Preclinical studies of adenovirus-specific T-cells for adoptive transfer to haemopoietic stem cell transplant recipients

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Abstract

Allogeneic stem cell transplantation (SCT) is the only curative treatment option for many haematological malignancies. Adenovirus (Ad) infections are a significant cause of morbidity and mortality post SCT. Lack of effective anti-viral treatment for Ad disease has led to the development of adoptive immunotherapy of Ad-specific T-cells as a promising therapeutic option for patients in this setting. The aim of this project was to establish preclinical criteria for the development of a clinical trial comparing two T-cell enrichment methods- multimer selection and cytokine secretion selection to enrich Ad-specific T-cells for the purposes of adoptive transfer directly without the need for *in vitro* culture.

Eight pHLA tetramers containing HLA class I restricted Ad epitopes were generated and their ability to identify and enrich Ad-specific T-cells investigated. HLA A*01 TDL tetramer consistently detected T-cells in all (13/13) healthy adult donors screened. Frequency and enrichement of Ad-specific T-cells by cytokine secretion and selection was also investigated. Despite the low frequency of Ad-specific T-cells, clinical grade enrichment was feasible by both methods. T-cells selected by both methods were then characterised for homing and proliferative potential. Ad-specific T-cells identified by either method had a high proliferative potential, possessed a novel minimally differentiated memory phenotype, were cytotoxic towards Ad species responsible for infections in SCT recipients and capable of limiting virus replication. In conclusion, Ad-specific T-cells enriched by multimer selection or cytokine secretion selection are suitable for adoptive transfer to patients with Ad infection following HSCT. Both methods also allow the monitoring of Ad-specific immune reconstitution after adoptive transfer.

Publications related to this thesis

Adenovirus vector-specific T-cells demonstrate a unique memory phenotype with high proliferative potential and co-expression of CCR5 and integrin $\alpha_4\beta_7$. Chakupurakal G, Onion D, Cobbold M, Mautner V, Moss PAH AIDS 2010 Jan 16;24(2):205-10.

HLA-peptide multimer selection of adenovirus specific T-cells for adoptive T-cell therapy of adenovirus infections post haematopoietic stem cell transplantation. Chakupurakal G, Onion D, Cobbold M, Mautner V, Moss PAH; Internationational Congress of Immunology, Japan, August 2010. Invited for oral and poster presentation (WS/PP-069-07)

Abstract: Adenovirus infection following haematopoietic stem cell transplantation affects 5-28% patients, with a mortality rate of 10%. There are currently no pharmaceutical agents licensed for treatment in this setting. T-cells are critical for the control of adenovirus infection and thus adoptive T-cell therapy is an attractive therapeutic option. The role of CD8 T-cells in clearing adenovirus infection is not fully understood as their low frequency hinders studies in healthy donors. We generated HLA-peptide multimers (tetramer) for 8 HLA class I restricted epitopes from the adenovirus hexon protein, which are highly conserved across adenovirus species. Epitope specific T-cells from healthy donors were characterised in terms of frequency, phenotype and functionality. These cells have a minimally differentiated central memory phenotype; $CD45RA^+$, $CD45RO^+$, $CCR7^+$, $CD62L^{low}$, $CD27^+$, $CD28^+$ and $CD57^-$. Upon antigen stimulation (peptide or virus) Ad specific T cells produce IFN-γ and proliferate *in vitro* on average 450 fold over 10 days. Following proliferation, phenotype switches to $CD45RO^+$, $CCR7^-$, $CD62L^{high}$, $CD27^+$, $CD28^+$ and $CD57^-$, consistent with an effector memory phenotype. Despite their low frequency

(<0.2% of CD8 T-cells), we have established that HLA-peptide multimer stained cells can be enriched for adoptive immunotherapy using magnetic bead technology and that they retain their response to antigen and effector function post selection. Their minimally differentiated phenotype and high proliferative capacity should permit rapid establishment *in vivo* post adoptive transfer, increasing therapeutic options for patients with adenovirus infection. The efficacy of these cells needs to be evaluated in the setting of a clinical trial.

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Abbreviations

aa	Amino acid
Ab	Antibody
Ad	Adenovirus
ACT	Adoptive cell therapy
ADA	Adenosine deaminase
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
AP	Alkaline phosphatase
APC	Antigen presenting cell
APC	Allophycocyanin
APS	Ammonium persulphate
ATG-	Anti-thymocyte globulin
ATP	Adenosine-5'-triphosphate
ATT	Adoptive transfer of T-cells
BAC	Bacterial artificial chromosome
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAR	Coxsackievirus and adenovirus receptor
CCS	Cytokine capture system
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxy fluorescein diacetate, succinimidyl ester
CLIP	Class II associated invariant chain peptide
CMV	Cytomegalovirus
Con A	Concanavalin A
Ct	Cycle threshold
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte antigen-4
DBP	DNA binding protein
DC	Dendritic cell

dH_2O	De-ionised water
DLI	Donor lymphocyte infusion
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
E1	Adenovirus early region 1
E3	Adenovirus early region 3
EBV	Epstein-Barr virus
EBMT	European bone marrow transplant
EDTA	Ethylenediamine tetra acetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
ER	Endoplasmic reticulum
env	Envelope
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Acceleration due to gravity
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTP	Guanosine triphosphate
G	Gray
GvHD	Graft versus host disease
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid); 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HEK	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hr	Hour(s)

HS	Human Serum
HSV	Herpes simplex virus type
HHV	Human herpes virus
HSCT	Haemopoietic stem cell transplantation
HTLV	Human T lymphotrophic virus
ICS	Intracellular staining
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
iCasp9	Inducible caspase 9
i (NOS)	Inducible nitric oxide synthase
IPTG	Isopropyl- β-D-1-thiogalactopyranoside
ITR	Inverted terminal repeat
IV	Intravenous
IU	International Units
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
LB	Luria broth
LAMP	Lysosome-associated membrane protein
LCL	Lymphoblastoid cell line
LDA	Limiting dilution assay
Luc	Luciferase
M	Molar
mAb	Monoclonal antibody
MDDC	Monocyte derived dendritic cell
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
Mn. Fl.	Mean fluorescence

Mock	Mock infected cells
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
$MgCl_2$	Magnesium chloride
MUD	Matched unrelated donor
NaCl	Sodium chloride
nef	Negative factor
ng	Nanogram
NK	Natural killer
nM	Nanomolar
nm	Nanometer
NPC	Nuclear pore complex
NO	Nitric oxide
nt	Nucleotide
ntr	Nitroreductase
OD	Optical density
ORFs	Open reading frames
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerytherin
PEG	Polyethylene glycol
pfu	Plaque forming unit
pg	Picogram
PHA-L	Phytohaemagglutinin-leucoagglutinin
pHPMA	Poly-[N-(2-hydroxypropyl) methacrylamide]
PI	Post infection
PI3K	Phosphoinositol 3 kinase
PKB	Protein kinase B
PKC	Protein kinase C
pM	Picomolar
pMHC	peptide major histocompatibility complex
ppm	Parts per million
pol	Polymerase

PTLD	Post transplant lymphoproliferative disorder
pTP	Precursor terminal protein
QPCR	Quantitative polymerase chain reaction
RID	Receptor internalisation and degradation
RNA	Ribonucleic acid
RNAi	Interfering RNA
rRNA	Ribosomal RNA
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-QPCR	Real time- Quantitative polymerase chain reaction
SEB	Staphylococcal enterotoxin B
SDS	Sodium dodecyl sulphate
SFC	Spot forming cell
SKALP	Skin-derived antileukoproteinase
SLPI	Secretory leukoprotease inhibitor
$T_{10}E_1$	10 mM Tris, 1 mM EDTA buffer
T-ALL	T-cell acute lymphocytic leukaemia
TAP	Transporters associated with antigen processing
TBI	Total body irradiation
TCR	T-cell receptor
TEMED	N, N, N', N'-tetramethylethylenediamine
Th1	T-cell helper type 1
Th2	T-cell helper type 2
T_m	Melting temperature
TNF-α	Tumour necrosis factor alpha
TP	Terminal protein
TRAIL	TNF related apoptosis-inducing ligand
TRM	Transplant related mortality
T_{reg}	Regulatory T-cell
Tris	Tris (hydroxymethyl) methylamine
Tween	Polyoxyethylene-sorbitan monooleate
U	Units
UV	Ultraviolet light
V	Volts

v/v	Volume by volume
WMNBS	West Midlands National Blood Service
WT	Wild type
w/v	Weight by volume
XMP	Xanthine monosine phosphate
β-gal	Beta-galactosidase
λ	Wavelength
µg	Microgram
µl	Microlitre
µM	Micromolar

