing cells. The opposite appears to be true for the expression of CXCR3 with the levels of CXCR3 being similar to controls in the IL-12_{OVCAR} stimulated population whereas 4-$1BBL_{OVCAR}$ clearly results in an increased expression of CXCR3, which is partially reversed by the addition of IL-12 as seen in the dual stimulated cultures. As can be seen in Figure 4-10 MFI was used to assess expression of CXCR3 while percentage of the NK population was used for CCR5, this was necessary as staining of CXCR3 did not generally produce a clear positive and negative population rather showing a shift of the population as a whole.

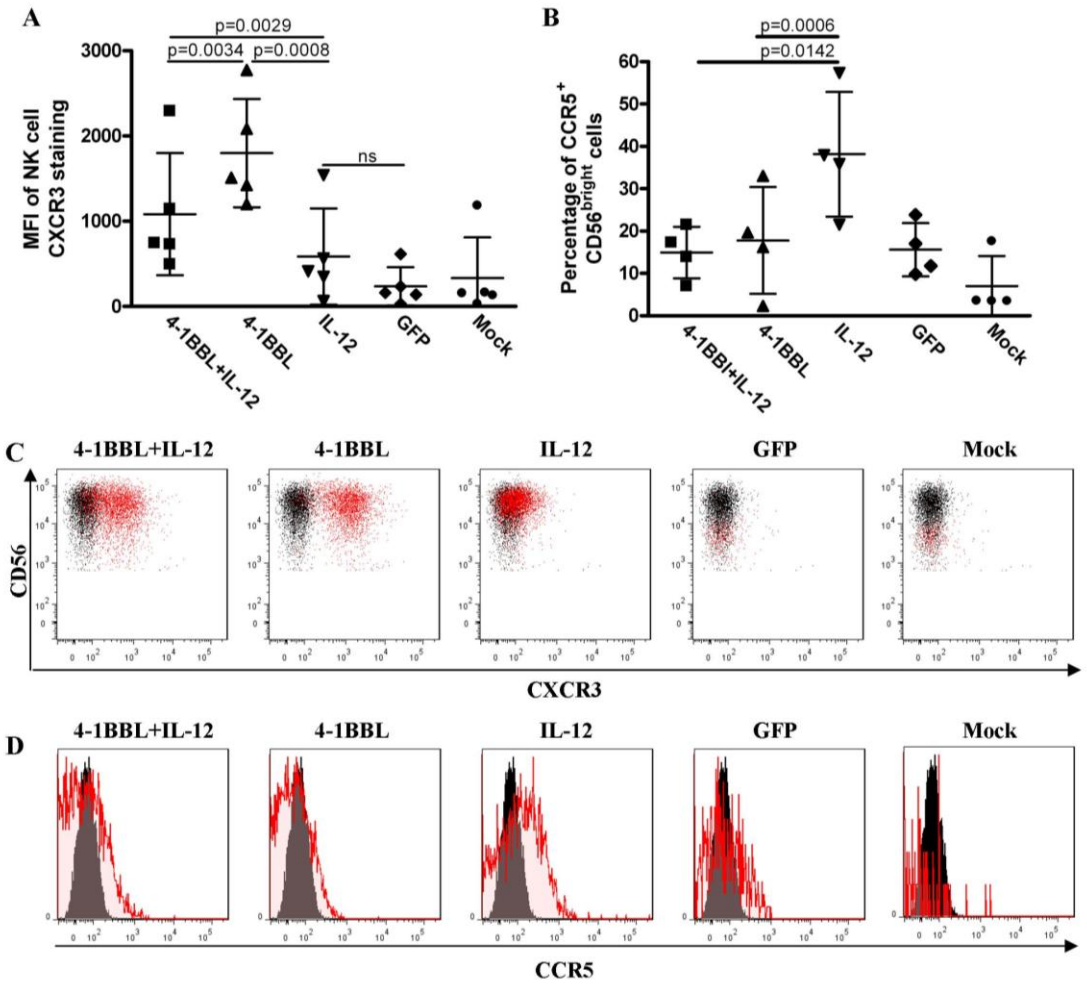

Figure 4-10 Expression of CCR5 and CXCR3 following short term culture. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells (infected with adenoviral vectors expressing costimulation as indicated) for 7 days. Expression of CXCR3 was determined and presented as MFI of the NK population ($CD3^-CD56^+$) (A) which is normalised to the background staining of the 2^o antibody control. Expression of CCR5 (B) by the $CD56^{bright}$ population was also determined by flow cytometry. Mean, SD, paired t-test. C shows a clear example of CXCR3 expression in response to stimulation, (gated on the NK population ($CD3^-CD56^+$)). D shows CCR5 staining of the $CD56^{bright}$ population following culture, red indicates staining, black shows isotype control staining.

These data show that the majority of NK cells following stimulation with the active costimulatory molecules are of the $CD56^{bright}$ subset. The lack of CD27 however is in contrast to other markers of the $CD56^{bright}$ subset which were tested. As CD27 is a marker of effector function this may be reflective of the activation of these cells rather than as a subset marker (Appay et al., 2002; Hayakawa and Smyth, 2006; Tomiyama et al., 2004; Marzo et al., 2007). The expression of homing markers such as CCR7 and CD62L is in accordance with the $CD56^{bright}$ phenotype, and also suggests that *in vivo* cells stimulated with 4-1BBL and/or IL-12 may be able to home to secondary lymphoid tissue, where NK cells may have important roles in the activation of T cell based immune responses (Mailliard et al., 2003; Marcenaro et al., 2005; Garcia-Sastre and Biron, 2006). The expression of CXCR3 by 4-1BBL stimulated cultures and the ability of 4-1BBL to partially retain CXCR3 expression with IL-12 stimulation is a novel finding and as CXCR3 has also been implicated in homing to tumour (Wendel et al 2008), including renal cell carcinoma, potentially important to function (Cozar et al., 2005).

4.1.7 Natural Killer cell proliferation characteristics

4.1.7.1 Natural Killer Proliferation is delayed following stimulation

The lack of CD16 expression by cultured cells and other markers, including the acquisition of a $CD56^{bright}$ state, would suggest that $CD56^{bright}$ NK cells are being produced in response to stimulation. There were therefore two possibilities; either these stimulatory conditions were selectively inducing proliferation of the $CD56^{bright}$ population or a transition of $CD56^{dim}$ NK cell to a $CD56^{bright}$ NK cell was taking place.

To try and identify the subset of NK cells in the original samples which were proliferating in response to 4-1BBL and IL-12, non-adherent PBMC were labelled with CFSE and cultured for 5 days. Cells were stained for CD56 and CFSE dilution analysed on day 1, 3 and 5 after stimulation. However, surprisingly the data did not show proliferation of a specific subset following stimulation. As Figure 4-11 shows, no proliferation took place by day 3 of stimulation rather proliferation was delayed until between day 3 and day 5 of stimulation. In the period between stimulation and proliferation the level of CD56 expression can be seen to be increasing in response to 4-1BBL and IL-12, prior to the production of $CD56^{bright}$ cells following proliferation.

This suggests that a population of $CD56^{dim}$ cells is able to transition to express similar levels of CD56 as $CD56^{bright}$ NK cells. The subsequent proliferation of $CD56^{bright}$ cells may suggest that a population of $CD56^{dim}$ cells can become $CD56^{bright}$ and proliferate. This is interesting as $CD56^{dim}$ cells are suggested to be a mature subset derived from the $CD56^{bright}$ subset which is regarded as immature (Cooper et al., 2001**,** Juelke et al., 2010). Therefore the production of $CD56^{bright}$ cells from $CD56^{dim}$ required further investigation.

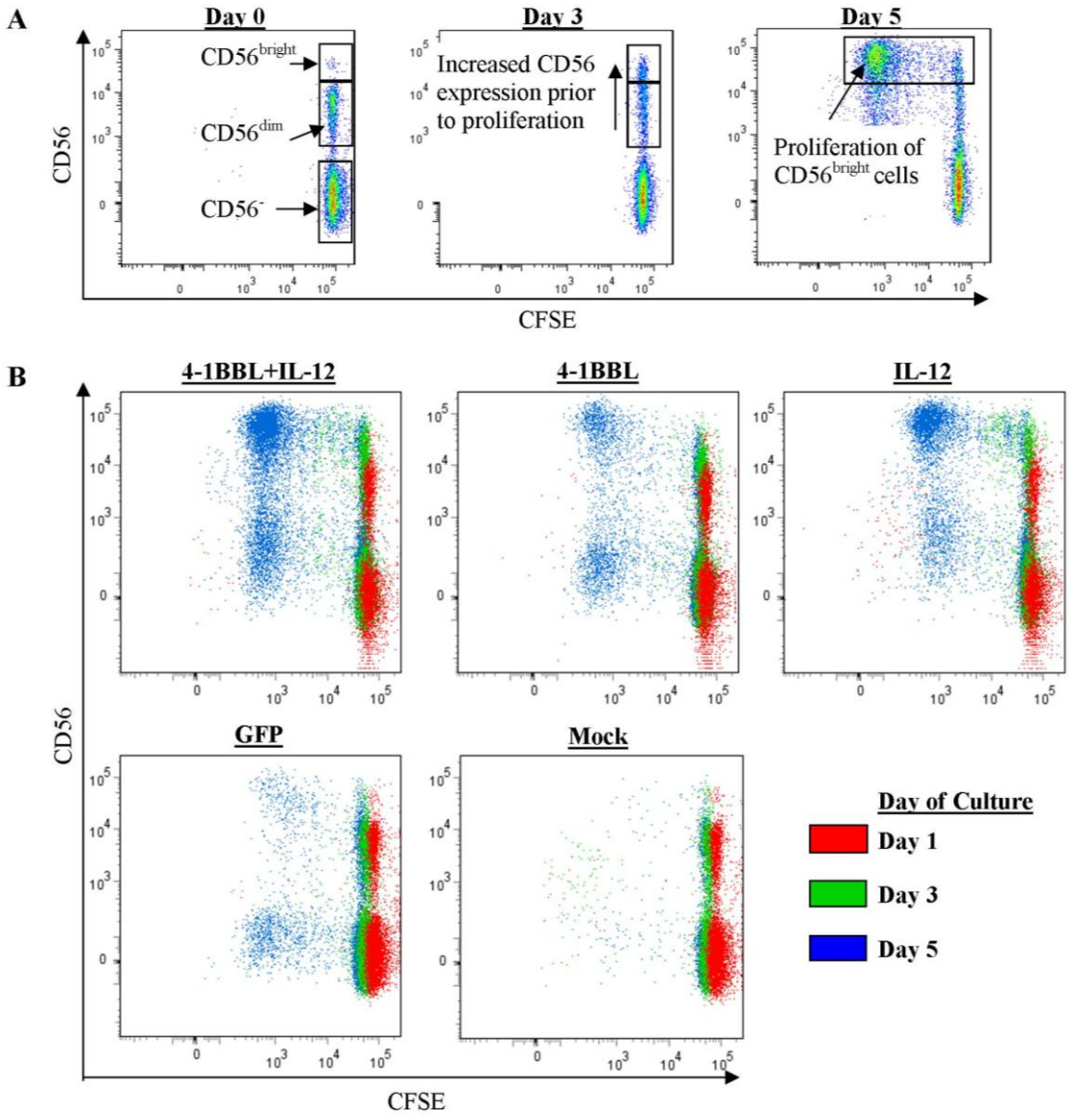

Figure 4-11 Time course of NK cell proliferation by CFSE. $0.5x10^6$ Adherent cell depleted PBMC isolated from a healthy lab donor were labelled with CFSE and stimulated with $1x10^5$ pre-infected OVCAR-3 cells (infected with adenoviral vectors expressing costimulation as indicated). Panel A provides explanation of CD56 expression levels and changes which occur following culture. B shows expression of CD56 and CFSE dilution analysed on day 1 (red), 3 (green) and 5 (blue) of culture by flow cytometry. Shown is CFSE (X axis) and CD56 (Y axis), representative plots of at least 3 donors.

4.1.7.2 Acquisition of a CD56^{bright} phenotype prior to proliferation

In order to assess the phenotypic changes of stimulated NK cells further the dynamics of the NK cell population phenotype during the first five days of culture (i.e. up to the start of proliferation) expression of CD56 and CD16 was analyses at days 1, 3 and 5 of culture. As shown in Figure 4-11A there is a population of cells appearing to transition to a CD56^{bright}CD16^{-} phenotype, firstly by loss of CD16 then increased CD56 expression in response to stimulation with either IL-12±4-1BBL. A significantly increased population of CD56^{bright}CD16^{-} is present following proliferation at day 5 of culture, again showing that the main subset of cells produced in response to 4-1BBL+IL-12 are of the CD56^{bright} subset. Note Mock stimulated cultures did not differ significantly from GFP stimulated cultures therefore are not shown for clarity.

Examining expression of CD56 further reveals that NK cells stimulated with IL-12$_{OVCAR}$ or 4-1BBL+IL-12$_{OVCAR}$ show as a population an increase in the expression of CD56 prior to proliferation of CD56^{bright} cells as seen in Figure 4-11. In contrast to this there appears to be a very modest increase in the levels of CD56 following stimulation with 4-1BBL $_{OVCAR}$, prior to proliferation, but following proliferation, there is a notable increase in CD56 levels (Figure 4-12A and B).

Analysis of the expression of CD16 during the same time period, as shown in Figure 4-12C shows a steady lost of CD16 in response to stimulation with IL-12$_{OVCAR}$ alone or in combination with 4-1BBL, with a notable population of cells lacking CD16 prior to significant proliferation. However with 4-1BBL$_{OVCAR}$ stimulation there is again

little change in CD16 expression until after the onset of proliferation. This again suggests that a population of $CD56^{dim}$ cells is able to transition to a $CD56^{bright}$ NK cell and may proliferate. In order to investigate this further $CD56^{dim}$ cells were separated and CD56 expression and proliferation analysed in subsequent experiments (4.1.7.5).