1 Introduction

1.1 T-cells

1.1.1 T-cell development

Pluripotent stem cells in the bone marrow and foetal liver give rise to common lymphoid and myeloid progenitors. Common lymphoid progenitors (CLP) differentiate into B-cells within the bone marrow but those committed for T-cell differentiation enter into the blood circulation. These T-lineage CLPs embark on a proliferation differentiation programme, i.e, thymopoiesis which occurs in the thymus, a primary lymphoid organ located in the mediastinum. Within the thymic cortex CLPs are initially phenotypically triple negative due to absence of the cell surface receptors $CD3^-$, $CD4^-$, $CD8^-$. CLPs undergo massive antigen-independent expansion facilitated by IL-7 supplied by the thymic cortical cells (Bhandoola & Sambandam, 2006; Godfrey et al, 1993) and differentiate to $CD3^+$, $CD4^-$, $CD8^-$ double negative (DN) $CD44^{high}CD25^{low}$ thymocytes. These thymocytes then become $CD25^{high}$ $CD44^{high}$ but retain their ability to differentiate to T-cells, NK-cells and dendritic cells. The next developmental subset is DN $CD25^{high}$ $CD44l^{ow}$ when they become T-cell lineage committed to become a pro T-cell (Bhandoola & Sambandam, 2006). The pro-T-cell undergoes TCR β gene rearrangement and expresses the TCR β chain with pre-TCR α chain called the pre-TCR on the cell surface to become a pre-T-cell. Failure to express the pre-TCR results in programmed cell death and the remaining T-cells after several rounds of proliferation achieve TCR rearrangement and express the complete TCR on cell surface as well as become double positive (CD4+, CD8+), *i.e.* the immature T-cells [extensively reviewed by (Res & Spits, 1999)].

1.1.1.1 *Central tolerance*

Central tolerance is the mechanism by which the thymus maintains a population of T-cells in the periphery with minimal autoreactivity and maximal recognition of foreign antigens

(Mathis & Benoist, 2004). The immature T-cells enter the thymic medulla and differentiate into single positive cells, *i.e* CD4+ CD8- or CD4- CD8+ T-cells. The immature T-cells encounter dendritic cells which enable positive and negative selection. The thymic epithelium expresses a transcription factor called autoimmune regulator (AIRE) which allows the promiscuous expression of tissue specific antigens (Hogquist et al, 2005; Palmer, 2003). Immature T-cells with receptors that bind to self major histocompatibility complex molecules with low avidity are positively selected. Negative selection is a mechanism by which the thymus eliminates T-cells that bind with a high avidity to self-antigen or fails to bind at all and thereby maintaining a population of selected T-cells tolerant to self peptides as well as removing a significant proportion of T-cells that bind to a ubiquitous protein in the periphery with high avidity. Following weak recognition of class I MHC molecules CD4 is downregulated and conversely CD8 if class II MHC molecules are recognised. The surviving pool of selected weakly self-reactive single positive mature T-cells (Naïve T-cells) are capable of recognising foreign peptide antigens displayed by self MHC molecules on antigen presenting cells (APCs) in the peripheral tissues. Naïve T-cells are released into the peripheral blood circulation where they home to secondary lymphoid tissues (lymph nodes, spleen, skin and mucosa) See Figure 1-1.

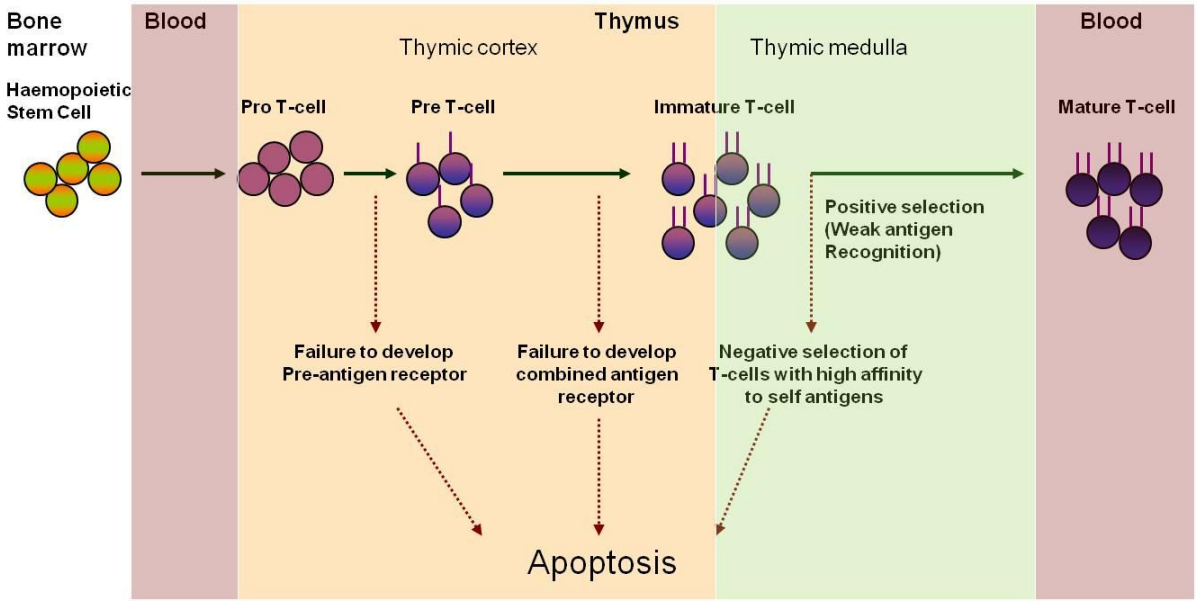

Figure 1-1 T-cell development of haemopoietic stem cells in the thymus

Haemopoitic stem cells from the bonemarrow undergo various stages of differentiation within the thymic cortex and attain a TCR and become double (CD4 and CD8) positive. These T-cells become single (CD4 or CD8) positive by positive selection. Central tolerance mediated by the thymus also results in the negative selection of T-cells with high affinity to self antigens.

1.1.1.2 *Peripheral tolerance*

Although thymic negative selection deletes T-cells of high avidity for self-antigen resulting in the release of <5% of thymocytes, some low avidity T-cells that recognise self antigens may be spared (Redmond et al, 2005; Redmond & Sherman, 2005). Peripheral tolerance is the mechanism by which these self-reactive T-cells become unresponsive to tissue antigens achieved mainly by anergy, deletion or suppression of the T-cells.

Different mechanisms are involved in peripheral tolerance including

1. The most important is probably the role of regulatory T-cells ($CD4^{+}$ $CD25^{high}$, $CD127^{low}$, Foxp3 +) which inhibit autoimmune responses [extensively reviewed by (Feuerer et al, 2009)].

2. Repeated exposure of T-cells to self antigen in the absence of costimulation may result in activation induced cell death triggered by the expression of death receptors and its ligands such as Fas and Fas ligand (Walker & Abbas, 2002)

3. T-cells become cytotoxic only following priming with an antigen presenting cell. The TCR binds with high affinity to the HLA-peptide complex but in the absence of costimulatory and cytokine signals the T-cell does not attain an effector function (Chai et al, 1999).

4. Antigen presenting cells that are not activated by cell death or microbial products will not become activated and hence self antigen presented in this setting may result in anergy manner (Dhodapkar et al, 2001; Levings et al, 2005; Redmond et al, 2005)

5. AIRE may be expressed in small amounts in secondary lymphoid organs (Gardner et al, 2009)

6. Immune privileged sites such as testis, central nervous system and eye. These sites may be difficult to access i. e. blood-ocular or blood brain barrier or the organ may produce immunosuppressive cytokines such as TGF-β. Another mechanism is by facilitating regulatory T-cell development (Stein-Streilein, 2008).

1.1.2 Antigen processing

All class I HLA molecules have a conserved heterotrimeric structure of highly polymorphic HLA protein (heavy chain; 44-47kD), peptide and non-polymorphic β_2 microglobulin (β_2m) (light chain; 12kD). The non polymorphic βchain is encoded by genes on the β_2 microglobulin gene on chromosome 15. The HLA protein or α chain has 5 domains:- 2 peptide binding domains (α_1 and α_2), 1 immunoglobulin like domain (α_3), the trans-membrane region and the cytoplasmic tail. The function of Class I MHC molecules is to

report intracellular events (such as viral infection, presence of intracellular bacteria, cellular transformation) to CD8 T-cells.

The majority of antigenic peptides are products of virus translation, derived from either defective ribosomal products (DRiPs) or short- lived ribosomal products (SLiPs) (Dolan et al; Princiotta et al, 2003). These peptides are then rapidly degraded by cytosolic amino peptidases in the proteasome but a minority bind to the Transporter associated with Antigen Processing (TAP) and are translocated into the ER. The TAP is a heterodimer comprised of TAP1 and TAP2 which bind to peptides with some selectivity to peptides thus reducing the repertoire which are pumped into the endoplasmic reticulum (ER). Once in the ER peptides may be trimmed to optimum length by the aminopeptidase ERAP1 (York et al, 2006).

Newly synthesized empty HLA class I molecules are tethered via tapasin and calreticulin to the TAP where peptides are brought into close proximity to the HLA proteins facilitating the binding of the peptides to the peptide binding grove. β_2m stabilizes the HLA-peptide complexes. The complexes are transported to the Golgi apparatus and reach the cell surface. It has been suggested that only one in a million proteasome – derived peptide reach the cell surface bound to HLA proteins (Yewdell & Bennink, 1992; Yewdell & Princiotta, 2004). A typical somatic cell has several hundred thousand peptides for immune surveillance by CTLs (Vyas et al, 2008; Yewdell, 2002).

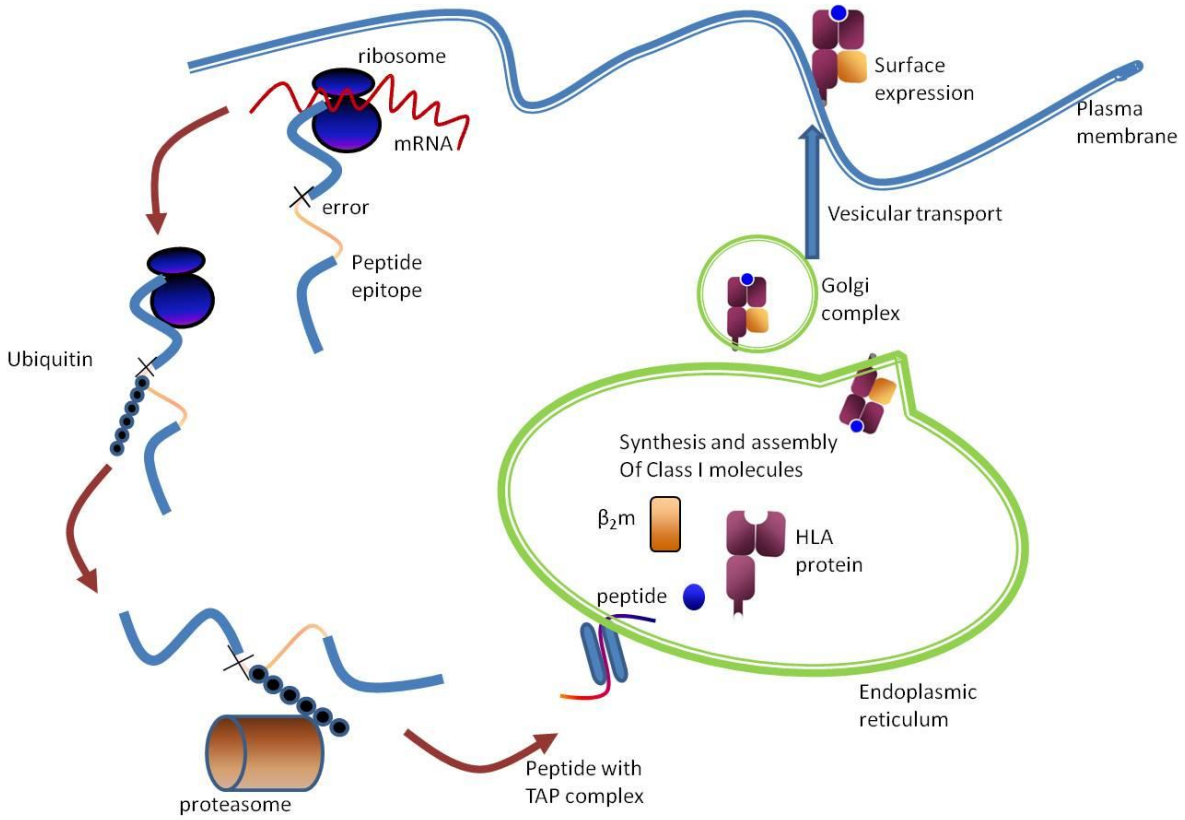

Figure 1-2 Class I antigen presentation

Biosynthesis of Class I MHC peptide complexes involves 6 main steps- Acquisition of antigenic peptides from proteins that are not folded due to premature termination or misincorporation, tagging of the misinterpreted peptide for destruction by the ubiquitin enzyme, Ubiquitylation is followed by degradation by proteolysis in the proteasome, the peptides are delivered to the endoplasmic reticulum (ER) (where the HLA Class I molecule is generated) via the transporter associated with antigen processing (TAP) complex, peptides bind to the MHC class I molecules followed by the display of the peptide-MHC class I complex transported by Golgi bodies on the the cell surface. β_2m, β_2-microglobulin. Adapted from Nature reviews Vyas (Vyas et al, 2008)

Class II MHC molecules have a conserved structure comprising a hetrotrimer of (32-34kD), β chain (29-32kD) and a peptide, homologous to Class I MHC molecules except that the binding grove is open, allowing longer peptides to bind. Each of the class II α and β chains has four domains: the peptide-binding domain (α_1 or β_1), the immunoglobulin-like domain (α_2 or β_2), the transmembrane region, and the cytoplasmic tail. Class II peptides from degraded proteins in endocytes are degraded in a manner similar to the Class I molecules, α and β chains are synthesized in the ER. αβ MHC class II dimer associates with an invariant chain (Ii), aided by ER chaperones such as calnexin (Benaroch et al, 1995; Lindner, 2002). The Ii

chains are degraded on transfer within the Golgi apparatus and the MHC class II molecules are loaded with antigenic peptides. The resulting peptide-MHC complexes are then delivered to the cell surface (Busch et al, 2005).

1.1.3 Antigen presentation

Antigen presenting cells (APCs) such as dendritic cells and macrophages capture and display antigens to 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. 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 antigens presented via MHC class I molecules to CD8 T-cells are approximately 8-10 aminoacids long as opposed to those presented by MHC class II molecules to CD4 T-cells are 12-15 aminoacids in length. All somatic cells possess MHC class I and can therefore present antigen to CD8 T-cells, in contrast predominantly antigen presenting cells such as NK-cells, dendritic cells and B-cells express MHC class II molecules. CD8 T-cells participate in cell mediated immunity by active killing of host cells whereas CD4 T-cells secrete cytokines maintaining CD8 T-cell memory responses, facilitating B-cell differentiation as well as macrophage activation (Zhu & Paul, 2008). An additional costimulatory signal (signal two) provided by the APCs followed by antigen presentation results in T-cell activation proliferation and acquisition of effector functions [extensively reviewed by (Sharpe & Freeman, 2002)].

Costimulatory receptor: ligand pairs belong to two main families

a) Immunoglobulin superfamily such as CD28: CD80/CD86

b) Tumor necrosis factor receptor family such as 4-1BB: 4-1BBL

Additional cytokine signals (signal three) such as IL-12 may be required for attaining full effector functions (Croft, 2003b; Curtsinger et al, 2003; Schmidt & Mescher, 2002).