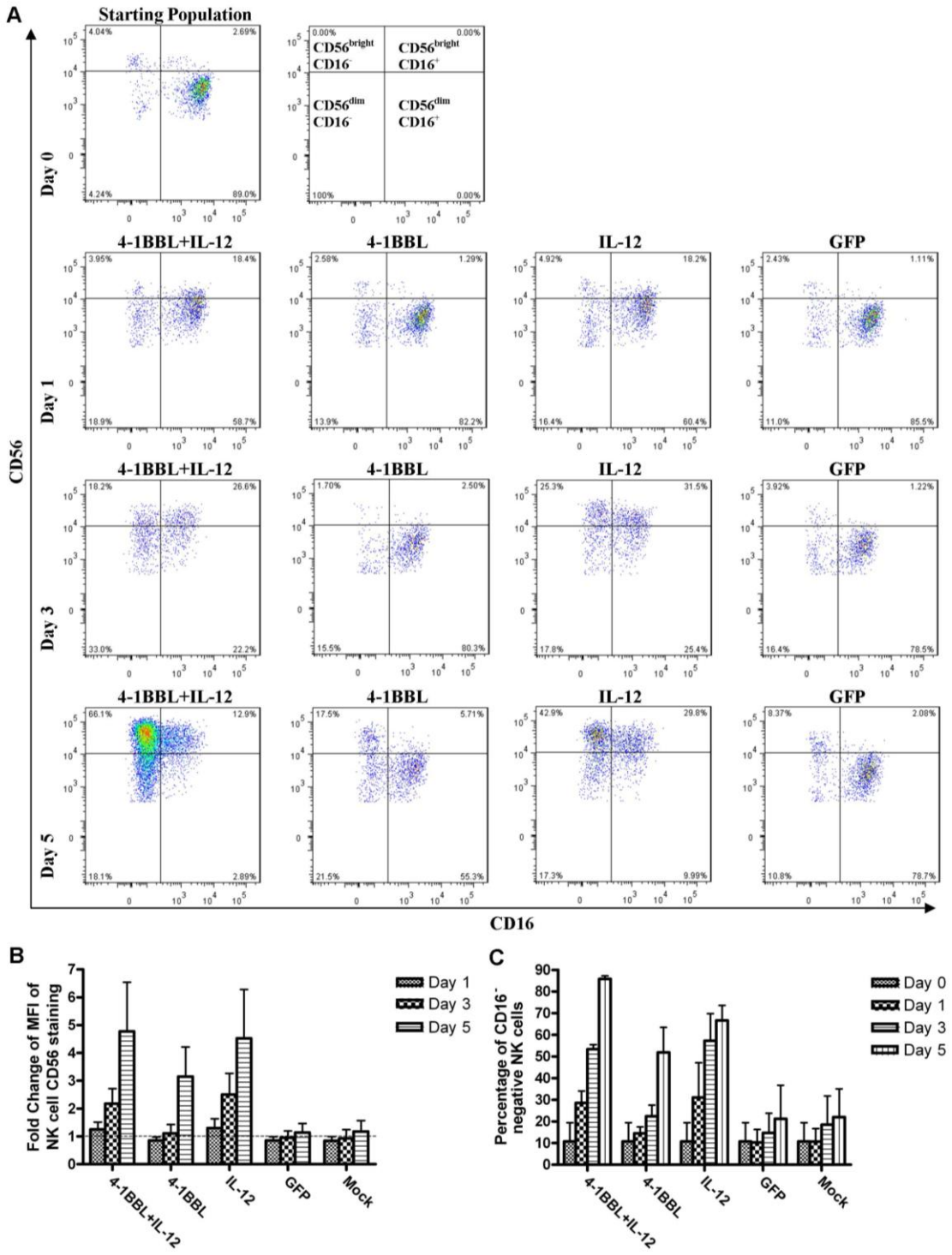

Figure 4-12 Time course of CD56 increase and loss of CD16 by cultured NK cells. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells expressing costimulation as indicated. Expression of CD56 and CD16 was measured at day 0, 1, 3 and 5 of culture by flow cytometry (A), (gated on NK cells ($CD3^-CD56^+$)) upper right panel indicates gates shown. Expression levels of CD56 (B) were also measured as MFI of the NK population ($CD3^-CD56^+$) by flow cytometry and the fold change from the starting population (day 0) determined at day 1, 3 and 5 of culture (N=6, SD). The loss of CD16 expression was also determined at days 1, 3, and 5 of culture (C) as a percentage of the NK population appearing $CD16^-$ by flow cytometry (N=3, SD).

4.1.7.3 Natural Killer cells are rapidly activated

To test whether NK cells were firstly showing a delay in activation and secondly whether NK cells were able to respond to stimulation during the period prior to the proliferation, NK cells were stained for CD25 and 4-1BB expression, both of which are activation markers.

As Figure 4-13A and B shows, CD25 is rapidly induced by 4-1BBL and IL-12 alone or in combination. The dual combination rapidly induces CD25 by day 1 to a level which is maintained on day 3 prior to proliferation, however the dynamics of expression appear to be slower in single stimulation, IL-12_{OVCAR} begins to induce CD25 on day 1 which increases by day 3 to a level similar to the combination. A similar pattern is seen with 4-1BBL_{OVCAR} stimulated cells albeit to a lower level.

Figure 4-13A and C show the dynamics of 4-1BB expression to be similar to expression of CD25 across all stimulations, with induction by day 1 which is increased by day 3. IL-12 stimulation alone or in combination induces similar levels of 4-1BB expression. As with CD25, the percentage of 4-$1BB^{+}$ cells induced by stimulation with 4-1BBL_{OVCAR} is lower than that induced by IL-12_{OVCAR}.

Therefore while NK cells do not proliferate during the initial period of stimulation this is not due to an inability to respond to stimulation. This may suggest that the proliferation of NK cells is delayed due to a requirement to attain a 'proliferative' state rather than the primary effector function of NK cells. As a report has suggested NK cells degranulate prior to 4-1BB expression (Lin et al., 2008) and NK cells 24hrs

post-4-1BBL stimulation show reduced effector function (Baessler et al., 2010), the response to and effects of 4-1BB stimulation require further investigation.

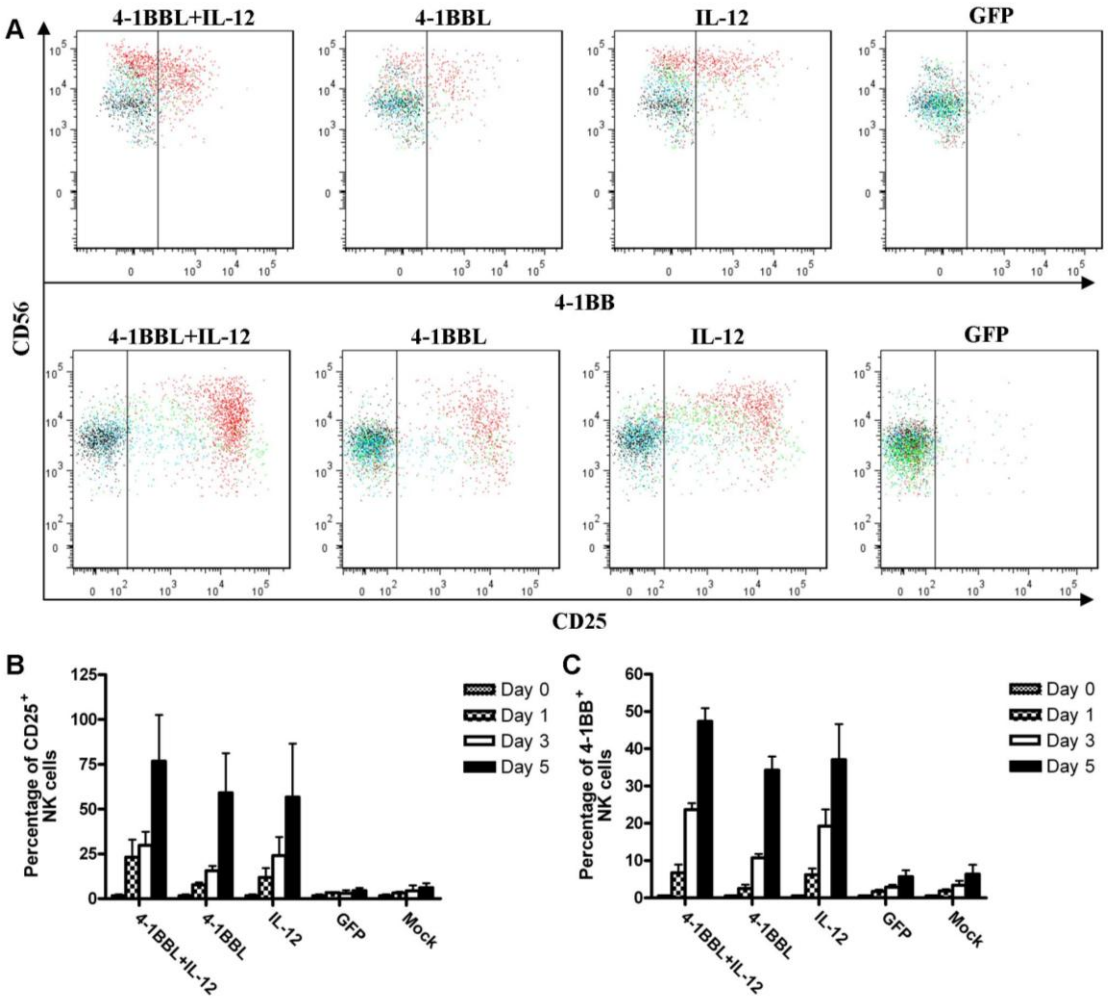

Figure 4-13 Time course of CD25 and 4-1BB expression by cultured NK cells. $0.5x10^6$ Adherent cell depleted PBMC were stimulated with $1x10^5$ pre-infected OVCAR-3 cells (infected with adenoviral vectors expressing costimulation as indicated), Expression of CD25 and 4-1BB was then determined by flow cytometry at day 0 (black), 1 (blue), 3 (green) and 5 (red) of culture (A), plots are gated on NK cells ($CD3^-CD56^+$). The percentage of the NK cell population expressing CD25 (B) or 4-1BB (C) in response to stimulation was also assessed for three healthy lab donors SD.

4.1.7.4 Natural Killer Cells require additional 'Help' in order to respond to 4-1BBL

It appeared paradoxical that 4-1BBL stimulation was able to induce phenotypic changes, including down regulation of CD16, and up regulation of CD56, CD25 and its own receptor 4-1BB, even though at the outset the proportion of cells expressing 4-BB was negligible. Therefore I wondered whether NK cells may express undetectable levels of 4-1BB allowing them to respond to 4-1BBL$_{OVCAR}$ or whether there may be a role for an accessory cell, capable of responding to 4-1BBL$_{OVCAR}$, stimulating the expression of 4-1BB by NK cells.

In order to test this NK cells were separated from PBMC by negative isolation with a Miltenyi kit, purified NK cells or total PBMC were then stimulated with OVCAR-3 infected with Ad-4-1BBL or Ad-IL-12 as a positive control for three days. As can be seen in Figure 4-14A, purified NK cells do not express 4-1BB to levels seen in PBMC cultures following stimulation with 4-1BBL$_{OVCAR}$, and more closely resemble the control culture, suggesting the response of a secondary cell type is required for 4-1BB induction.

As this suggested NK cells were inducing 4-1BB expression through a secondary cell type, possibly by the production of cytokine, I wished to assess whether NK cells were inducing 4-1BB in response to IL-12 stimulation through activation by IL-12 or whether IL-12 was allowing NK cells activated by targets to express 4-1BB. PBMC were therefore stimulated with either OVCAR-3, GFP$_{OVCAR}$, or without additional targets in the presence or absence of 20ng/ml rhIL-12, or IL-12$_{OVCAR}$ as a positive

control. Cells stimulated in the absence of rhIL-12 do not express 4-1BB in response to OVCAR-3 with or without Ad-GFP (as was shown previously), nor is 4-1BB expression induced in the presence of rhIL-12 alone; however NK cells in the presence of both targets and rhIL-12 are able to express 4-1BB (Figure 4-14B).

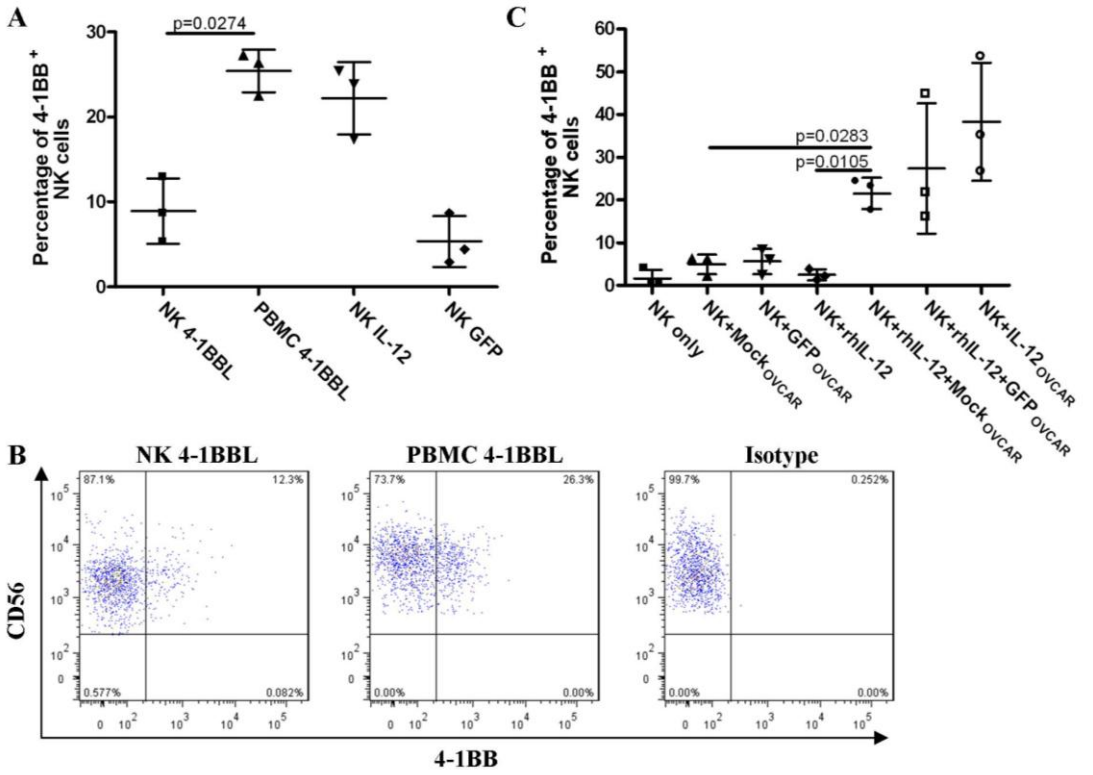

Figure 4-14 4-1BB expression by isolated NK cells requires help. $0.5x10^5$ negatively isolated NK cells (NK) or $0.5x10^6$ adherent cell depleted PBMC (PBMC) were cultured with $1x10^5$ OVCAR-3 cells (pre-infected with the appropriate adenoviral vectors) for 3 days and percentage of the NK cell population ($CD3^-CD56^+$) expressing 4-1BB determined (A and B). Isolated NK cells were also cultured alone or in the presence or absence of recombinant human IL-12 (rhIL-12) or OVCAR-3 cells without adenoviral vector infection ($Mock_{OVCAR}$) or with Ad-IL-12 ($IL\text{-}12_{OVCAR}$) or Ad-GFP ($Ad\text{-}GFP_{OVCAR}$) infection, for 3 days and expression of 4-1BB by the NK cell population analysed by flow cytometry (C). SD, paired t-test.

These data therefore show that in contrast to the induction of 4-1BB on T cells in response to strong TCR stimulation, NK cells require a combination of signals to promote 4-1BB expression. In this system target recognition, likely provided by the allogenic nature of OVCAR-3 cells or potentially ligation of activating receptors by ligands expressed by OVCAR-3, is likely to be providing a 'first signal'. A secondary signal in the form of IL-12 or another cytokine produced by accessory PBMC (possibly IL-15 for example) is then required for full activation and responsiveness to 4-1BBL stimulation as shown in Figure 4-15. This could be an important consideration for the use of 4-1BB stimulation of NK cell for cancer immunotherapy. The actual activating signals which are required for NK cell 4-1BB responsiveness and the role and requirement for different cytokines in the activation of NK cells is an interesting point requiring further investigation.