1.1.4 T-cell subsets

a) Naïve and Memory T-cells

Memory is the characteristic by which T-cells can mount a rapid response to subsequent antigen encounter. On antigen exposure, T-cells undergo clonal expansion followed by the contraction phase when ~90% of the responding T-cells apoptose and a few cells enter into the memory phase, dependent on the initial response (Obar & Lefrancois, 2010; Seder & Ahmed, 2003). CD4 and CD8 T-cells have been shown to develop memory as a linear process, i.e. naïve to effector to memory governed by signals during the immune response (Bannard et al, 2009; Harrington et al, 2008). As commitment to memory formation has been shown to occur during the initial stages of T-cell division the earliest signalling events may dictate the final outcome of memory and effector T cell development (Chang et al, 2007).

b) T_{H1} and T_{H2}

Naïve T-cells on antigen recognition differentiate into two distinct subsets of T-cells (Amsen et al, 2009; O'Garra, 1998; Zhu & Paul, 2008). Once polarized the T-cells are committed to the T_{H1} or T_{H2} type activity and they do not revert to the naïve state, though plasticity between T_{H1} and T_{H2} T-cells has been recognised (Murphy et al, 1996; O'Shea & Paul).

i. T_{H1}T-cells are characterised by IFN-γ production, involved in cellular immunity against intracellular pathogens such as viruses and tumours. These cells are polarised by IL-12 and IFN-γ through induction of STAT4 and STAT1.

ii. T_{H2} T-cells produce IL-4, IL-5, and IL-13 and are required for humoral immunity as well as critical for immunity to extracellular pathogens such as helminths. Polarisation requires IL-4 mediation as well as induction of STAT6 followed by GATA3 expression (Mosmann & Coffman, 1989).

c) Regulatory T-cells (T-regs)

T-regs are actively engaged in peripheral tolerance and prevention of autoimmune disorders [extensively reviewed by (Campbell & Koch; Feuerer et al, 2009; Sakaguchi et al, 2010)]. They are characterised by expression of CD4, $CD25^{high}$ and $Foxp3^{high}$ (Forkhead boxp3) $CD127^{low}$. They are of two main types; a) Naturally T-regs occurring produced in the thymus, b) Induced T-regs produced in the periphery following exposure to antigen in the presence of cytokines such as IL-2 or TGF-β (Chen et al, 2003). Constitutive CTLA-4 expression enable T-regs to suppress T-cell activation by binding to CD80 and CD86 on the APC, thereby blocking further T-cell activation (Wing et al, 2008). T-regs can recognise a range of antigens such as self-antigens targeted in autoimmune disease, tumour associated antigens and allogeneic transplantation antigens there by suppressing autoimmunity, hamper tumour immunity and suppress graft rejection.

d) T_{H17}

These are distinct subsets of CD4 T-cells that differentiate in the prescence of cytokines such as IL-23 but in the absence of cytokines of transcription factors required for differentiation of

T_{H1} or T_{H2} cells (Harrington et al, 2005). They are thought to play a role in combating extracellular pathogens such as bacteria and fungi as well as in suppressing autoimmunity (Chen & O'Shea, 2008).

1.1.5 T-cell effector functions

Following antigen recognition and activation, CD8 T-cells actively kill host cells by differentiating into effector cytotoxic T-cells or secrete cytokines that suppress virus replication. CD4 T-cells on the other hand secrete cytokines that support CD8 T-cell memory responses, facilitate B-cell differentiation as well as macrophage activation (Zhu & Paul, 2008). Apart from CD8 T-cells, natural killer (NK) cells, NK T-cells, γδ T-cells and CD4 T-cells are capable of target recognition and cytolysis (Lefrancois & Obar).

Cytolysis occurs via two major mechanisms-

a) The perforin / granzyme pathway results in granule exocytosis following pMHC recognition (Catalfamo & Henkart, 2003). Cytotoxic T-cells primarily use this pathway. They release perforin present as a monomer in the granules of CTLs and NK cells (Waterhouse et al, 2004). This pore forming protein undergoes polymerization in the membrane of the target cell and forms an aqueous channel. Granzymes secreted by the CTL enter the target cell through these channels (Metzgar et al, 2005; Shresta et al, 1998). The advantage of this method of killing is that the CTL is able to administer the cytotoxic granule proteins, detach from the target cell to to attach to another infected cell.

b) The Fas/FasLigand (FasL) pathway where the cells surface receptor Fas on T-cells binds to the FasL on the target cell (Brown et al, 2009). This method is used by CD4 T-cells primarily and Fas mediated killing occurs in the absence of TCR stimulation

("bystander killing"). Antigen presenting B-cells express FasL and are susceptible to Fas mediated cytolysis.

The caspase cascade is activated by either pathway resulting in apoptosis of the target cells (Berke, 1995). In summary T-cells with their effector functions maintain a constant check on foreign antigens as well as dampen immune responses to self antigens with their tolerance mechanisms.

1.2 Haemopoietic stem cell transplantation

Haemopoietic stem cell transplantation (HSCT) can be defined as the transfer of haemopoietic stem cells harvested from recipient to the recipient (autologous HSCT) or donor to recipient (allogeneic HSCT). HSCT is now an established treatment modality for a variety of diseases including leukaemia, lymphoma, myeloproliferative diseases, myelodysplasia, bone marrow failure syndromes, congenital immune deficiencies, enzyme deficiencies and haemoglobinopathies [reviewed by (Chinen & Buckley, 2010)]. It not only restores normal function in inherited or acquired deficiencies of the haemopoietic or immune system but also can rescue haemopoiesis after high-dose chemo or radiotherapy for malignancy and promote powerful graft versus leukaemia effect (Horowitz et al, 1990; Kolb et al, 1990; Ljungman et al, 2010; Mathe et al, 1965).

The goal of HSCT is lifelong engraftment of administered cells, resulting in some or all of the recipient's haemopoietic system being repopulated and replaced by the HSCT graft. This is referred to as full donor engraftment. These stem cells are capable of establishing long term stable haematological and immunological function (Thomas & Blume, 1999). However, 'mixed chimerism', wherein elements of both donor and recipient haemopoietic system

survive, may be sufficient to cure the underlying condition in some clinical settings (Gaziev et al, 2008; Huss et al, 1996).

The first report of allogeneic HSCT in humans was in 1957 (Thomas et al, 1957) but it took another 11 years before a successful outcome was observed (Gatti et al, 1968). The pioneering work of Nobel laureate E. Donnell Thomas, whereby radiation doses, bone marrow harvest techniques and post transplant support were defined, using canine models, was fundamental to this success [reviewed by (Perry & Linch, 1996)]. When Jean Dausset recognised the human leukocyte system in 1965 another major leap in HSCT was made (Dausset, 1974). In the 2007 European Bone Marrow Transplant (EBMT) Registry report, 25,563 HSCT were performed of which 10,072 (39%) were allogeneic, 15,491 (61%) autologous and 3606 additional transplantations for relapsed or planned second transplants (Gratwohl et al, 2009). The main indications for transplantation were lymphoproliferative disorder (57%) and leukaemia (32%).

Though allogeneic HSCT can cure or improve outcome in a wide spectrum of diseases, it is associated with significant morbidity and mortality due to regimen-related toxicity. The main risk factors affecting the outcome of a HSCT are the stage of the disease, age of the patient, the time interval from diagnosis to transplant and for allogeneic HSCT the donor/recipient histocompatibility and the donor-/recipient sex combination (Ljungman et al, 2010).

1.2.1 Conditioning regimens

The main objective of conditioning is the generation of space within the bone marrow for successful engraftment, immunesuppresion and disease eradication (Bacigalupo et al, 2009). A myeloablative conditioning results in complete eradication of bone marrow cells and regeneration does not occur in the absence of stem cells. The Seattle Marrow Transplant Team began HLA-compatible sibling marrow transplants for advanced hematological

malignancies with a myeloablative conditioning regimen consisting of cyclophosphamide or total body irradiation (TBI) (Thomas et al, 1977). High incidence of relapse led to the combination of TBI and cyclophosphamide (Devergie et al, 1991), the current gold standard. Fractionated TBI, initially in canine models and then in the clinic, had fewer long-term sequelae (Clift et al, 1982). Busulphan —an alkylating agent that kills cells by cross-linking DNA—was introduced as an alternative to TBI as early as 1983 (Santos et al, 1983). Randomised control trials demonstrated comparable results when combined with cyclophosphamide but the combination of cyclophosphamide and TBI was superior (Clift et al, 1994). These regimens have a high transplant related mortality (TRM), particularly in patients >45 years of age (Bornhauser et al, 2003; Giralt et al, 1997). Non-myeloblative regimens, introduced in 1994, were primarily used for non-malignant diseases such as haemoglobinopathies and aplastic anaemia (Clift et al, 1994). T-cell depletion of the stem cells though highly effective in the prevention of acute and chronic GvHD increases the risk of graft failure and disease relapse (Delain et al, 1993). The role of immunological tolerance in a state of mixed chimerism led to reduced intensity (RI) conditioning regimens using busulfan in combination with cyclophosphamide or fludarabine (Slavin, 2000). This led to transplantation being offered to patients above 45 with significantly reduced TRM.

1.2.2 Donor sources and categories

HSCs may be derived from three sources, bone marrow (Thomas et al, 1975b) (Thomas et al, 1975a), peripheral blood (Schmitz et al, 1995) or umbilical cord (Gluckman et al, 1997). Peripheral blood stem cells (PBSC) are currently used primarily for HSCT and comprise 98% of the stem cell products in the autologous and 71% in the allogeneic setting. Cord blood is being increasingly used and represents 5% of all allogeneic and 10% of all unrelated HSCTs, as per the EBMT registry.

Donor type is categorised as autologous, syngeneic, HLA-identical sibling, other family donor or unrelated donor (Ljungman et al, 2010). A well matched unrelated donor is defined as a 9/10 or 10/10 identical donor based on HLA- high resolution typing for MHC class I and class II alleles (HLA-A, B, Cw, DRB1 and DQB1 alleles) (Yakoub-Agha et al, 2006). A mismatched unrelated donor is defined as a 6-8/10 matched donor or a less than 8/8 match (not including DQB1) (Lee et al, 2007; Weisdorf et al, 2008).

1.2.3 Immune reconstitution following HSCT

T-cell development in a healthy individual is discussed in section 1.1. Innate immunity (e.g., epithelial barriers, phagocytes, natural killer (NK) cells) typically recovers within weeks post-transplant whereas adaptive immune reconstitution takes longer (Storek, 2008; Storek et al, 2008). In general NK-cells are the first lymphocyte subset to recover, followed by CD8 T-cells (which often reach above normal levels within 2-8 months after HSCT), B cells and CD4 T-cells. Despite adequate count recovery, lymphocyte function is often impaired for years post transplant (Mackall et al, 2000; Nordoy et al, 2001).

Regeneration of lymphocytes following a myeloablative allograft involves two distinct pathways- thymus dependant and independent [extensively reviewed by (Krenger et al) Figure 1-3). After conditioning and stem cell infusion the surviving recipient naive and memory T-cells as well as mature donor T-cells in a T-replete graft undergo thymus independent peripheral expansion. Type of conditioning, degree of T-cell depletion and dose of T-cells in the donor graft are factors that affect this reconstitution (Baron & Sandmaier, 2006; Dumont-Girard et al, 1998; Roux et al, 2000; Roux et al, 1996). Homeostatic T-cell expansion in this pathway is limited by the starting repertoire of T-cells as well as the pathogens encountered resulting in the oligoclonal expansion of antigen-specific T-cells

(Bourgeois & Stockinger, 2006). Mature T-cells in the graft expand in vivo in response to T-cell lymphopenia as well as inflammatory cytokines such as IL-7 and IL-15 released by stromal cells within the lymphoid tissue. Peripheral homeostatic expansion is more efficient for CD8 T-cells compared to CD4 T-cells (Mackall et al, 1997). Following expansion, the T-cells contract due to replicative senescence and activation induced apoptosis (Muraro & Douek, 2006). As the immuneprotection provided by the T-cells regenerating independent of the thymus is transient and limited the thymus dependant reconstitution plays a vital role in the long term adaptive immunity.

In the thymus dependent pathway, lymphocytes regenerate from the BM lymphoid progenitors, thus recapitulating ontogeny and regenerating a naïve immune system similar to that found in a newborn child (Lewin et al, 2002). This pathway is reliant on the stem cell dose, the quality of the engrafted stem cells, mesenchymal stem cells and stroma of the bone marrow, migration of CLPs to the thymus followed by differentiation and maturation as well as release of naïve T-cells into the peripheral circulation. The microenvironment of the thymus is affected by age, therapy and Graft versus Host Disease (GvHD) resulting in the thymic dependant regeneration of naïve cells over months to years post transplant (Storek et al, 2002; Storek et al, 2001b; Weinberg et al, 1995). CD4 counts provide one of the most readily available and predictive markers for the restoration of immune competence. CD4 recovery is associated with diminished risk of infection and improved transplant outcome (Berger et al, 2008; Storek et al, 1997; Talmadge, 2008). B-cell recovery appears to follow a similar path (Small et al, 1990) but is reliant on the functional bone marrow environment which may have been damaged by the preparative regimen (Storek et al, 2001c). Complete humoral reconstitution will eventually restore the naïve and memory B-cell pool.

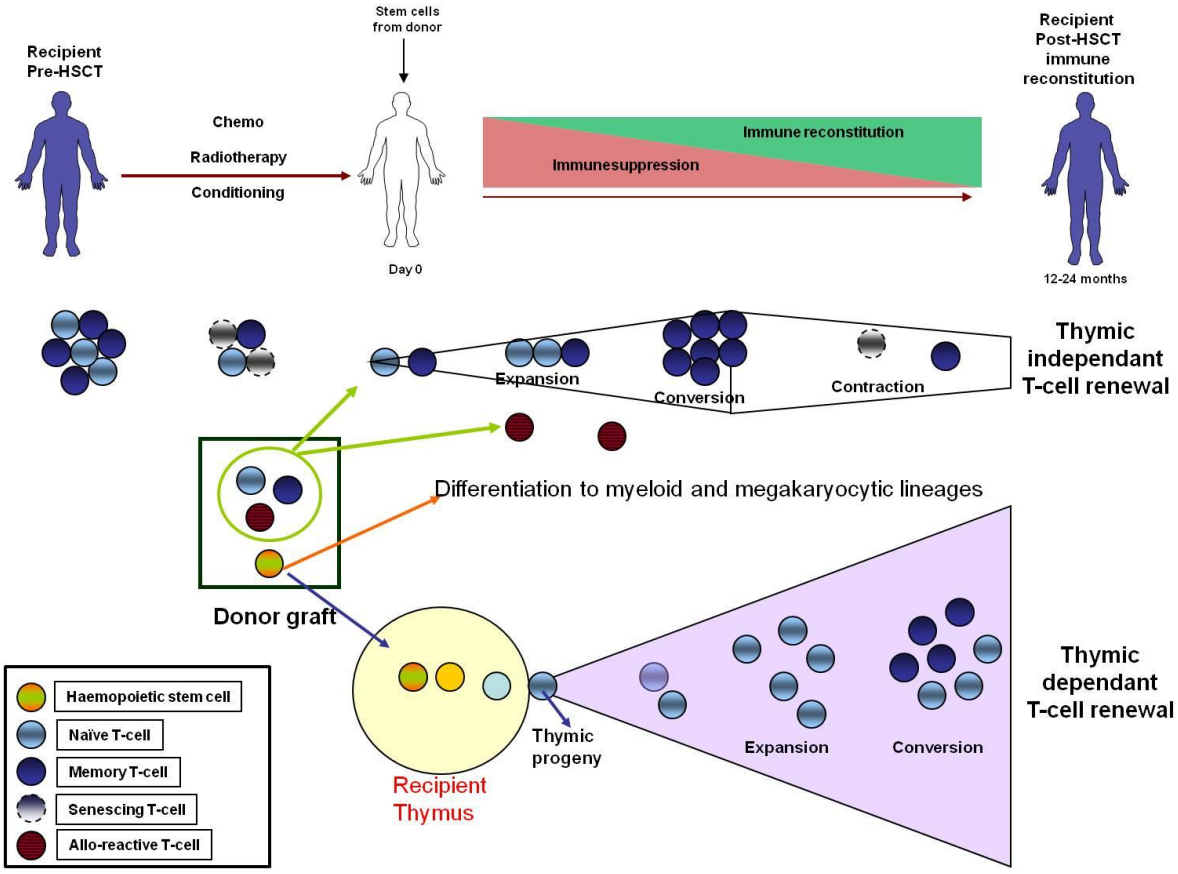

Figure 1-3 Adaptive immune reconstitution following an allogeneic HSCT

The host repertoire of naive and memory T-cells is affected by pre transplant conditioning. These along with mature T-cells from the donor graft expand in response to homeostatic signals and cognate antigen and contribute to the thymic independent T-cell reconstitution. New naive T-cells are generated in the thymus from the engrafted donor stem cells committed to the lymphoid lineage. Thymus dependant T-cell renewal is a slow process and can take up to 12-24 months post transplant adapted from (Krenger et al)

Despite a milder degree of myelosuppression in non-myeloablative conditioning regimens, the severity of lymphodepletion tends to be similar in myeloablative conditioning regimens. In both cases additional iatrogenic immunesuppression to control GvHD impairs immune recovery. Factors determining lymphocyte recovery post HSCT include:

1. GvHD: Higher grade GvHD correlates with the degree of immunesuppression and infectious complications (see section 1.2.4.1)

2. Recipient's age, co-morbidities and infectious exposure prior to transplant (Baron et al, 2006; Storek et al, 2002; Storek et al, 2003)

3. Donor source and conditioning regimen (Maris et al, 2003)
4. Graft associated factors: Count recovery and immune reconstitution following GCSF (granulocyte colony stimulating factor) mobilised peripheral blood stem cells is quicker in comparison to marrow or umbilical cord blood grafts (Storek et al, 2001a; Thomson et al, 2000)
5. CD34 cell count above $3x10^6$ CD34 cells are associated with improved haemopoietic recovery, decreased incidence of fungal infections and improved overall survival (Bittencourt et al, 2002)

1.2.4 Complications post stem cell transplant

HSCT is associated with significant morbidity and mortality (Thomas et al, 1975b). This varies depending on stem cell source, conditioning regimens and underlying diagnosis. High dose chemotherapy and radiotherapy included in conditioning regimens affect all organs and tissues, producing early and late secondary effects of variable intensity. Early complications include nausea, vomiting, mucositis, pain, GvHD, graft failure, infections and multiple organ dysfunctions. Late complications include GvHD related issues, cataract, Sicca syndrome (autoimmune destruction of salivary and lacrimal glands), avascular necrosis, osteoporosis, endocrinopathies as well as secondary malignancies. GvHD and infectious complications are discussed in detail in the following sections.