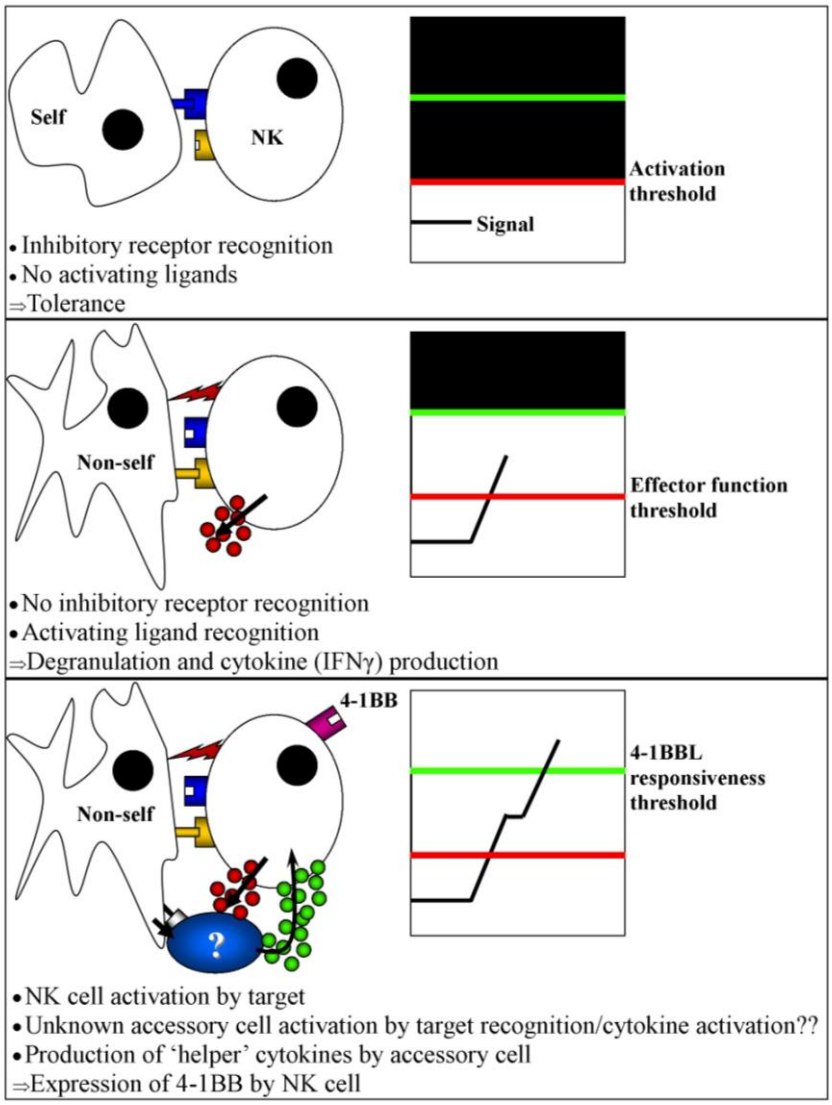

Figure 4-15 Requirements for 4-1BB expression by NK cells. In the absence of sufficient activation signals (either activating ligands or missing self) tolerance is maintained. When sufficient activating signals are achieved NK cells perform effector functions, however further activation is not achieved. Through the recognition of target and provision of 'help' by an unknown accessory cell increased activating signals are achieved and 4-1BB is expressed allowing further activation. In the absence of accessory cells 'help' can be provided by IL-12.

4.1.7.5 Proliferation of CD56^{dim} NK

In order to evaluate the proliferation of the two subsets of NK cells (CD56^{bright} and CD56^{dim}) a Miltenyi CD56^{+}CD16^{+} NK Cell Isolation Kit was used to isolate cells which were classically CD56^{dim} (i.e. CD3^{-}CD56^{dim}CD16^{+}) (Figure 4-16) and also allowed the enrichment of the CD56^{bright}CD16^{-} population. The isolated cells were subsequently CFSE labelled and cultured with OVCAR-3 expressing 4-1BBL+IL-12 or IL-12 alone. OVCAR-3 with 4-1BBL alone was not included as I have shown additional stimulation is required for isolated NK cells to be responsive to 4-1BBL.

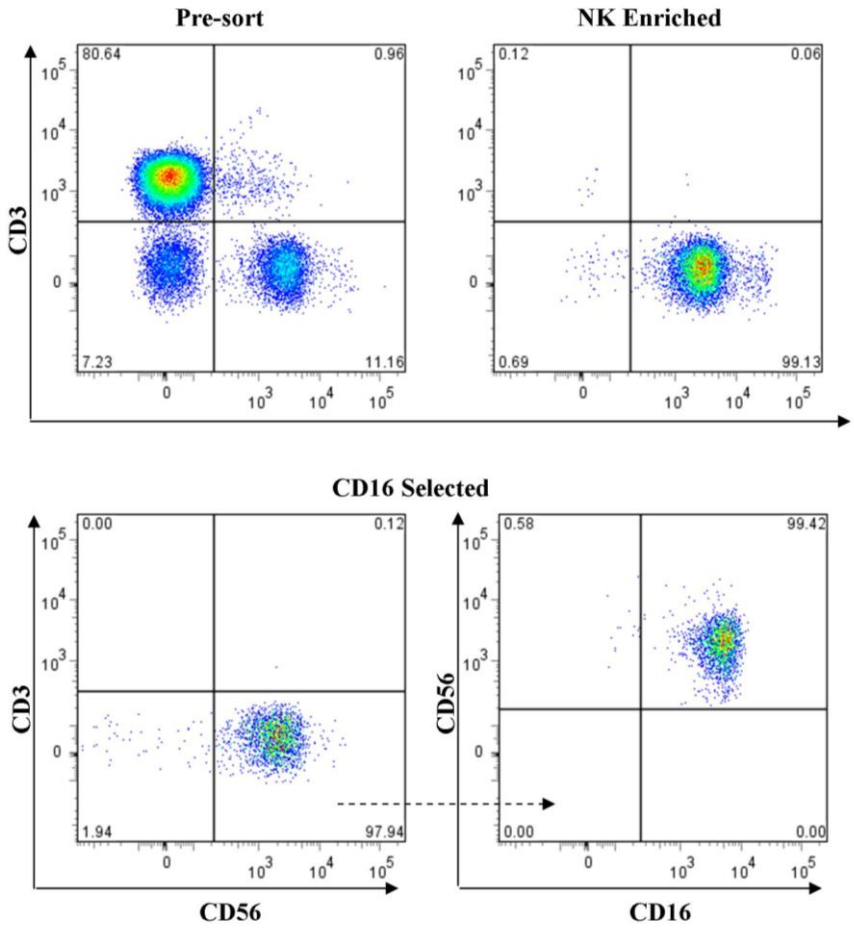

Figure 4-16 Purification of CD56^{dim} NK cells. Using a Miltenyi CD56^{+}CD16^{+} cell isolation kit NK cells were first enriched by negative isolation. Subsequently cells were positively selected by CD16 microbeads with a Miltenyi CD56^{+}CD16^{+} cell isolation kit. Shown are CD3 and CD56 staining of the lymphocyte population and CD16 staining of the NK (CD3^{-}CD56^{+}) population following positive selection of CD16^{+} cells.

I was first interested to assess whether CD56^{dim} cells were able to proliferate in response to IL-12$_{OVCAR}$ with or without 4-1BBL. When NK cells of the CD56^{bright} and CD56^{dim} subset are compared by CFSE the cells dividing in both groups appear to also undergo CFSE dilution (Figure 4-17A). Whether CD56^{dim} cells could transition to a CD56^{bright} state was also examined, as can be seen in Figure 4-17B there is a small minority of CD56^{dim} cells which appear to increase levels of CD56 expression following stimulation with IL-12$_{OVCAR}$ with or without 4-1BBL on the third day of stimulation. Subsequently the proportion of the CD56^{bright} cells increases by the

seventh day of culture with IL-12$_{OVCAR}$ and is markedly increased following stimulation with 4-1BBL+IL-12$_{OVCAR}$.

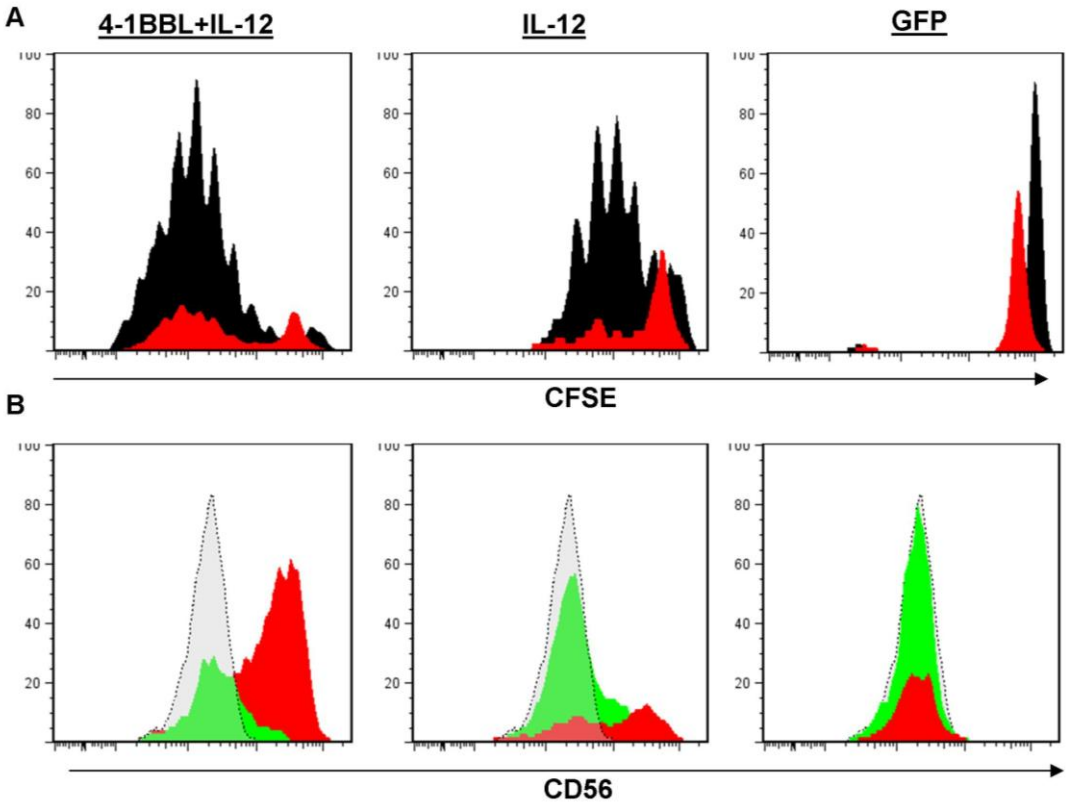

Figure 4-17 Analysis of CD56^{dim} NK cell proliferation and expression of CD56 in culture. NK cells were negatively isolated and further selected on the basis of CD16 expression to provide CD56^{bright} (CD16^{-}) and CD56^{dim} (CD16^{+}) NK cell populations which were labelled with CFSE. 0.5x10^{5} NK cells were then cultured with 1x10^{5} OVCAR-3 cells (pre-infected with adenoviral vectors) for 7 days. Dilution of CFSE by isolated CD56^{bright} (Black) and CD56^{dim} (red) NK cells was then analysed by flow cytometry (A). The expression of CD56 by isolated CD56^{dim} NK cells was analysed on days 0 (shaded grey), 3 (green), and 7 (red) of culture (B).

In order to validate the transition of a $CD56^{dim}CD16^{+}$ cell to a $CD56^{bright}$ cell type, the presence of $CD56^{bright}CD16^{-}$ cells was assessed in cultures following 7 day stimulation. As can be seen in Figure 4-18 there is a notable population of $CD56^{bright}$ cells following stimulation with IL-12_{OVCAR}, while stimulation with 4-1BBL+IL-12_{OVCAR} results in the majority of cells becoming $CD56^{bright}$. The expression of CD16 shows a similar pattern with the combination of 4-1BBL+IL-12_{OVCAR} producing a large population of $CD16^{-}$ cells, while IL-12_{OVCAR} alone does not show such marked production of $CD16^{-}$ cells. A similar pattern is also seen with expression of CD62L. Therefore these data suggest that 4-1BBL+IL-12_{OVCAR} is facilitating a switch from a $CD56^{dim}$ to a $CD56^{bright}$ cell. The ability of 4-$1BBL_{OVCAR}$ alone or the requirement for IL-12 in this process however could not be assessed.

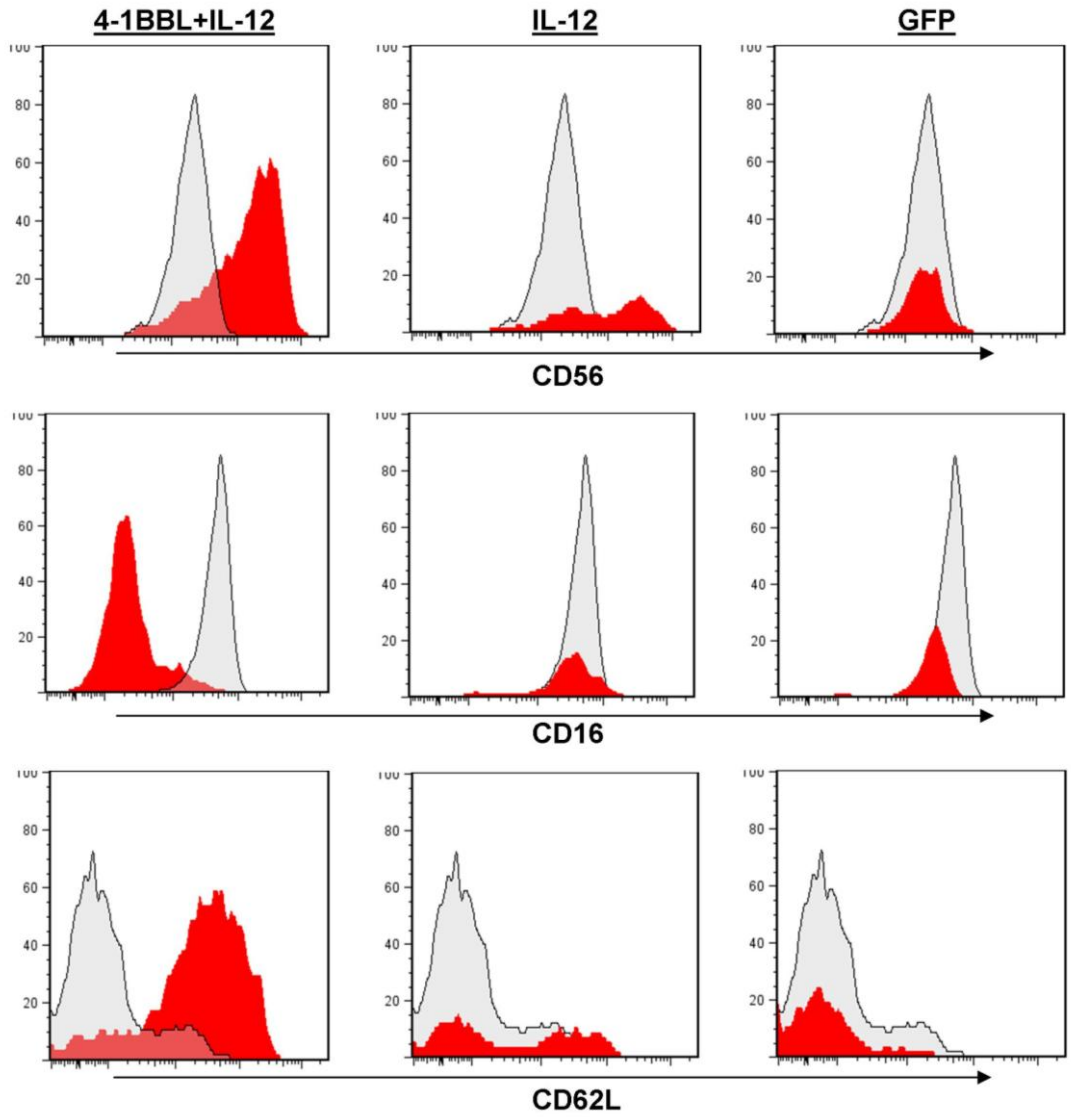

Figure 4-18 CD56^{bright} NK cells are produced in response to 4-1BBL and IL-12. NK cells were negatively isolated and CD56^{dim} (CD16^{+}) NK cells positively selected on the basis of CD16 expression. 0.5x10^{5} CD56^{dim} NK cells were then cultured with 1x10^{5} OVCAR-3 cell (pre-infected with adenoviral vectors) for 7 days. Expression of CD56, CD16 and CD62L were then determined by flow cytometry at day 7 of culture, expression by the starting population at day 0 is shown as shaded grey.