1.2.4.1 *GvHD*

GVHD occurs when donor T-cells respond to host peptides presented by HLA molecules. The incidence of acute GvHD is directly related to the degree of mismatch between HLA proteins (Loiseau et al, 2007) and hence ideally donors and recipients are matched for 8

alleles (HLA-A, B, C and DRB1) (8/8 matches). The risk of GvHD is further increased by the level of genetic disparities outside the HLA locus e.g.: minor histocompatibility antigens (Edinger et al, 2009).

GvHD occurs when the graft contains immunologically competent cells, the recipient expresses tissue antigens not present in the transplant donor and the recipient is incapable of mounting an effective response to eliminate the transplanted cells (Billingham criteria) (Billingham, 1968). It is mainly classified as acute (occurring prior to 100 days) (Martin et al, 1991) or chronic (occurring after 100 days) (Sullivan et al, 1991) based on the early Seattle experience. The latest National Institute of Health (USA) classification includes overlap syndromes with features of both acute and chronic GvHD (Loiseau et al, 2007).

Acute GvHD manifestations are mainly in the skin (81%), gastrointestinal tract (54%) and liver (50%) (Martin et al, 1991). The incidence and severity of acute GvHD, assessed by Glucksberg's criteria (Glucksberg et al, 1974; Weisdorf et al, 1990), is determined by the extent of involvement of these 3 principal target organs; Table 1-1. Severe GvHD has a poor prognosis; with 25% long term survival for grade III and 5% for grade IV (Cahn et al, 2005). Symptoms include fever and-/or a morbilliform rash and/or severe diarrhoea (Glucksberg et al, 1974). The rash becomes progressively confluent and can involve the entire body surface. It is both pruritic and painful and can lead to marked exfoliation. Eosinophilia and lymphocytosis are followed shortly by hepatosplenomegaly, exfoliative dermatitis, protein-losing enteropathy, bone marrow aplasia, generalised oedema, increased susceptibility to infections and death (Deeg & Antin, 2006). Skin biopsy specimens reveal basal vacuolar degeneration or necrosis, spongiosis, single-cell dyskeratosis, eosinophilic necrosis of the epidermal cells and a dermal perivascular round cell infiltration. Similar necrotic changes can occur in the liver, intestinal tract and eventually most other tissues. Chronic GvHD has a

progressive, quiescent or de novo presentation (Carlens et al, 1998) and is the major cause of late non-relapse death following HSCT (Lee et al, 2002). It affects almost every organ and the manifestations are mostly autoimmune in nature.

Many regimens have been used to mitigate GvHD and in allogeneic HSCT it is necessary to use GvHD prophylaxis. Patients are usually given a combination of methotrexate, corticosteroids and a calcineurin inhibitor daily for 3-6 months depending on whether the donor was a sibling or unrelated donor and also the degree of HLA compatibility (Carnevale-Schianca et al, 2009; Finke et al, 2009; Ogawa et al, 2002). When GvHD becomes established it is extremely difficult to treat. Anti-thymocyte globulin (ATG), steroids, cyclosporine, tacrolimus, anti-IL-2Rα chain antibodies, mycophenolate mofetil, anti-TNF-α inhibitors have been used in this setting with varying degrees of success (Ferrara et al, 2009).

Organ	**Skin**	**Liver**	**Gut**
Grade I	Rash over <25% of body area	Bilirubin 2-3mg/dl	Diarrhoea <500ml/day
Grade II	Rash over 25-50% of body area	Bilirubin 3.1-6mg/dl	Diarrhoea 500ml-1000ml/day
Grade III	Generalised erythroderma	Bilirubin 6.1-15mg/dl	Diarrhoea >1500ml/day
Grade IV	Generalised erythroderma with bullous formation	Bilirubin >15mg/dl	Diarrhoea >2000ml/day or severe abdominal pain with or without illeus

Table 1-1 Acute GVHD staging by the affected organ systems

(Glucksberg et al, 1974)

1.2.4.2 *Infectious complications post HSCT*

The aggressive induction regimens utilised in HSCT may result in multiple organ toxicity. Time to engraftment as well as use of immunesuppressive agents to avoid GvHD increases the risk of bacterial, viral and fungal infections (Frassoni et al, 1996). Despite improvements in therapy and supportive care, infections remain a significant cause of transplant related

mortality accounting for 8% and 17% of deaths following an autologous and allogeneic transplant respectively (CIBMTR, 2009). The use of growth factors for rapid haemopoietic engraftment has reduced the aplastic phase and consequently the febrile neutropenic episodes (Hughes et al, 2002). Better therapeutic knowledge as well as supportive care post transplant has reduced the incidence of infections as well as secondary mortality and morbidity (Hiemenz, 2009; Kontoyiannis et al, 2009).

Infectious complications after a HSCT with myeloblative conditioning can be divided into 3 phases (Mackall et al, 2009). During phase I or pre-engraftment period (<15-45 days after HCT), prolonged neutropenia and breaks in the mucocutaneous barrier result in substantial risk for bacteremia and for fungal infections involving candida species. If neutropenia is prolonged then aspergillus infections and herpes simplex virus (HSV) reactivation can occur. In phase II or period of early immune recovery (30-100 days post HSCT), infections are secondary to impaired cell mediated immunity. The scope and impact of the infections is related to degree of immune suppressive therapy for GVHD. Herpes viruses (particularly cytomegalovirus (CMV)), pneumocystis carini and aspergillus species are common pathogens. In phase III or period of late immune recovery (>100 days post HSCT), patients with chronic GvHD and recipients of allogeneic transplants are at risk. The common pathogens are CMV, HSV and encapsulated bacteria such as pneumococci and streptococci. The risk of infection is directly proportional to the severity of GvHD, status of disease and the degree of immune suppression. Community acquired respiratory viruses are a threat throughout the post-transplant period (see Figure 1-4).

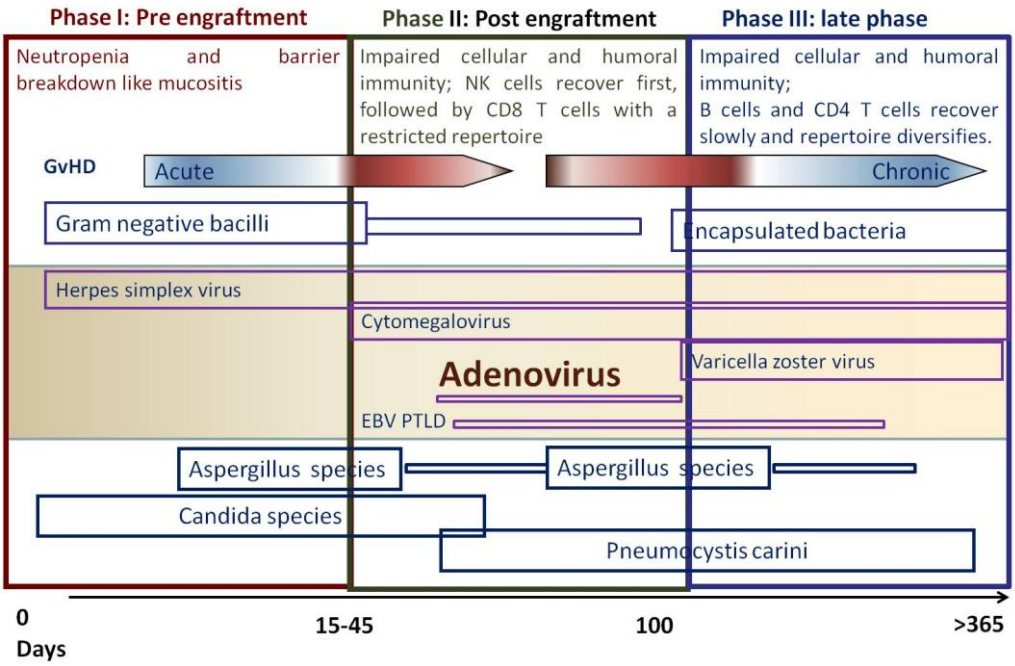

Figure 1-4 Phases of opportunistic infections among allogeneic HSCT recipients

HHV, human herpes virus; NK, natural killer; PTLD, post transplant lymphoproliferative disease. This figure highlights the risk of virus, bacteria and fungus through the different phases post HSCT. Adapted from Mackall (Mackall et al, 2009)