These data definitively show a population of $CD56^{dim}$ NK cells can proliferate and become $CD56^{bright}$. Therefore these data are important for the understanding of NK cell biology, showing that the differentiation of $CD56^{dim}$ to $CD56^{bright}$ is not a unidirectional process. Therefore the $CD56^{bright}$ and $CD56^{dim}$ NK cells cannot be regarded as immature and mature stages of the same cell population.

4.2 Long Term Proliferation of Natural Killer Cells

4.2.1 4-1BBL allows Long Term Proliferation of Natural Killer Cells

As 4-1BBL is known to promote the long term survival and proliferation of T cells, it was of considerable interest to investigate whether the use of adenovirus directed 'costimulation' with 4-1BBL alone or in combination with IL-12 would also permit the long term expansion of Natural Killer cells. Following the initial 7 day stimulation with Ad-infected OVCAR-3, the lymphocytes were counted and redistributed with the inclusion of 20U/ml IL-2. Following a further 7 days of culture populations were again counted and analysed, cultures were then re-stimulated with OVCAR-3 pre-infected with appropriate adenovirus vectors for a further 7 days. This system of stimulation was settled on to give the best proliferation of NK cells while avoiding the use of excessive recombinant cytokines. IL-2 was found to improve proliferation; however the population was able to proliferate at a reduced level in the absence of IL-2 (data not shown). As NK cells were by this stage beginning to dominate the 4-1BBL+IL-12_{OVCAR} cultures and as NK cells are incapable of producing IL-2, it

seemed reasonable to add exogenous cytokine as *in vivo* T cells would presumably be present in a true immune response to provide a source of IL-2.

Figure 4-19 shows the proliferation of Natural Killer cells over a period of 3 weeks. IL-12_{OVCAR} stimulated cultures were unable to continue to proliferate to a significant degree (mean 64 fold expansion) following the initial burst of proliferation in the first week. Cultures stimulated with 4-$1BBL_{OVCAR}$ proliferated to a similar level to IL-12_{OVCAR} stimulated cultures over the first 7 days however the 4-$1BBL_{OVCAR}$ stimulated cultures continued to proliferate (mean 259 fold expansion at day 21). As with short term cultures, the combination of 4-1BBL+IL-12_{OVCAR} again showed a significant enhancement of proliferation in long term culture, compared with either alone (mean 1076 fold expansion at day 21). The control cultures generally diminished to untenable levels by day 14. These data clearly show 4-1BBL is required and capable of inducing the long term expansion of NK cells. This is the first clear demonstration of the ability of 4-1BBL alone to induce the long term expansion of NK cells, and the first demonstration of the long term expansion of NK cells in response to 4-1BBL+IL-12.

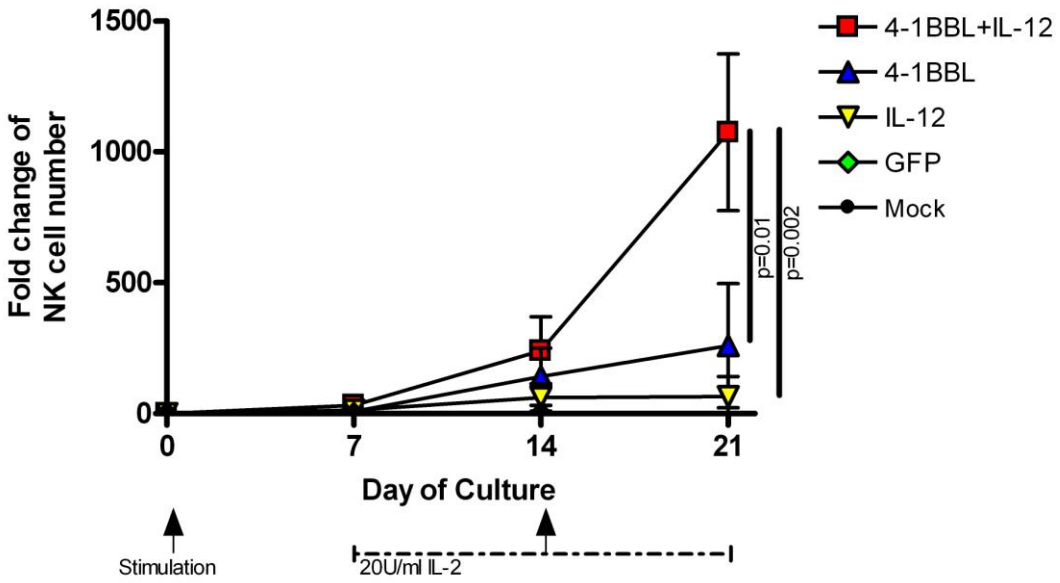

Figure 4-19 Long term proliferation of NK cells in response to costimulation. $0.5x10^6$ non-adherent PBMC were cultured for 7 days, with $1x10^5$ OVCAR-3 cells (pre-infected with adenoviral vectors) and expansion of NK cells calculated by flow cytometry and haemocytometer. Cultures were then redistributed and IL-2 added to a final concentration of 20U/ml. Expansion was then determined again at day 14 of culture by haemocytometer count and flow cytometry. Cultures were then re-stimulated with $1x10^5$ OVCAR-3 cells pre-infected with the appropriate adenoviral vectors in the continued presence of IL-2 and expansion determined again at day 21 of culture. N=5, SD, paired t-test.

4.2.2 Cytokine Production and Cytotoxicity of Long Term Cultures

Following culture for 21 days the ability of the cultured cells (predominantly NK cells) to show effector functions was again characterised.

Cultures were again adjusted to $0.5x10^6$cells/ml and cultured overnight. The production of IFNγ was then assessed by ELISA. As can be seen in Figure 4-20A 4-1BBL stimulated cultures continue to produce IFNγ although this is overshadowed by the amount of IFNγ produced by IL-12 stimulated cultures, as was seen in short term culture. As control cultures diminish by this time point no direct comparison can be made to these cultures, however the pattern for the stimulated cultures remains similar to that in short term culture.

The cytotoxicity of cultures against K562 targets was also characterised by Cr release assay, as shown in Figure 4-20B the cultures continue to be cytotoxic with the presence of IL-12 in cultures allowing a higher degree of cytotoxicity to be attained.

As mentioned in section 4.1.5 the assessment of the ability of 4-1BBL to support NK cell effector functions is highly pertinent. It is of interest and importance that stimulated cultures are able to maintain effector function during long term stimulation.

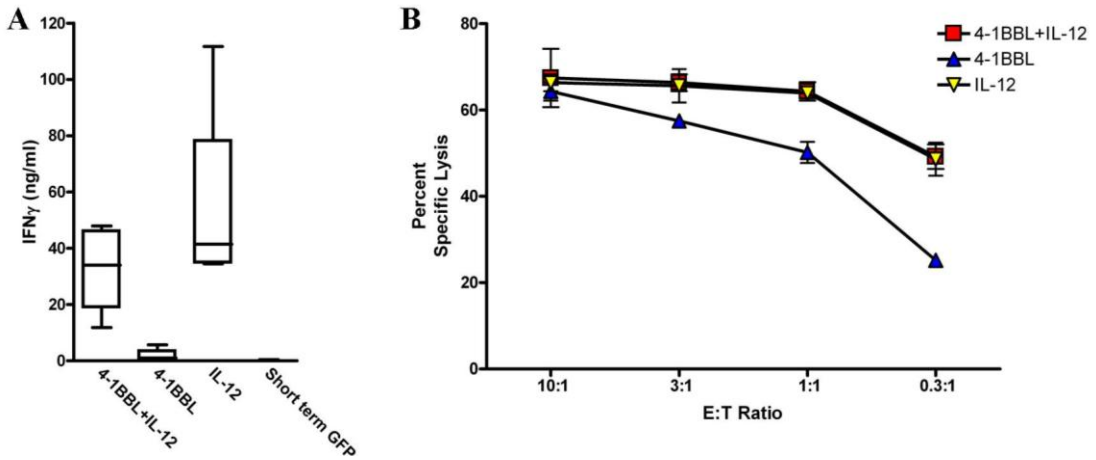

Figure 4-20 IFNγ production and cytotoxicity by long term cultured cells. PBMC (predominantly NK cells) cultured for 21days with stimulation with OVCAR-3 cells pre-infected with adenoviral vectors at day 0 and 14 were re-suspended in fresh media, cells were then cultured o/n at $0.5x10^6$ cells/ml and production of IFNγ determined by ELISA (N=5, SD), production of IFNγ by day 7 GFP stimulated cultures is also shown for comparison (A). Cytotoxic function of day 21 cultured cells was also tested in a standard 5hr chromium release assay against K562 targets (SD) (B).

4.2.3 4-1BBL does not enhance survival of Natural Killer cells

As we have shown the ability of 4-1BBL to facilitate the long term proliferation of NK cells and given 4-1BBL is known to facilitate the long term expansion and maintenance of T cells by allowing increased survival I chose to examine whether 4-1BBL was facilitating the long term expansion of NK cells through a reduction in cell death.

To do this I examined the presence of live, apoptotic and necrotic NK cells within cultures at day 7, 10 and 14 with the use of Syto 16 and PI staining. The SYTO dyes are cell permeable but essentially non-fluorescent until bound to nucleic acids. Thus when combined with PI staining allows the identification of live cells which have a full DNA content and intact cell membranes, Syto $16^{bright}PI^{-}$. While apoptotic cells have reduced or absent Syto 16 staining but retain membrane integrity excluding PI, and necrotic cells are Syto $16^{-}PI^{+}$. The gating strategy used for Syto-16 staining is shown in Figure 4-21A.

Initial staining at day 7 revealed a stark reduction in the survival of control cultures compared with those stimulated with 4-1BBL and/or IL-12 which were also actively proliferating at this stage (Figure 4-21B and Figure 4-21C). There is however no difference seen between the $4\text{-}1BBL_{OVCAR}$ stimulated cultures and the $IL\text{-}12_{OVCAR}$ stimulated culture. Comparing $4\text{-}1BBL_{OVCAR}$ stimulated cultures to $IL\text{-}12_{OVCAR}$ cultures at day 10 and 14 there is again no difference between these cultures (Figure 4-21C).

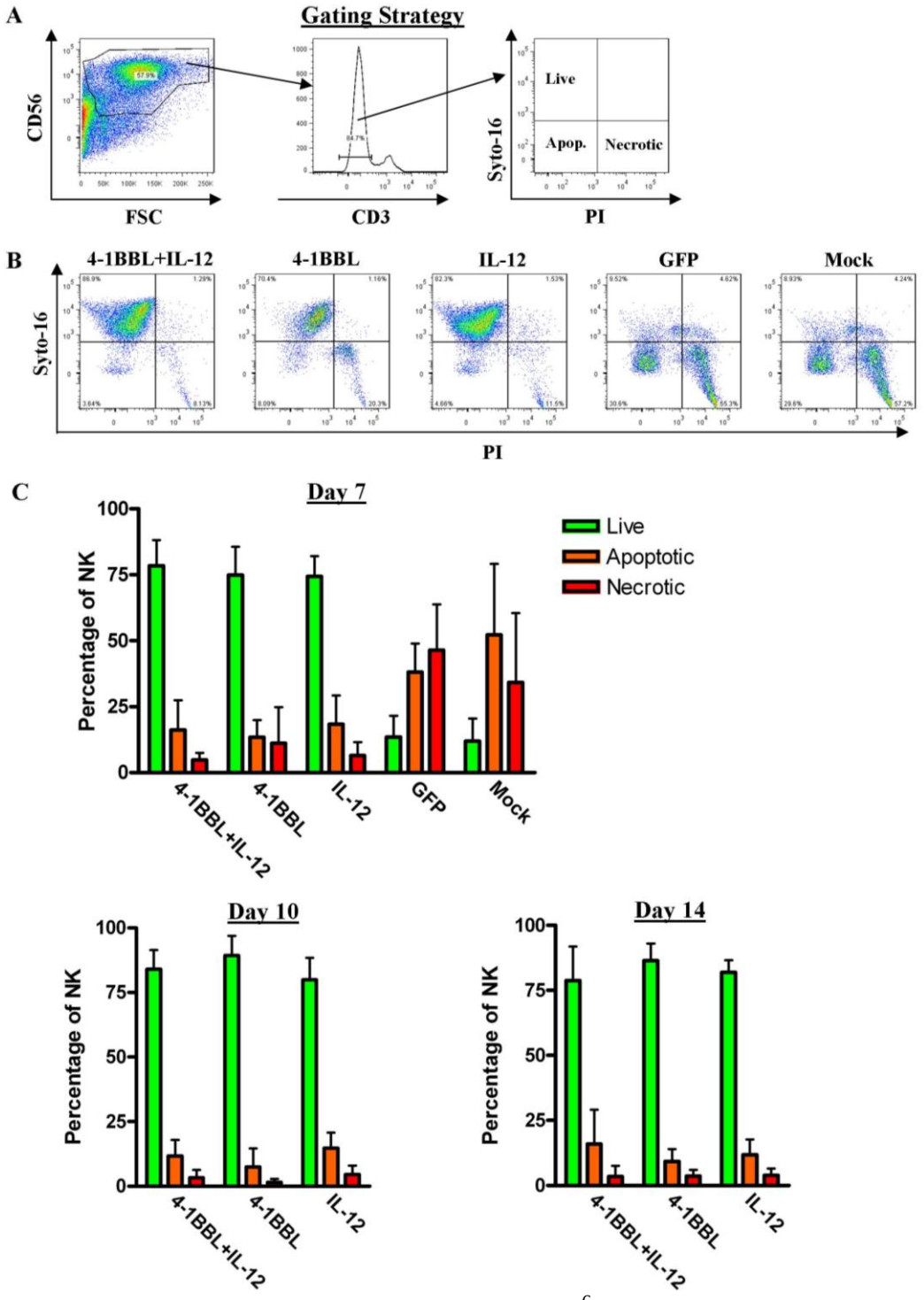

Figure 4-21 Viability of cultured NK cells. $0.5x10^6$ adherent cell depleted PBMC were cultured with $1x10^5$ OVCAR-3 cells (pre-infected with the appropriate adenoviral vectors) for 7 days, after which time 20U/ml IL-2 was added to cultures. The viability of NK cells was determined by Syto-16 and PI staining flow cytometry assay, as shown in A. cells were first selected by forward scatter and $CD56^+$ cells and subsequently gated on the $CD3^-$ population. NK cells staining Syto-16^+PI^- were termed live, double negative cells were proposed to be apoptotic and Syto-16^-PI^+ cells necrotic. An example of day 7 staining from one donor is shown in B. Results from six healthy lab donors from day 7, 10 and 14 of culture are shown in C. Cultures were supplemented with 20U/ml at day 7-14 of culture. SD.

As these cultures had been cultured in accordance with the long term expansion model with the addition of 20U/ml IL-2 at day 7, I also tested whether the presence of IL-2 may be masking a protective effect for 4-1BBL in NK cell survival. However as shown in Figure 4-22, IL-2 improved the viability of the control cultures, but had no significant effect on the viability of cultures stimulated with IL-12 and 4-1BBL alone and in combination, therefore suggesting 4-1BBL does not increase the survival of NK cells in contrast to T cells.

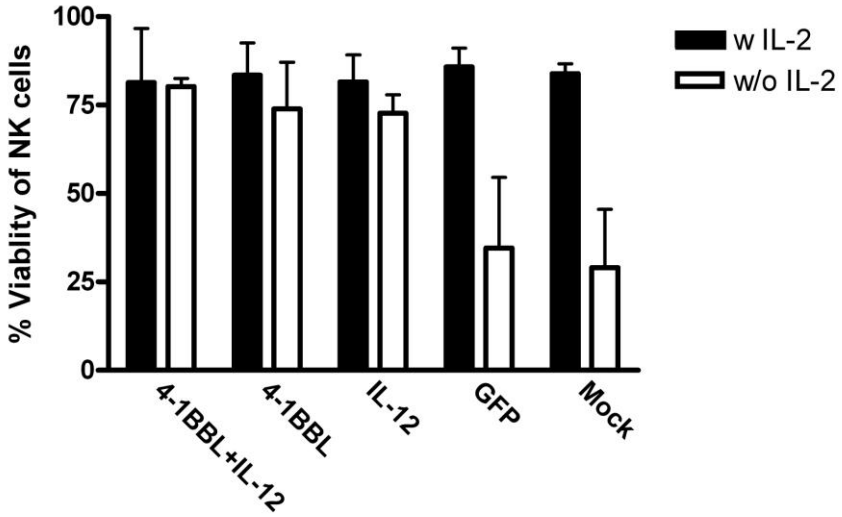

Figure 4-22 Viability of expanded NK cells in the presence or absence of IL-2. $0.5x10^6$ adherent cell depleted PBMC were cultured with $1x10^5$ OVCAR-3 cells (pre-infected with the appropriate adenoviral vectors) for 7 days, after which time cells were cultured in the presence or absence of 20U/ml IL-2. NK cells viability was then determined by Syto-16 and PI staining at day 14 of culture. N=2, SD.

This shows 4-1BBL does not provide a survival advantage to NK cells, allowing their continued expansion. This may suggest that in contrast to T cells, NK cell stimulation by 4-1BBL allows increased or continued proliferation. This would be in accordance with the data of Fujisaki et al. (2009) who showed 4-1BBL+IL-15 stimulation of NK cells does not increase Bcl-2 family members. However it should be noted that as cells are gated on CD56 it may be that a proportion of necrotic cells may be missed during gating, a purified NK cell population may allow a more accurate measurement of necrotic cells in culture to be obtained, i.e. remove the requirement to gate on an antibody stained NK cell population.

4.3 Expansion of Natural Killer cells from Renal Cell Carcinoma patients

4.3.1 Introduction

We have shown the ability of 4-1BBL and IL-12 to activate and induce the short and long term proliferation of NK cells, and the beneficial effects of a combination of both 4-1BBL and IL-12. However this has been based on the responses of NK cells derived from healthy lab donors. I therefore wished to investigate the potential applicability of this finding for the stimulation of anti-tumour immune responses in cancer patients.

Renal cancer is the seventh most common cancer diagnosis in men and the ninth most common in women, with the peak incidence between 60-70 years of age. The incidence of renal cancer has increased over the past 65 years by 2% per year with an

estimated 54,390 new cases and 13,010 deaths expected in 2008. Prognosis is poor, 30% of patients present with metastatic disease, and 20 to 30% of patients with clinically localized disease will develop metastatic disease after nephrectomy, having a variable 5-year survival rate that is generally less than 2% and a median survival of less than 1 year. However, even with the licensing of small molecule drugs such as the tyrosine kinase inhibitor Sorafenib, and the use of targeted therapies such as Bevacizumab, the treatment choice is still limited. IL-2 therapy remains the first choice therapy for most patients following nephrectomy, producing response rates between 10 to 20%. Therefore new therapies for the treatment of renal cancer are important and desirable (Reeves and Liu 2009; Rini, 2009).

NK cells are present in the majority of RCC tumours. Conversely tumour infiltrating NK cells lack cytotoxic function, however following *in vitro* stimulation with IL-2 NK cells derived from tumour are capable of killing autologous tumour (Schleypen et al., 2003; Schleypen et al., 2006). Therefore there is rational for the activation of NK cells for immunotherapy of RCC. Additionally the use of adenoviral vector delivered costimulation may be particularly appropriate for RCC therapy as the majority of TAA epitopes identified are expressed uniquely by individual tumours (Geiger et al., 2009).

In order to test the ability of our system to produce an immune response from renal cell carcinoma patients, blood, tumour and normal kidney tissue was collected from such patients at the time of first treatment (i.e. nephrectomy). Non-adherent PBMC were isolated and stimulated with OVCAR-3 infected with adenoviral vectors as for health controls.

4.3.2 Short term expansion of Natural Killer Cells derived from RCC patients

Examining the short term expansion of NK cells derived from RCC patients the pattern of proliferation seen in healthy lab donors is maintained as shown in Figure 4-23, albeit to a lower level than seen in healthy donors, with 4-1BBL$_{OVCAR}$ and IL-12$_{OVCAR}$ able to induce proliferation of NK cells (mean 4 and 5 fold respectively), while the combination 4-1BBL+IL-12$_{OVCAR}$ enhances this proliferation (mean 18 fold expansion).

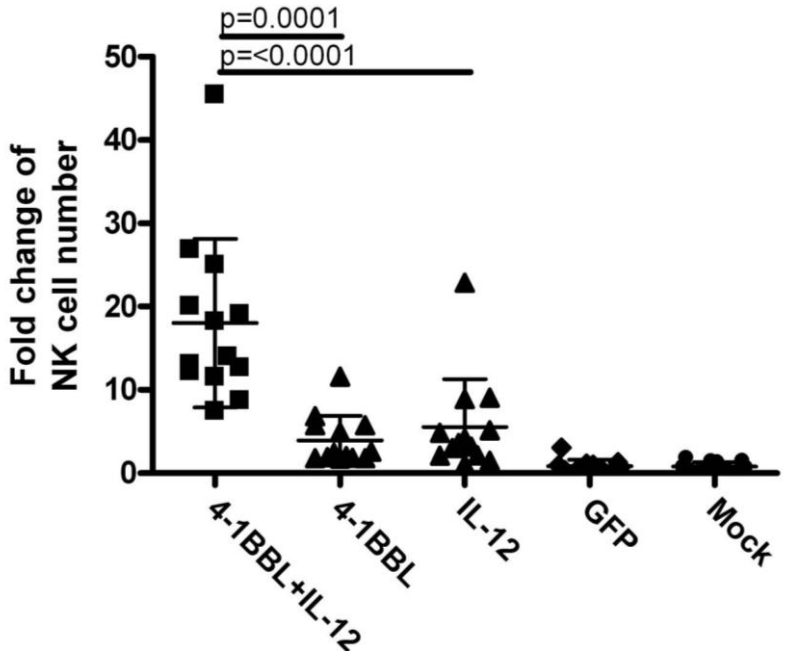

Figure 4-23 Short term proliferation of RCC patient NK cells in response to costimulation. 0.5x10^6 adherent cell depleted PBMC from different RCC patients were cultured with 1x10^5 OVCAR-3 cells (pre-infected with the appropriate adenoviral vectors) for 7 days. NK cell expansion was determined by haemocytometer count and flow cytometry and expressed as fold change compared to the starting population. SD, paired t-test.

4.3.2.1 Comparison of Healthy and RCC Patient Natural Killer Cell Responses

Comparison of the proliferation of NK cells derived from healthy donors and RCC patients reveals a significant defect in the proliferation of NK cells derived from RCC patients compared to healthy donors in response to stimulation, as shown in Figure 4-24.

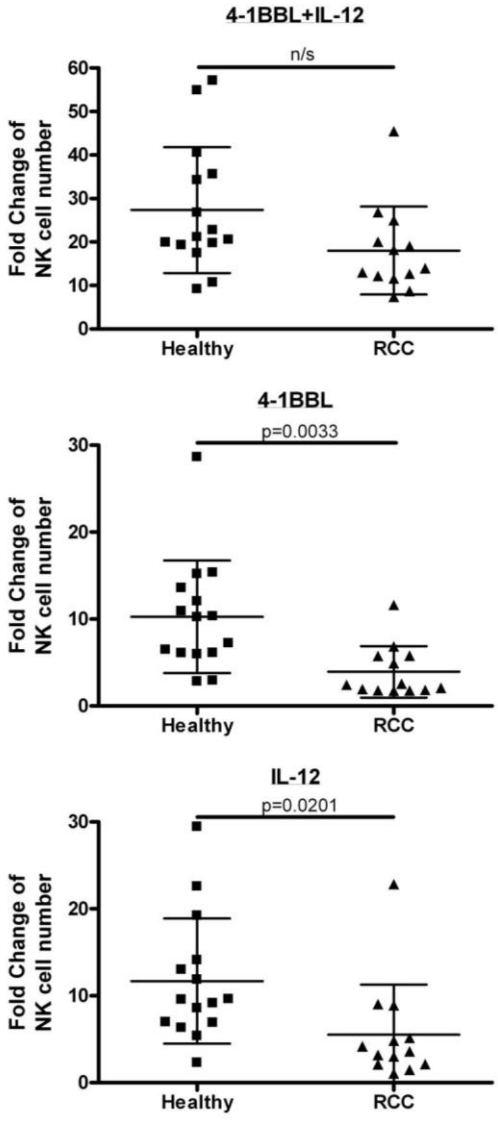

Figure 4-24 Comparison of RCC patient and healthy lab donor NK cell expansion in short term culture. Data from section 4.1.4 and section 4.3.2 are presented. SD, student t test.

4.3.3 Functionality of Short Term Culture

Having shown that our system could expand NK cells from RCC patients we wished to determine whether these stimulated cultures were also capable of effector functions. To do this we again assessed the production of IFNγ by ELISA and cytotoxicity by killing of K562 targets in a standard five hour chromium release assay.

As shown in Figure 4-25A cultures which had been stimulated for 7 days then re-suspended at $0.5x10^6$ cells/ml and cultured overnight produced large amounts of IFNγ in response to stimulation with IL-12_{OVCAR} or 4-1BBL+IL-12_{OVCAR}, however stimulation with 4-$1BBL_{OVCAR}$ alone did not induce IFNγ production to levels which were significantly different to control cultures. This latter finding was in contrast to the production seen in healthy controls.

In contrast to IFNγ production, stimulation with either 4-1BBL or IL-12 alone or in combination resulted in similar levels of killing of K562 targets, which were significantly greater than the control stimulated cultures (Figure 4-25).

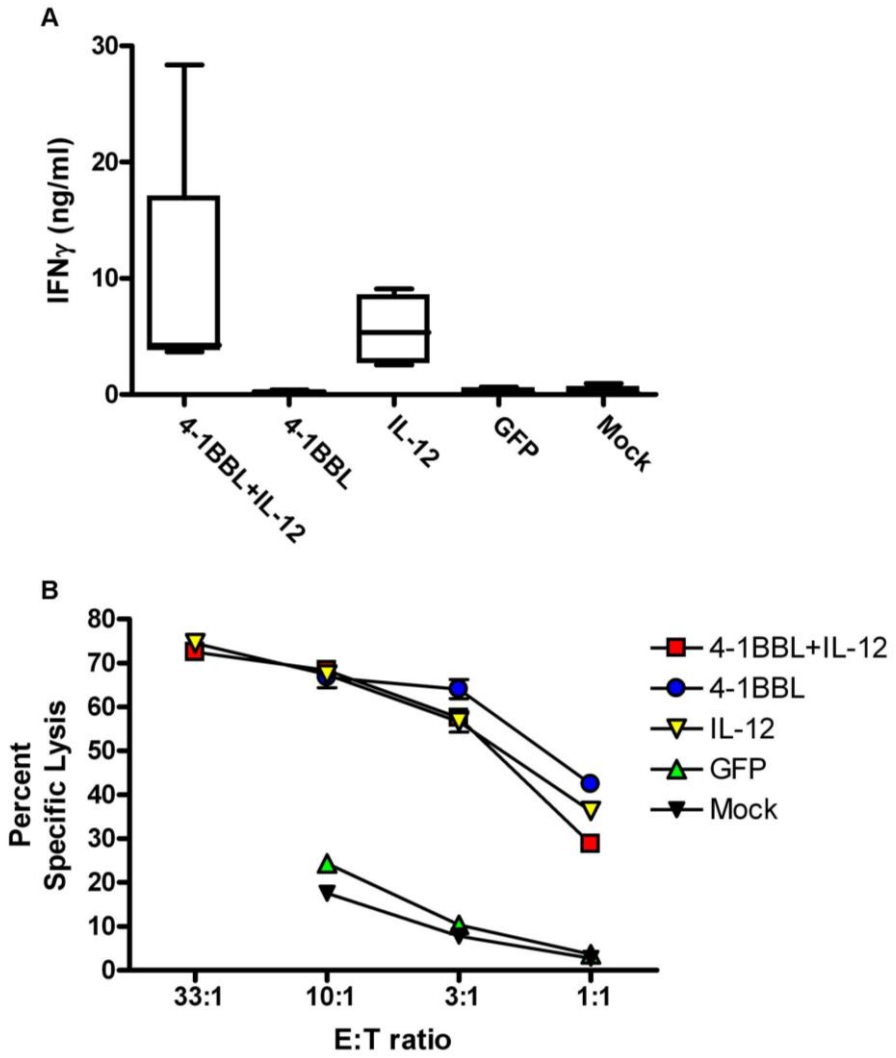

Figure 4-25 IFNγ production and cytotoxicity by short term cultured RCC patient PBMC. $0.5x10^6$ adherent cell depleted PBMC derived from RCC patients were cultured with $1x10^5$ OVCAR-3 cells (pre-infected with the appropriate adenoviral vectors) for 7 days. Cells were re-suspended in fresh media, and tested for either overnight production of IFNγ at $0.5x10^6$ cells/ml by ELISA (A) (N=5), or cytotoxicity in a standard 5hr chromium release assay against K562 targets (B).

4.3.4 Short term Expansion of Natural Killer Cells Derived from an Ovarian cancer patients

As shown for one ovarian cancer patient in section 3.2 our model system allowed the expansion of TAL from the ascitic fluid of an ovarian cancer patient. I was also able to test the expansion of NK cells derived from there ovarian cancer patient, as shown in Figure 4-26, NK cells can be expanded from these donors in a pattern similar to the expansion seen in healthy and RCC donors.

Another patient sample was also tested during the initial stages of the NK expansion work using autologous adherent cells isolated from ascitic fluid. An increase in the percentage of $CD3^{-}CD56^{+}$ cells was seen in agreement with later data, however the actual expansion of these cells could not be enumerated due to technical issues with the CD56 antibody used (data not show).

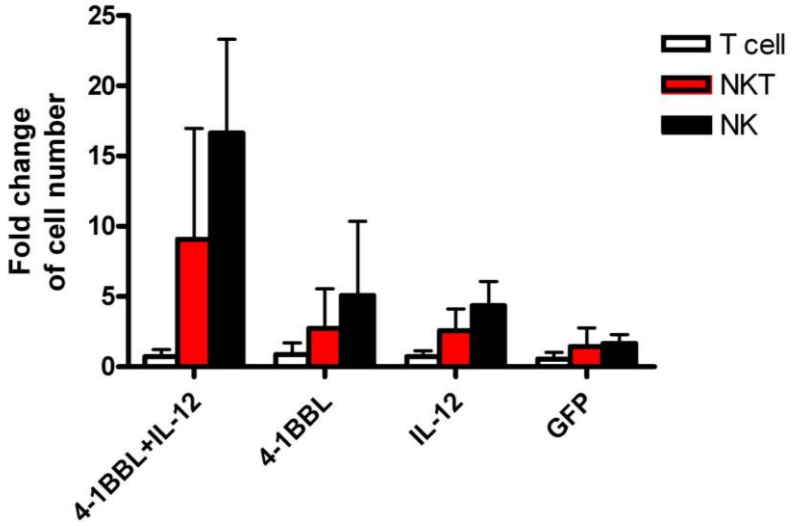

Figure 4-26 Expansion of tumour-associated T, NKT and NK cells isolated from ovarian cancer patients. $0.5x10^6$ TAL isolated from the ascitic fluid of three ovarian cancer patients were cultured with $1x10^5$ OVCAR-3 cells (pre-infected with appropriate adenoviral vectors), for 7 days. Expansion of T, NKT and NK cells was determined by haemocytometer cell count and flow cytometry at day 7 of culture, and expressed as a fold change compared to the starting population.