In summary infectious complications following a HSCT are determined by a number of factors including donor-host histocompatibility, disease status, graft type, graft contents, conditioning intensity, neutrophil engraftment, time from transplant and presence or absence of GvHD (Junghanss et al, 2002; Meijer et al, 2004; van Burik & Brunstein, 2007). These are summarised in Table 1-2.

Factor	Risk of infection
Type of transplant	Allogeneic> autologous> syngeneic, depending on graft manipulation, clinical setting and previous therapies
Time from transplant	Higher in the immediate post transplant period
Pre-transplant factors	immunosuppressive therapy, neutropenia, infections
GvHD	Higher with grade III or IV GvHD or chronic extensive GvHD
HLA match	Haplo-identical donors> HLA mismatched unrelated donors> HLA matched unrelated donors> HLA mismatched sibling donors> HLA matched sibling donors> Syngeneic donors
Disease status	Higher with advanced disease at the time of transplant
Donor type	Marrow MUD> Marrow Sibling> PBMC MUD> PBMC sibling
Graft type	Umbilical cord> Marrow> PBMC. Higher with T depleted marrows
Immune suppression post transplant	Higher with immune suppressants e.g.; corticosteroids, ATG, Alemtuzumab
Conditioning intensity	Myeloablative> reduced intensity
Neutrophil engraftment	Increased risk with delay or graft failure

Table 1-2 Risk factors for infection post transplant

Adapted from Mackall (Mackall et al, 2009)

1.2.4.3 *Viral infections post HSCT*

Viruses pose a significant risk following HSCT, especially in patients receiving extensively manipulated products or who require intensive and prolonged post-transplant immune suppression. Persistent herpes viruses like Epstein Barr virus and CMV cause main concern, accounting for up to 20-30% of TRM (Cohen et al, 2007; Jancel & Penzak, 2009). Increasing numbers of viral pathogens have been implicated in infectious complications due to a combination of intensive screening and improved detection methods (Fischer, 2008). Other viruses which have been reported include adenovirus (Ad), BK virus, bocavirus, human

herpes virus (HHV)-6, metapnuemonia virus, para-influenza and respiratory syncitial virus. Though pharmacological agents are available against some of these viruses, most have substantial toxicities and are not effective against all viruses [reviewed by (Leen et al, 2010)]. Adenovirus infections in HSCT recipients is discussed in detail in section 1.4.7.1.

1.3 Adoptive cell therapy

Immunotherapy can be active (vaccine or cytokine therapy) or passive (intravenous immunoglobulin, monoclonal antibody therapy or adoptive cell therapy). The advantages and disadvantages of immunotherapy are summarised in Table 1-3. Adoptive transfer involves transfer of sensitised or insensitised immunologic agents (cells or serum) to non-immune recipients. When cells are used as a therapy, it is called adoptive immunotherapy or adoptive cell therapy (ACT) and was first described in 1988 (Rosenberg et al, 1988). ACT can be performed using the patient's own (autologous) or donor (allogeneic) cells. T-cells with specificity for diseased cells; virus or tumour, can be engineered for the purposes of the targeted therapy of human viral and malignant diseases. This includes the isolation, with or without *ex vivo* activation and proliferation, and reinfusion into the recipient. ACT may have a role not only in replacing, repairing or enhancing the immune function damaged as a consequence of cytotoxic therapy but also in tumour elimination directly by anti neoplastic effects or indirectly by immune mediated destruction of elements supporting tumour growth such as angiogenesis (Dudley & Rosenberg, 2007). Tumour and NK cell-specific immunotherapy will not be discussed in detail [reviewed by (Grupp & June, 2010)].

Characteristic of response	Clinical benefit	Disadvantages
Antigen specificity	Minimal toxicity	Infusional adverse effects including cytokine storm Other complications like graft aplasia, GvHD
Homing to sites where antigen is present	Disease elimination irrespective of site	Time consuming to generate adequate number of cells
Proliferation and survival in the presence of antigen	Minimal need for repeated treatments	May induce autoimmunity
Immunological memory	Lack of recurrence	May stimulate regulatory T-cells and negatively regulate their own proliferation

Table 1-3 Advantages of Immunotherapy

1.3.1 Virus-specific Anti T-cell Therapy

Conditioning regimens in HSCT allow the treatment of malignancy and facilitate engraftment but in turn impairs immune reconstitution which is vital for effective control and elimination of pathogens and tumour (Dunn et al, 2002). Multivariate analysis highlight infection as a dominant factor associated with non relapse mortality (Mackall et al, 2009). An impaired immune function following HSCT renders recipients susceptible to both reactivating and community acquired viruses (Storek et al, 2000) and most have at least one late infection (>50 days to 2 years) after transplant (Ochs et al, 1995).

Adoptive immunotherapy aims at restoring antigen-specific immunity in immunocompromised individuals by the transfer of antigen-specific T-cells. Selected populations of virus specific T-cells offer the advantage of being antigen-specific and are as a consequence minimally alloreactive, reducing the risk of severe GvHD or graft aplasia. These T-cells can restore virus-specific immunity and complement antiviral agents. A recent 10 year review of adoptive transfer of 381 T-cell products to 180 recipients documented no

grade 3-4 infusion reactions. Common side effects included nausea, vomiting and hypotension immediately after infusion and culture negative fever and chills within 24 hours (Cruz et al, 2010). Methods used to restore virus-specific immunity in HSCT recipients by adoptive transfer are discussed in the following section.

1.3.1.1 *DLI*

Donor lymphocyte infusions are defined as the infusion of lymphocytes, obtained from the HSCT donor, to the recipients for the purpose of enhancing donor engraftment, preventing rejection or treating disease relapse (Ljungman et al, 2010). The role for DLI in restoring anti-tumour immunity was demonstrated as early as 1995 (Kolb et al, 1995). This method has shown success in virus infections such as EBV (Epstein barr virus) (Papadopoulos et al, 1994) and adenovirus (Hromas et al, 1994b) but is limited by the low frequency of virus-specific T-cells within the DLI product. The high ratio of alloreactive T-cells to virus-specific T-cells increases the risk for GvHD. Grade II or greater GvHD has been observed in 40% of patients after infusion of $1x10^5$ unmanipulated T-cells/kg (Mackinnon et al, 1995; Shiina et al, 2009). This limits the tolerable dose of DLI, thereby restricting the dose of virus-specific T-cells that can be delivered. Thus methods to reduce the number of alloreactive T-cells in the selected T-cell pool have been developed to enhance the antiviral effect and alleviate GvHD.

1.3.1.2 *Alloreactive T-cell depletion*

1.3.1.2.1 Antigen-specific T-cell anergy

T-cells require 2 signals to become activated; signal 1 involves TCR engagement with peptide loaded MHC molecules whilst signal 2 is mediated by co-stimulatory receptors on T-cells which engage their ligands on antigen presenting cells (APC). The interaction between

CD28 on T-cells and its ligands, B7-1 (CD80) and B7-2 (CD86) on APCs, can be blocked by anti-CTLA-4, anti-CD80 and anti-CD86 monoclonal antibodies. Antigen-specific T-cell anergy can be induced ex vivo by T-cell receptor signaling in the absence of costimulation. In a HSCT setting to ameliorate symptoms of GvHD, CTLA-4 Ig has been used to prevent B7 proteins and CD28 interaction thereby preventing costimulatory signaling (Guinan et al, 1999). This strategy resulted in anergising the infused T-cells reducing alloreactivity thereby reducing the risk of GvHD with no impact on haemopoietic reconstitution. CTLA-4 monoclonal antibodies have been explored in melanoma where it enhances tumour immunity (Callahan et al). PBSCs with higher numbers of regulatory T-cells (T-regs) have been associated with a reduced incidence of GvHD in the HSCT recipient (Rezvani et al, 2006). T-regs can be selected *ex vivo* and expanded for purposes of adoptive transfer (Hoffmann et al, 2006; Trenado et al, 2004). Recently, this method showed promise in one patient (Trzonkowski et al, 2009) and needs to be investigated further in a clinical trial setting.