4.3.5 Long Term Expansion of Natural Killer Cells from RCC Patients

Having shown that our system could expand NK cells from RCC patients, albeit to a lower level than healthy donors, we wished to assess whether RCC patient NK cells could continue to expand in long term culture and whether there would also be a defect in long term proliferation. NK cells were therefore cultured as for healthy donor long term culture. As shown in Figure 4-27 the combination of 4-1BBL+IL-12 was able to allow long term expansion of RCC patient NK cells (mean 431 fold expansion at 21 days), however in contrast to healthy donors 4-1BBL alone was unable to allow long term proliferation of RCC patient NK cells, expanding to levels not dissimilar to $IL\text{-}12_{OVCAR}$ stimulated cultures (33 fold and 15 fold expansion at 21 days respectively).

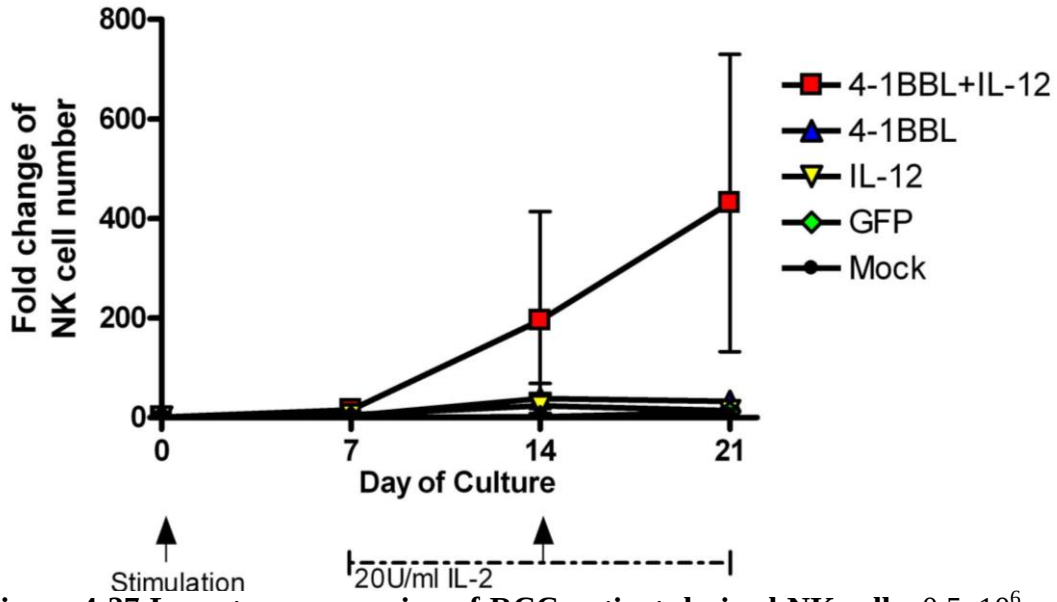

Figure 4-27 Long term expansion of RCC patient derived NK cells. $0.5x10^6$ non-adherent PBMC from six RCC patients were cultured for 7 days with $1x10^5$ OVCAR-3 cells (pre-infected with appropriate adenoviral vectors), and expansion of NK cells calculated by flow cytometry and haemocytometer. Cultures were then redistributed and IL-2 added to a final concentration of 20U/ml. Expansion was then determined again at day 14 of culture by haemocytometer count and flow cytometry. Cultures were then re-stimulated with $1x10^5$ OVCAR-3 cells (pre-infected with the appropriate adenoviral vectors) in the continued presence of IL-2 and expansion determined again at day 21 of culture. N=6, SD.

4.3.5.1 Comparison of Healthy and RCC Patient Natural Killer Cell Responses

Figure 4-28 compares the expansion of NK cells from healthy donors and RCC patients, in response to long term culture as previously described. Stimulation of RCC patient PBMC with 4-1BBL$_{OVCAR}$ gave only a mean ~33 fold expansion, almost ~10% that seen in healthy donors, likewise IL-12$_{OVCAR}$ also gave very little expansion. The combination of 4-1BBL+IL-12 gave a mean expansion of ~430 fold of NK cells from RCC patients; this was ~40% that of the expansion achieved in healthy donors. The advantage of the combination of 4-1BBL+IL-12 for stimulation of NK cell proliferation was therefore greater in the RCC patients.

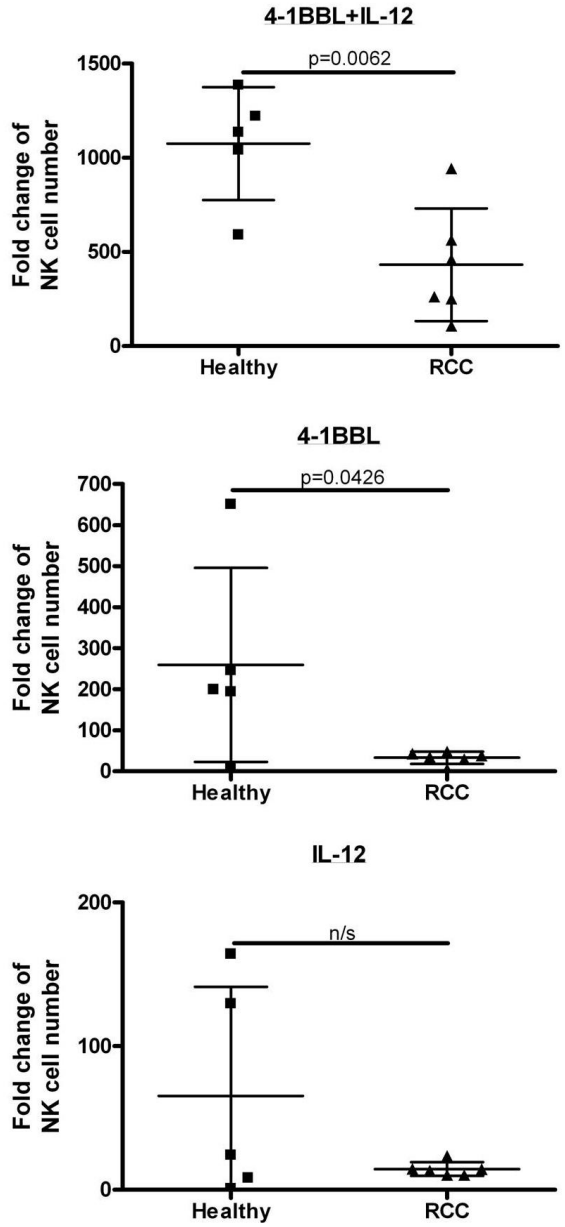

Figure 4-28 Comparison of RCC patient and healthy lab donor NK cell expansion in long term culture. Data from section 4.2.1 and section 4.3.5 are presented. SD, student t test.

4.3.6 RCC Patient NK can express 4-1BB

As RCC patient NK cells show relatively poor responses to 4-1BBL$_{OVCAR}$ alone in short or long term culture we wished to determine whether RCC patient NK cells were capable of expressing 4-1BB in response to stimulation.

RCC patient PBMC were cultured with OVCAR-3 as for standard short term culture, then analysed at day 3 for the expression of 4-1BB on NK cells in response to stimulation. As shown in Figure 4-29 NK cells are able to induce expression of 4-1BB. Expression was induced to the greatest extent following stimulation with IL-12$_{OVCAR}$, while expression in response to 4-1BBL+IL-12$_{OVCAR}$ or 4-1BBL$_{OVCAR}$ showed variation between the donors tested, but overall showed a slightly reduced expression of 4-1BB, perhaps due to the ligation of the receptor in these cultures.. Therefore the reduced responsiveness of RCC patient NK cell to 4-1BBL$_{OVCAR}$ cannot be attributed to a lack of expression of 4-1BB.

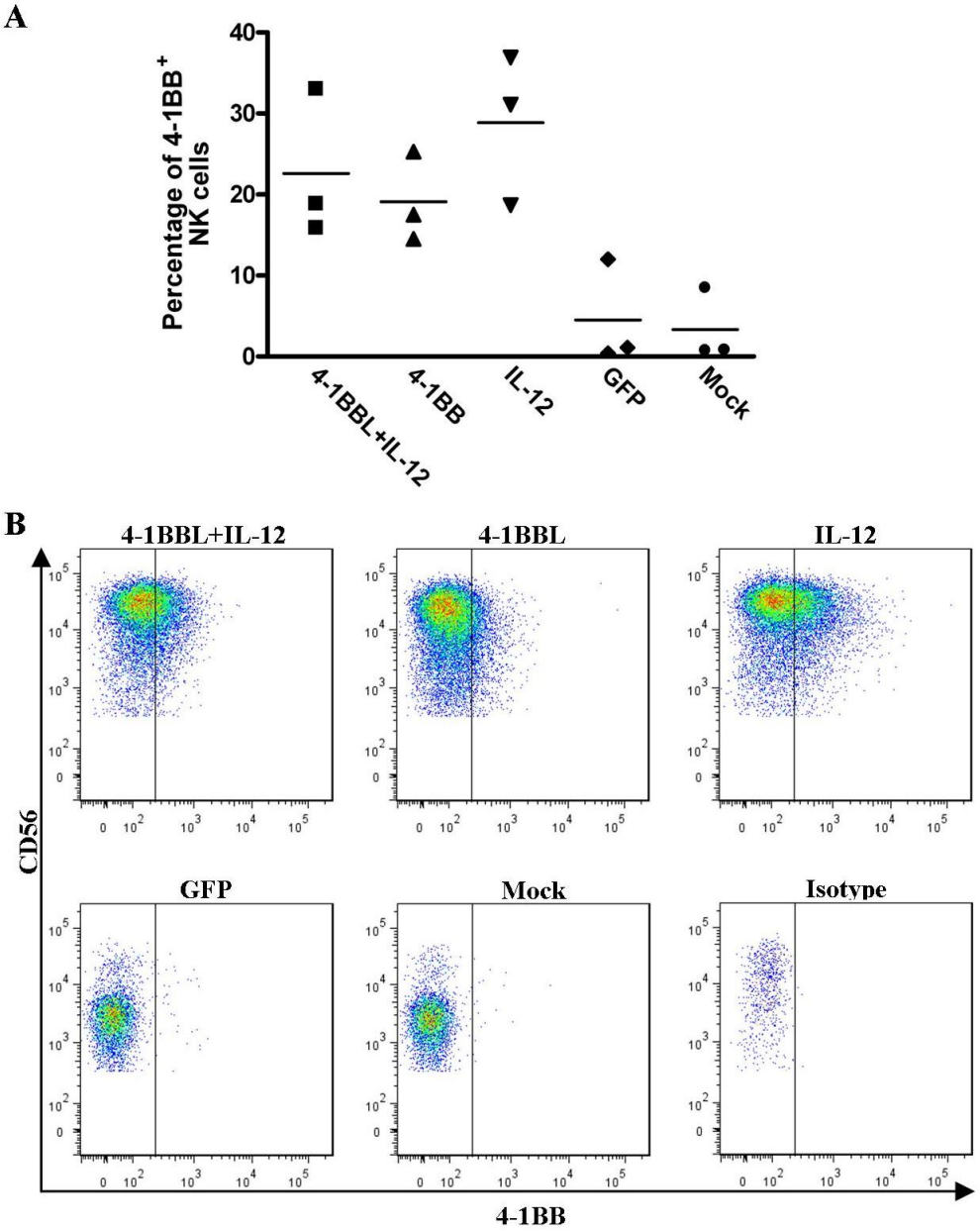

Figure 4-29 Expression of 4-1BB by RCC patient NK cells. $0.5x10^6$ adherent cell depleted PBMC isolated from RCC patients were stimulated with $1x10^5$ OVCAR-3 cells (pre-infected with appropriate adenoviral vectors). Expression of 4-1BB was determined by flow cytometry as a percentage of the NK population ($CD3^-CD56^+$) after 3 days of culture (A), mean is shown. B shows plots of 4-1BB staining at day 3 of culture, gated on NK cells ($CD3^-CD56^+$).

These data show that in a similar manner to healthy donors RCC patient NK cells are able to respond to 4-1BBL+IL-12 stimulation for short and long term expansion; although at a reduced level compared to healthy donors. Whether the reduction in expansion is due to the age of these donors or whether the immunosuppressed nature of cancer patients has reduced the responsiveness of these cells has not been determined. The requirement for 4-1BBL and IL-12 interestingly agrees with results from a murine model system using a poorly-immunogenic tumour model where only the combination of 4-1BBL+IL-12 could reject tumour, in an NK cell dependent manner (Xu et al., 2004). Therefore suggesting that 4-1BBL in combination with IL-12 or another cytokine may be required for efficacy as an immunotherapy, and therefore warrants further investigation.

4.3.7 Killing of autologous tumour cells

As we have been able to show that the combination of 4-1BBL+IL-12 is able to induce the expansion of functional NK cells from RCC patients, assessing the ability of these NK cells to recognise and kill autologous tumour was of significant importance.

At the time of blood collection tumour and normal margin was also collected from patients therefore it was possible to match patients to autologous tumour and a negative control, while K562 could provide a positive control. PBMC were cultured as before for 7 days, at which point tissue which had been dissociated and cryopreserved could be used in a standard five hour chromium release assay. Unfortunately due to the small number of tumour cells recovered, only an

effector:target ratio of 10:1 could be used for tumour and ‘normal’ margin, whereas the K562 control was performed at a 1:1 ratio to conserve effectors.

As shown in Figure 4-30A, cells cultured with costimulation were able to efficiently kill K562 targets, while little cytotoxicity was seen with GFP and mock controls. Reassuringly no cultures showed detectable levels of killing of ‘normal’ cells. However due to wide variation in results the level of tumour cell killing could not be reliably determined.

The assay involving tumour was confounded by a high background level of ^{51}Cr release, resulting in a wide error margin in which low levels of killing could be masked. Also the population may contain cells which are not tumour cells, therefore reducing the number of valid targets. We therefore chose to attempt to grow out tumour cells *in vitro*. Following culture for 2-3 weeks, cells were used as targets for a five hour chromium release assay. Following culture we were able to detect cytotoxicity against cultured tumour cells by 7 day cultured PBMC, with cells cultured in the presence of 4-1BBL providing the highest level of killing, however following culture there was also a similar level of killing of ‘normal’ cells (Figure 4-30B).

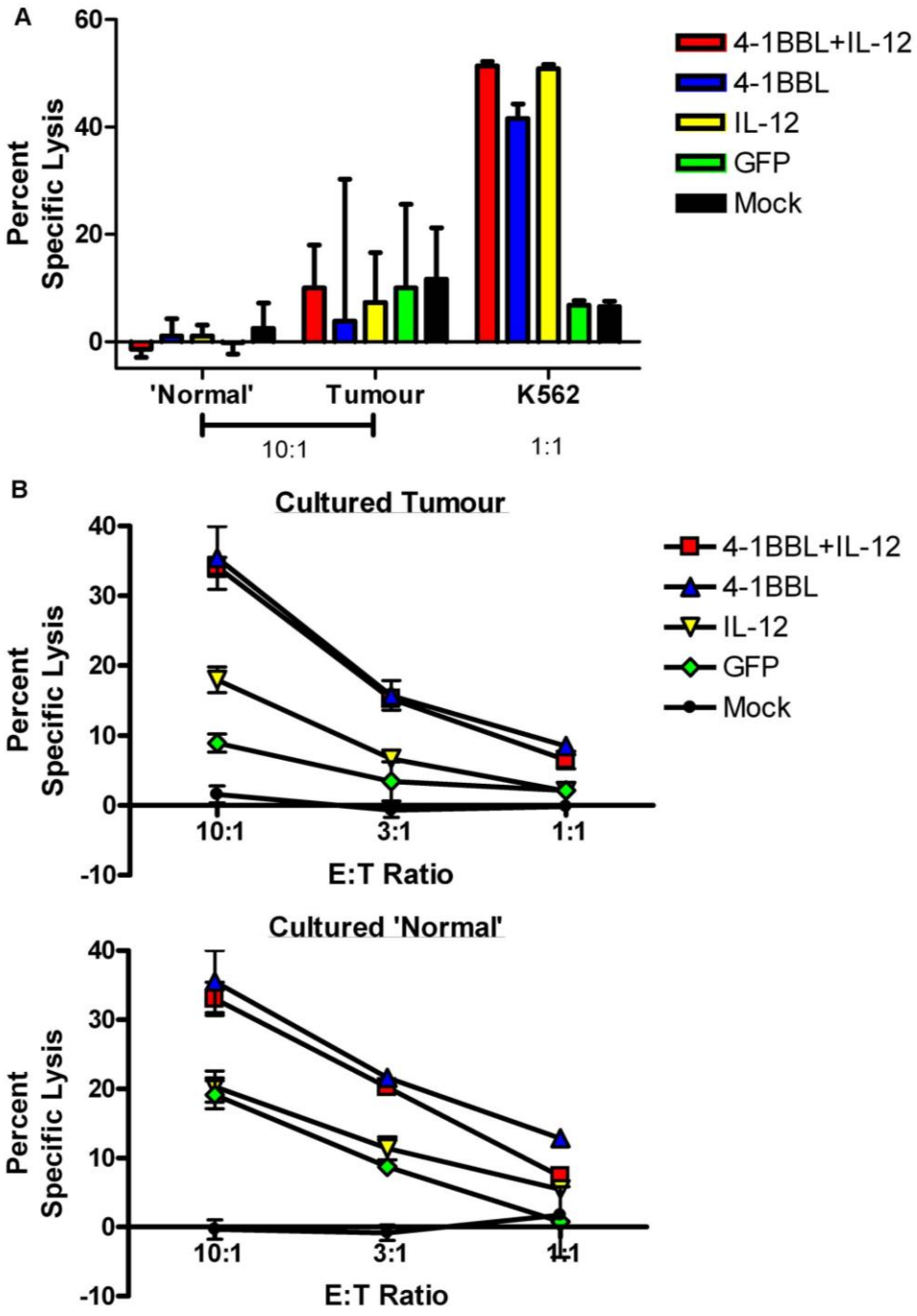

Figure 4-30 Killing of autologous target cells by short term cultured RCC patient derived PBMC. $0.5x10^6$ adherent cell depleted PBMC isolated from RCC patients were stimulated with $1x10^5$ OVCAR-3 cells (pre-infected with appropriate adenoviral vectors). After 7 days cultures were used as effectors in standard chromium release assays against autologous cells isolated from normal and tumour tissue used as targets. Target cells were either cryopreserved (A) until the 7^{th} day of culture of PBMC or cultured alongside PBMC cultures (B). SD.

The ability of cultured NK cells in my experiments to retain self tolerance against 'freshly' isolated normal tissue is encouraging, it is unfortunate that for technical reasons the ability of these cells to recognise 'freshly' isolated autologous tumour could not be reliably tested. Further experiments will be required to investigate this further.

4.3.8 Discussion

These experiments are the first to study the responses of NK cells to a combination of 4-1BBL+IL-12 within a human system, and add to the limited data available regarding the response of human NK cells to 4-1BB stimulation. I have been able to show that 4-1BBL or IL-12 alone can induce the expansion of NK cells derived from healthy and RCC patients in short term culture, additionally 4-1BBL is able to promote long term expansion of healthy donor NK cells. The combination of 4-1BBL+IL-12 however has been shown to be superior to either stimulation alone in short and long term culture in healthy lab donors and RCC patient samples. In contrast to healthy lab donors RCC patient NK cells were shown to require a combination of 4-1BBL+IL-12 to promote long term proliferation. Importantly the expanded NK cells have also been shown to be functional; the combination of 4-1BBL+IL-12 stimulation resulting in superior cytokine production. 4-1BBL+IL-12 stimulated NK cells also possessed potentially beneficial homing potential, i.e. the potential to home to tumour via CXCR3 and also secondary lymphoid tissue through expression of CCR7 and CD62L. These data highlight the requirement for a combination of stimuli for effective immunotherapy strategies.

I have also shown the favoured expansion of a $CD56^{bright}$ NK cell population, including the differentiation of $CD56^{dim}$ NK cells to $CD56^{bright}$ NK cells. These data add to the knowledge of the complex relationship of the $CD56^{bright}$ and $CD56^{dim}$ subsets.

4.3.8.1 Proliferation and phenotype of NK cells

From phenotyping data it has been shown that the majority of expanded NK cells are of the $CD56^{bright}$ subset. These data are in contrast to those reported by the Campana group using a K562 model system stably expressing 4-1BBL and IL-15. No preference was shown in that system in terms of subset or receptor diversity, however CD56 levels were increased (Fujisaki et al., 2009b; Imai et al., 2005). In contrast to data from the Campana group Loza and Perussia (2004) showed IL-12 could induce the acquisition of a $CD56^{bright}$ phenotype from NK cells cultured for 5 days; this phenotype was stable in the presence of IL-12. Therefore the use of IL-12 rather than IL-15 may explain the difference in reported phenotypes following culture.

Of particular interest I have also provided data which show that a proportion of $CD56^{dim}$ NK cells are able to transition to a $CD56^{bright}$ phenotype. The derivation and relationship of these two subsets is an area which has yet to reach a consensus. Currently there are a number of studies showing, in different systems, the conversion of $CD56^{bright}$ to $CD56^{dim}$ and vice versa, such as the work mentioned by Loza and Perussia (2004), or work by Chan et al. (2007) showing differentiation to a $CD56^{dim}$ phenotype following culture with synovial fibroblasts. A number of markers have been suggested to identify different subsets of NK cells, such as the presence or absence of CD27. CD27 has been identified to be differentially expressed by human

NK cells with $CD56^{bright}$ NK cells predominantly expressing CD27 while it is absent in most $CD56^{dim}$ NK cells. CD27 was initially identified to subdivide murine NK cells into functional groups analogous to those of $CD56^{dim}$ and $CD56^{bright}$ cells in humans (Hayakawa and Smyth, 2006; Vossen et al., 2008). However I found the NK cells which were expanded to be predominantly $CD27^{-}$ characteristic of the $CD56^{dim}$ phenotype, this is in contrast with other phenotypic makers tested. Loss of CD27 however is also a marker for the acquisition of effector function in T cells (Appay et al., 2002; Tomiyama et al., 2004; Marzo et al., 2007). However study of CD27 expression on NK cell subsets has been performed on resting NK cells; therefore as other markers are in accordance with the $CD56^{bright}$ phenotype it may be possible that these cells are losing CD27 not as a marker of the $CD56^{dim}$ subset but rather as a consequence of activation and cytotoxic potential. Indeed the absence of CD27 was shown in the previous study to be associated with increased cytolytic function (Hayakawa and Smyth, 2006).

The expression of high or low density CD94 was recently shown to divide the $CD56^{dim}$ subset into two distinct subsets, $CD94^{low}$ possessing classical $CD56^{dim}$ properties of cytotoxicity and minimal cytokine production, whereas the $CD94^{high}$ population showed increased IFNγ production (albeit lower than $CD56^{bright}$ NK cells) while retaining cytotoxicity. The $CD94^{high}$ $CD56^{dim}$ subset of NK cells also differentially expressed other markers such as CD62L. These data were suggested to show an intermediary state during the transition of the $CD56^{bright}$ cell to $CD56^{dim}$. However there is no evidence to exclude the possibility of transition in the alternate direction. Likewise, these data do not show an actual change toward a $CD56^{dim}$

phenotype, therefore the differential expression of CD94 may be marking further subdivisions in the NK cell population.

More convincingly Chan et al. (2007) showed a proportion (~30%) of a population of sorted $CD56^{bright}$ cells could become $CD56^{dim}$, with associated changes in function, following culture with synovial fibroblasts. This process was contact dependent, and was not seen with other culture conditions including IL-2 and IL-15. Intriguingly differentiation was partially inhibited by a FGFR-I blocking antibody; FGFR-I is able to bind NCAM i.e. CD56. Surprisingly the acquisition of a $CD56^{dim}$ phenotype was not associated with division.

The CD56 dim and bright subset have also been found to possess differential lengths of telomeres with the $CD56^{dim}$ population having shorter telomeres, which suggests a history of higher proliferation and given the lower proliferative potential ascribed to the $CD56^{dim}$ population these data have suggested $CD56^{dim}$ NK cells are a more mature form of NK cell than the $CD56^{bright}$ subset (Ouyang et al., 2007). However, the differential telomere length of $CD56^{dim}$ and $CD56^{bright}$ NK cells is in contrast with the data of Chan et al. (2007) showing differentiation to a $CD56^{dim}$ cell in the absence of proliferation, i.e. if $CD56^{bright}$ NK cells are becoming $CD56^{dim}$ without proliferation telomere length should be maintained.

The observation of a population of $CD56^{dim}$ NK cells which can transition to a $CD56^{bright}$ phenotype and proliferate may suggest 4-1BBL+IL-12 is capable of 'rejuvenating' $CD56^{dim}$ NK cells to allow further proliferation. This is particularly pertinent as 4-1BBL is capable of reactivating and inducing proliferation of

‘exhausted’ T cells (Bukczynski et al., 2003; Waller et al., 2007). 4-1BB signalling is able to induce the expression of telomerase by activated T cells, it would be interesting to determine if telomerase is expressed by NK cells in response to 4-1BBL stimulation; the expression of telomerase by NK cells could provide a mechanism by which 4-1BBL is able to promote expansion of NK cells in long term culture, with stimulation by IL-12 increasing the responsiveness of NK cells to 4-1BBL by increasing 4-1BB expression.

These data and the literature suggest human NK cells are a complex heterogeneous population. Likewise it could be suggested all NK cells are derived from a single progenitor with not only divergent activating and inhibitory receptor expression but also variable functional abilities, dependent upon signals received during development. As I show given appropriate signals NK cells are able to alter subtypes. Perhaps these data and the literature suggest clonal NK cells can acquire both ‘helper’ and ‘effector’ status in a similar manner as is ascribed to CD4 and CD8 T cells, whether these NK cells can also ultimately form memory however is currently debated (Cooper and Yokoyama, 2010). A potential model for this is show in Figure 4-31. The occurrence of subset transitions and the functional consequences during infection would be interesting to elucidate.

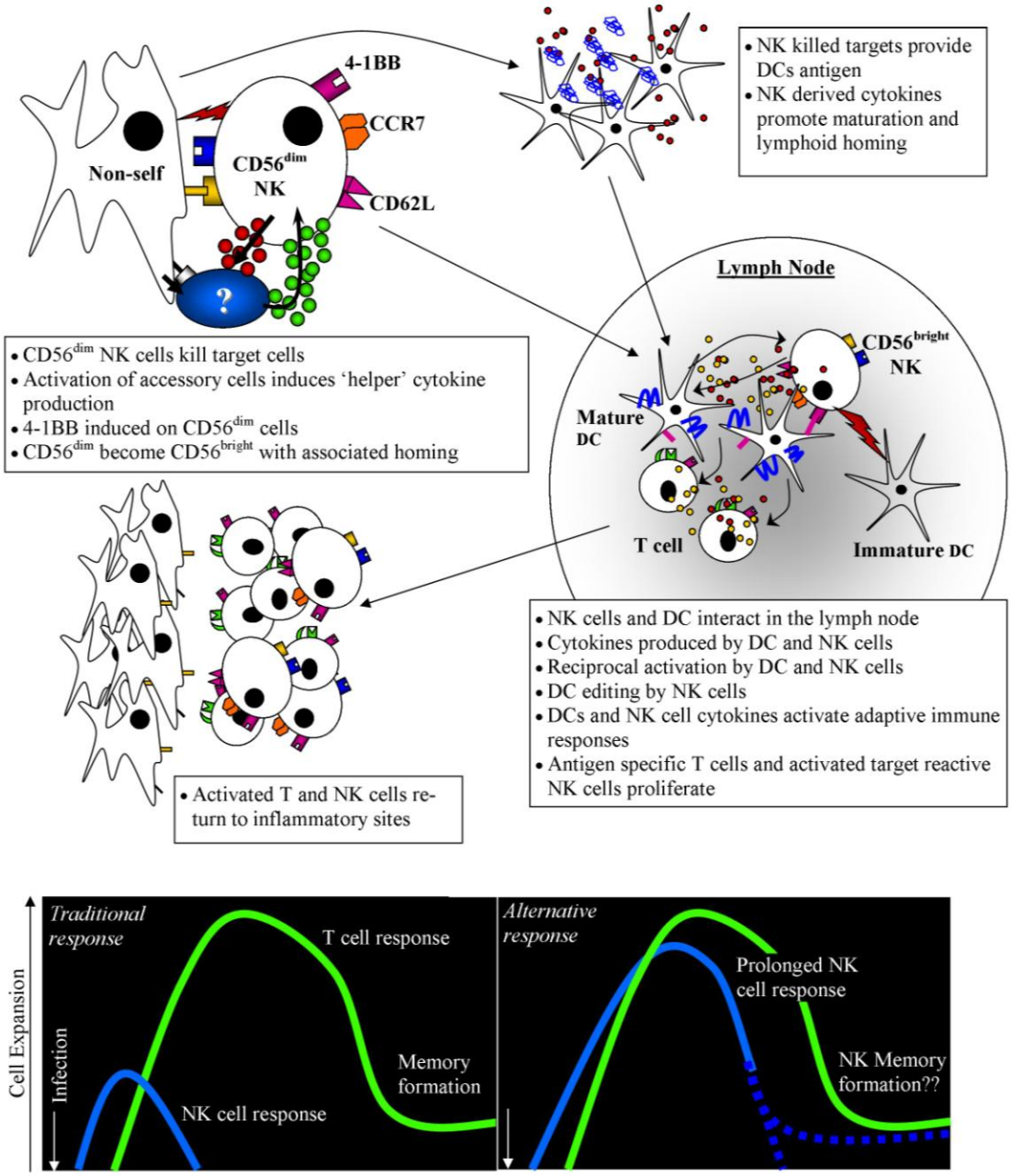

Figure 4-31 NK cell activation in an immune response. $CD56^{dim}$ NK cells kill targets, and become activated, 4-1BB stimulation promotes a $CD56^{bright}$ cell type transition. Following homing to lympnodes these NK cells could carry out immunoregulatory functions and proliferate, forming a population of NK cells with appropriate receptor diversity to recognise the current target. The expanded NK cells may persist and show enhanced secondary responses.

4.3.8.2 Expression of 4-1BB by NK cells

While it has been shown previously that NK cells can induce the expression of 4-1BB following activation, the model systems used have relied upon culture with IL-2 (Imai et al., 2005; Lin et al., 2007). Interestingly 4-1BB was shown in a recent study to be expressed in response to IL-2 or IL-15 stimulation alone. However the NK cells used in this study were a polyclonal population cultured for 10 days with 50U/ml IL-2 (Baessler et al., 2010). In my experiments the absence of exogenous cytokines during short term culture has allowed the identification of a requirement for 'help' from other PBMC (possibly T cells) in order for NK cells to express 4-1BB and therefore respond to 4-1BBL. Previously it has been documented that NK cells require $CD8^+$ T cell help in a murine model system, for the rejection of P815 mastocytoma tumours (Shanker et al., 2007). These data therefore add further to the knowledge of the interactions of the immune system and the importance of a comprehensive activation of the immune system to generate an effective immune response. It would be of interest to investigate this area further.

4.3.8.3 Long term expansion of NK cells

Consistent with short term culture data I have shown that a combination of 4-1BBL+IL-12 stimulates increased expansion of NK cells in long term culture (21 days). Importantly I have also shown the ability of 4-1BBL alone to allow long term expansion of NK cells, which has not been shown before, previous data used IL-2 and IL-15. Therefore these data show that in parallel with other reports of 4-1BBL driving long term T cell proliferation 4-1BBL alone is also sufficient to sustain long term NK cell responses.

The long term expansion of NK cells in this model system with 4-1BBL+IL-12 stimulation resulted in approximately 1000 fold expansion of the starting NK population, on average, during a 21 day culture, which is again in keeping with reports by Imai et al. (2005). Given that it is likely only a portion of NK cells were responding actual expansion of responding cells could be considerably higher. The ability of NK cells to continue to expand further than this may be limited due to telomere degradation. The Campana group studied the loss of telomeres during long term culture with stimulation using the K562-IL-15-4-1BBL expansion system. Cultures expanded to a similar level to those reported here, but subsequently proliferation waned. The ectopic expression of telomerase however was found to restore proliferation indefinitely (Fujisaki et al., 2009a). This would suggest if telomerase is induced by 4-1BBL stimulation of NK cells, that expression is transient. This may also suggest the effects or responsiveness of NK cells to 4-1BBL vary in a temporal manner determined by prior activation as subsequent 4-1BB stimulation does not allow further expansion, as shown by Fujisaki et al. (2009a). There are therefore many questions remaining regarding the function of 4-1BBL in NK cell activation.

NK cells derived from older individuals are likely to have a lower replicative potential as they are likely to posses shorter telomeres (Ouyang et al., 2007). This could contribute significantly to the reduced proliferation seen in RCC patients. However, as there is a difference in proliferation at the 7^{th} day of culture, the impact of shorter telomeres in the NK population would be unlikely to provide a comprehensive explanation for the reduced level of proliferation. It would be informative to directly compare the proliferation of RCC patient NK cells and healthy age matched controls.

However due to the incidence of RCC being most prevalent between 60-70 years of age this was not done during this project. Likewise the attenuated response to single costimulation and long term response to 4-1BBL alone would suggest further deficiencies in RCC patient derived NK cells. Differences in the response of other 'helper' cells in culture maybe responsible, however as NK cells induce similar levels of 4-1BB this is unlikely to be the sole cause. There is reported to be an increased level of Treg in RCC patient PBMC (Cesana et al., 2004; Griffiths et al., 2007), however as the NK cell population tends to dominate cultures in later stages this is again unlikely to be a sole cause. NK cells from cancer patients are know to show reduced responsiveness *ex vivo*, therefore the NK cells may not be able to or may show differing requirements for full activation. Further analysis of the signalling events following 4-1BB ligation may be warranted. While there are many potential contributing factor to the reduced response of RCC patient NK cells it is encouraging that 4-1BBL+IL-12 can continue to induce expansion and function of this cell population.

4.3.8.4 Effector functions and homing potential

I have also shown the cells expanded within this model system to be functional, (and retain function during culture), in terms of cytolytic activity towards K562 targets, and IFNγ and GM-CSF production. I have also shown differences in the expression of homing markers which may be important in the homing of NK cells to tumours.

IL-12 has previously been shown to reduce the expression of CXCR3 (Hodge et al., 2002), however I show 4-1BBL partially retained its ability to promote the expression of CXCR3 in the presence of IL-12. CXCR3 is also predominantly expressed by the

$CD56^{bright}$ subset, which is consistent with my observation that the NK cells produced in response to 4-1BBL+IL-12 are predominantly of the $CD56^{bright}$ subset.

While levels of IFNγ production show little difference between cultures stimulated with IL-12 alone or in combination with 4-1BBL, cytolytic function against K562 targets show some difference between IL-12 or 4-1BBL alone with the combination being superior. The fact that the combination of 4-1BBL+IL-12 also enhances the proliferation of NK cells should result in a qualitatively and quantitatively superior response *in vivo*.

Recently Baessler et al. (2010) have reported a negative effect of stimulating NK cells derived from AML patients or healthy donors with 4-1BBL; in these experiments 4-1BB stimulation was shown to reduce IFNγ production and cytotoxicity. Interestingly the NK cells used by Baessler et al. (2010) were polyclonaly activated with IL-2 for 10 days prior to stimulation with 4-1BBL, therefore these cells were effectively experiencing 4-1BBL stimulation following initial culture. My data from long term cultures of NK cells from RCC patients and healthy lab donors showed significant cytotoxicity following long term culture with stimulation either alone or in combination in contrast to the results reported by Baessler et al.. These data therefore highlight the need for a full understanding of the effects of costimulation for cancer immunotherapy and the potential requirement for a combination of costimulation for efficacy.

The results of adoptive transfer of NK cells (as discussed in section 1.2.6.3 and 1.2.6.4) and the lysis of freshly isolated autologous tumour by NK cells (Alici et al.,

2008) have been encouraging. These data firstly show the ability of NK cells to recognise and kill autologous tumour cells and secondly maintain tolerance to self, or tolerance for healthy tissue in the case of adoptive transfer of KIR mismatched NK cells. The ability of cultured NK cells in my experiments to retain self tolerance against 'freshly' isolated normal tissue is encouraging (Figure 4-30), it is unfortunate that for technical reasons the ability of these cells to recognise 'freshly' isolated autologous tumour could not be reliably tested. No real conclusions can be drawn from these data. It would be important and very interesting to study the killing of normal and tumour tissue further, including the direct isolation and potential culture of tumour cell lines, as at this point we cannot be certain the cells isolated or cultured as tumour are actually tumour cells. Unfortunately further investigation was not possible due to the time constraints of this project.

4.3.8.5 NKT-like cells

Alongside the short term expansion of NK cells I have also demonstrated the expansion of NKT-like cells (Figure 4-3). Further investigation of the expansion of NKT cells in response to 4-1BBL and IL-12 may be warranted. The activation of NKT cells by 4-1BBL has been previously shown in a murine model of airway hyper-responsiveness (Kim et al., 2008), while 4-1BB deficient mice have been shown to have a reduced number of NKT cells (Vinay et al., 2004), however to my knowledge these are the first data showing the expansion and activation of human NKT cells in response to 4-1BBL. As discussed however some questions remain at this stage whether these represent true NKT cells. Previously Loza et al. (2002) have shown the acquisition of CD56 is associated with maturation of NKT and T cells in response to IL-12, becoming polarised towards Th1 cytokine secretion. Interestingly the

expression of CD56 by NKT cells has also been shown to be a marker for the subset of NKT cells with variant TCR usage (Giroux and Denis, 2005). Whether these NKT-like cells are true NKT cells is an interesting question to explore further. If these cells are true NKT cells, i.e. invariant NKT (1.1.3), these cells could be easily defined by assessing invariant TCR usage.

4.3.8.6 Conclusion

I have therefore shown that a combination of 4-1BBL+IL-12 is superior to individual ligands for the expansion of NK cells in short and long term culture, and NKT-like cells in short term culture. Furthermore I have shown that the combination of 4-1BBL+IL-12 provides a beneficial effect in terms of effector functions and potential homing ability. I have also produced novel data showing the ability of $CD56^{dim}$ NK cells to differentiate to $CD56^{bright}$ phenotype.

These data show that a combination of 'costimulatory' molecules is required to support optimal proliferation of NK cells, in particular from RCC patients, and therefore also highlight the importance, and add to the understanding of the effects of combinations of costimulation for optimal activation of an immune response for immunotherapy. These data also therefore provide evidence to suggest the combination of 4-1BBL+IL-12 warrants further study as a novel immunotherapy strategy.

5 General Discussion

The stimulation of T cells through costimulatory receptors has aimed to overcome the ineffective presentation of TAA, inducing an effective T cell response. The tumour specific T cells generated however may not overcome the immunosuppressive environment present within the tumour. Therefore strategies which overcome the immunosuppressive environment of the tumour may provide significant benefits (Choi et al., 2004; Kilinc et al., 2006; King and Seagal, 2005; Valzasina et al., 2005). As the non-specific activation of the immune system (such as Coleys toxin, BCG or IL-2) has provided significant efficacy, comprehensive activation of the immune system may be required to enable an effective anti-tumour response. While the use of costimulation as an immunotherapy is still in its infancy, as the understanding of the immune system increases and optimal combination of costimulation for the activation of not only T cell based immune response, but the immune system as a whole are elucidated the potential for significant benefit is increased. Here I have shown 4-1BBL with the addition of IL-12 activates not only the adaptive T cell immune response but also the NK and NKT innate response possibly facilitating a comprehensive anti-tumour immune response.

The serendipitous discovery of the stimulatory effect of the combination of 4-1BBL and IL-12 on the proliferation of NK cells in healthy donors during the investigation of T cell responses is novel and has highlighted an important and potentially beneficial effect for this combination if used as an immunotherapy. These data have highlighted the potential of 4-1BBL to induce the proliferation and sustained expansion of NK cells, although 4-1BBL was shown not to increase survival of NK cells compared to IL-12 stimulation. Further study of the mechanisms responsible for these effects of 4-1BBL on NK cells is required. It would also be interesting to further

examine the differentiation of $CD56^{dim}$ NK cells to a $CD56^{bright}$ state; it would be particularly prudent to determine whether this phenotype was maintained in extended culture or whether these cells revert to a $CD56^{dim}$ state. Initial extended culture experiments suggested there may be a population of $CD56^{dim}$ cell appearing after long term culture, unfortunately these are only preliminary data and therefore further work is needed to characterise and validate these observations. If this were the case it could be suggested that a population of $CD56^{dim}$ cells cycle through a $CD56^{bright}$ state then return to a $CD56^{dim}$ phenotype, rather than being terminally differentiated.

It would also be of great interest to further examine the responses of NK cells following culture, given the suggested role of 4-1BBL in the generation of memory T cells and the recent studies showing the generation of a 'memory-like' NK cell population following initial stimulation by cytokines *ex vivo* or interactions with DC *in vivo* (Cooper et al., 2009; Shimizu and Fujii, 2009; Sun et al., 2009). However a consensus of whether the NK cells characterised as memory cells represent a true memory population, as defined in T cell biology, has yet to be reached. Additionally these studies were performed in murine models rather than human systems; the ability of human NK cells to form a memory-like cell has not yet been reported. As the size of the initial immune response dictates the development of T cell memory (Prlic et al., 2007; Seder and Ahmed, 2003) the ability of 4-1BBL to stimulate significant expansion of NK cells may facilitate the production of memory NK cell properties by expanding specific NK cell populations. It would be interesting to determine if the response of NK cells which received 4-1BBL costimulation during initial interaction with a target was either enhanced or skewed in any way following reencounter with a target cell.

In concert with the study of 'memory' responses it would also be interesting to study firstly the diversity of inhibitory and activating receptor repertoires which are expressed following expansion using this system and secondly test whether 4-1BBL+IL-12 stimulation with the use of recombinant agonist antibodies to provide specific activating or inhibitory signals could be used to expand NK cells with specific inhibitory and activating receptor repertoires; if this were possible this could provide a novel technique for the *ex vivo* generation of NK cells with defined repertoires for adoptive transfer.

Importantly I have also shown the adenoviral vector directed expression of 4-1BBL+IL-12 is capable and sufficient to induce the short and long term expansion of functional NK cells derived from RCC patients. Significantly these data also highlight the benefit of a combination of costimulatory molecules for optimal efficacy. Interestingly as mentioned previously the synergy of 4-1BBL+IL-12 was shown by Xu et al. (2004) to promote the rejection in mice of poorly immunogenic melanoma tumours, which was not rejected by stimulation with 4-1BBL or IL-12 alone. These data encouragingly correspond to data from the *in vitro* RCC patient results. The validity and insight of further *in vivo* studies in mouse however may be of limited use given that mice do not possess CD56 bright and dim NK subsets and the combination of 4-1BB stimulation and IL-12 has been shown to be superior or required in tumour models and to be dependent on NK cells (Chen et al., 2000; Martinet et al., 2000; Xu et al., 2004). Therefore work moving towards clinical trial of adenoviral vector delivery of 4-1BBL+IL-12 maybe warranted.

As discussed the Campana group has developed a similar model system utilising IL-15+4-1BBL expressed by K562 cells, allowing the *ex vivo* expansion of NK cells; the large scale expansion of clinical grade NK cells has been reported, as have *in vivo* mouse data (Fujisaki et al., 2009b). From these data I believe that a clinical trial utilising adoptive transfer of *ex vivo* expanded NK cells for the treatment of ALL/AML may soon be undertaken. Given the success of adoptive transfers of KIR mismatched NK cells for the treatment of AML the results of this trial will be of great interest. Further work to study the effects of 4-1BBL+IL-12 stimulation of NK cells for *ex vivo* expansion are therefore also warranted. Given the success of NK cell therapy for AML but relatively poor outcomes in ALL, it would also be of interest to examine the development of 4-1BBL+IL-12 stably transfected 'off the shelf' cell reagents or modified autologous tumour cells as a vaccine for lymphocytic leukaemia's. Whereby following chemotherapy/conditioning 4-1BBL+IL-12 could be used to stimulate NK cell expansion and function *in vivo*, either of autologous NK cells or KIR mis-matched donor NK cells.

It may also be warranted to explore methods to enhance the effect of 4-1BBL stimulation. 4-1BBL like other TNF superfamily ligands such as CD40L and FasL is subject to cleavage by metalloprotease (Elmetwali et al., 2009). Cleavage of 4-1BBL is believed to occur following interaction with 4-1BB (Salih et al., 2001). It is possible that soluble 4-1BBL is able to antagonise the function of 4-1BBL as cross-linking of 4-1BB by 4-1BBL is required to stimulate T cell responses (Rabu et al., 2005). The creation of a non-cleavable form of CD40L has increased its effect by retaining CD40L on the cell surface (Palmer et al., 2007). Therefore it maybe of interest to examine the effects of cleavage of 4-1BBL in this model system; the

production of a non-cleavable 4-1BBL may allow enhanced costimulation by prolonging the time 4-1BBL is available on the cell surface. However the cleavage of 4-1BBL may be a mechanism by which the levels of stimulation can be controlled during activation; it may be that some of the deleterious effects attributed to anti-4-1BB agonist antibodies are due to the quantitative and qualitatively differences in signals delivered by prolonged stimulation of 4-1BB by agonistic antibodies (Schabowsky et al., 2009). Initial studies I have performed did not show a significant difference in the levels of 4-1BBL expressed by adenoviral vector on OVCAR-3 and A549 with the addition of metalloprotease inhibitors; however this was done in isolation without the addition of PBMC (data not shown).

Likewise it may also be interesting to examine the use of other cytokines alone or in combination with IL-12, in order to determine if alternative cytokines alter the outcome of stimulation, and the cytokine requirements for 4-1BBL responsiveness. This is especially pertinent given the differences in subset proliferation in this system and the K562-4-1BBL-IL-15 system utilised by Imai et al. (2005). Therefore there is further scope to enhance this system, and the understanding of NK cell biology.

In conclusion I have developed a novel system for the activation and expansion of NK cells, with a preference for the expansion of the $CD56^{bright}$ subset, these cells have been shown to be functional. Notably I have shown that a combination of 4-1BBL+IL-12 is required to expand NK cells from RCC patients. Given the progression of 4-1BB stimulation into the clinic these data are of particular importance. The literature suggests there is a significant potential for the use of NK cells for the immunotherapy of cancer. Therefore these data are of value.

Consequently further study of the stimulation of NK cell responses and the ability to simultaneously enhance the innate and adaptive immune responses requires and warrants further investigation.

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