1.3.1.2.2 Alloreactive T-cell depletion with immunotoxins

Membrane proteins such as CD25 (IL-2Rα) expressed on alloreactive T-cells (Martin et al, 2004) can be conjugated with immunotoxins and used for the selective depletion of alloreactive T-cells. Clinical trials using T-cells prepared using the CD25 immunotoxin have been reported with good immune reconstitution and no GvHD prophylaxis (Andre-Schmutz et al, 2002). Amrolia and colleagues have found that infusion of such cells is safe but a minimum dose of $1x10^5$cells/kg is required to produce accelerated anti-virus T-cell recovery (Amrolia et al, 2006). Inducible suicide transgenes, e.g., thymidine kinase gene from herpes simplex virus I (HSV-tk) (Bonini et al, 1997) or human caspase-9 (iCasp9) (Tey et al, 2007) have been used to modify T-cells. The herpes simplex virus thymidine kinase (HSVtk) enzyme, for instance, mediates the conversion of ganciclovir to ganciclovir triphosphate

which is toxic to dividing cells; administration of ganciclovir efficiently eliminates HSVtk-modified T cells and abrogates acute GVHD. These suicide genes can facilitate the delivery of higher doses of allodepleted T-cells (>$8x10^5$ T-cells/kg). These methods are time consuming and limited by the availability of clinical grade immunotoxins. As infectious complications are more common in unrelated donor and haplo-identical HSCT the feasibility of obtaining donor cells is another hurdle.

1.3.1.3 *Selection of virus- specific T-cells*

1.3.1.3.1 *Ex vivo* expansion of virus -specific T-cells prior to reinfusion

The potential of virus-specific T-cells in adoptive transfer was initially demonstrated by Riddell *et al* (Riddell et al, 1994). CMV-specific CD8 T-cell clones were expanded and reinfused as pre-emptive therapy for CMV infection. They demonstrated that this treatment modality is safe and can restore anti-CMV immunity (Riddell et al, 1994). Einsele and colleagues reinfused *ex vivo* expanded CD4 and CD8 T-cells into patients with active therapy-refractory CMV disease and demonstrated disease clearance in 5/7 patients with very low cell doses (10^7 cells/m^2) (Einsele et al, 2002). Other groups have since then demonstrated successful *ex vivo* expansion of CMV-specific CD8 T-cells and subsequent reconstitution in recipients (Micklethwaite et al, 2008; Peggs et al, 2003).

EBV-specific T-cells have also been successfully used for the therapy of post transplant lymphoproliferative disorder (PTLD) (Comoli et al, 2007; Cruz et al, 2010; Gustafsson et al, 2000). Rooney *et al* established that $2x10^7$ CTL/ m^2 is safe and follow up studies could detect the transferred T-cells 9 years following infusion (Heslop et al, 1996; Heslop & Rooney, 1997). Due to problems with virus mutations and hence changes in antigen recognition (Gottschalk et al, 2001), polyclonal CTLs with broad antigen and epitope specificity minimise tumour immune evasion.

More recently the safety and efficacy of multivirus (CMV, EBV and Ad) specific CTLs has been demonstrated by Leen and colleagues (Leen et al, 2009). Antigen presenting cells were produced by expressing the immunodominant CMV-pp65 antigen in activated monocytes and EBV LCLs using a chimeric adenoviral vector (Leen et al, 2006) and utilised for tri-virus-specific CTL growth. Though expanded T-cells were safely transfused, only the CMV and EBV-specific T-cells expanded *in vivo* and Ad-specific CTLs were found only in donors with active infection at the time of infusion.

CTLs expanded *ex vivo,* although effective in providing broad spectrum antiviral immunity, have a number of limitations. Time to manufacture is around 3 months; [2-6 weeks for APC generation (longer for EBV- LCLs)] followed by an additional 4-6 weeks for CTL activation and expansion, and another 2 weeks for identity, sterility and potency testing] (Leen et al, 2010). It is also laborious and time consuming and hence can only be prepared for preemptive or prophylactic purposes. In addition the cost of setting up a GMP grade facility and maintaining the regulatory components such as quality assurance, quality control and data management are considerable. Personnel time for generation of the CTLs, cost of manufacturing GMP grade vectors, the reagents and medium required for CTL production and the release testing of these CTL lines prior to infusion also have to be factored in. In 2009, the cost for manufacturing, testing and infusing EBV-specific CTLs was $10,559, excluding professional time (Leen et al, 2010). In addition, there is also the concern that extensive *ex vivo* expansion of virus-specific T-cells may limit the proliferative and effector capability of these cells *in vivo* following adoptive transfer (Pahl-Seibert et al, 2005).

1.3.1.3.2 Rapid generation of antigen-specific CTLs

The direct isolation of peptide Major Histocompatibility Complex (pMHC) multimer binding T-cells (section 1.3.1.3.2.1) or the selection of IFN-γ expressing T-cells following stimulation

with virus or virus–specific protein or peptide in a cytokine secretion selection (CSS) system allows rapid selection of virus–specific T-cells for direct infusion into patients (Cobbold et al, 2005; Feuchtinger et al, 2006). A median of $8.6x10^3$ T-cells/kg of tetramer selected T-cells and 1.2-50 $x10^3$ T-cells/kg of IFN-γ selected T-cells has been shown to be clinically effective. These methods do not require long term expansion of T-cells and can be used therapeutically as well as prophylactically. pMHC multimer selections are mostly limited to CD8 T-cells and reliant on the knowledge of virus-specific epitopes (Cwynarski et al, 2001). Though expensive to generate, whole virus can be utilised as antigen in CSS.

1.3.1.3.2.1 pMHC Multimers

pMHC multimers were first used to detect antigen-specific T-cells in 1996 by Altman *et al* (Altman et al, 1996). This technology has since advanced to include a range of multimers from dimers (Dal Porto et al, 1993; Schneck et al, 2001) to dextramers (Batard et al, 2006), class I and class II MHC molecules (Scriba et al, 2005) and many species including mouse, monkey and man. They now play a key role in the characterisation of antigen (virus or tumour)-specific T-cells and can be important tools in immunotherapy (Bakker & Schumacher, 2005; Casalegno-Garduno et al; Guillaume et al, 2009).

Eukaryotic and prokaryotic expression systems have enabled easy generation of the recombinant HLA and β_2m proteins which are purified, denatured and subsequently refolded with the desired HLA-restricted peptide. T-cell receptors (TCRs) have very low affinity (Kd~10μM) to their cognate pMHC with an off rate in the order of a few seconds (Davis et al, 1998; Matsui et al, 1991). This knowledge resulted in the generation of tetramers where chemically or enzymatically biotinylated monomers (O'Callaghan C et al, 1999) are bound to fluorophore labeled tetravalent streptavidin molecules (Figure 1-5).

Tetrameric complexes have high structural stability and bind to the respective TCR with high avidity (Boniface et al, 1998). This binding has been shown to be exquisitely specific to the peptide sequence and is attributed to the slow dissociation rate of the pMHC TCR complex (Burrows et al, 2000).Though the term 'tetramer' is used for multimers generated in this manner due to the rigid tetrahedral configuration only 3 out of 4 available pMHC complexes are thought to bind simultaneously to the T-cell surface (McMichael & O'Callaghan, 1998). The fluorophore (phycoerythrin (PE) or allophycocyanin (APC)) conjugated streptavidin is prepared by cross-linking and hence contains multimers of streptavidin. This implies that the valency of standard PE or APC labelled streptavidin conjugates will be greater than 4 (Guillaume et al, 2009; Segura et al, 2008).

pMHC multimer technology now includes pentamers, octamers and dextramers (Casalegno-Garduno et al, 2010). Pentamers, contain pMHC complexes facing the same direction through the use of a five stranded coil as the oligomerisation domain, resulting in high avidity to the TCR (Duplan et al, 2007). Each pMHC pentamer also comprise 5 fluorescent or biotin tags for bright and efficient labelling (Hadrup & Schumacher, 2010). While streptavidin or avidin conjugates with 4 or 5 fluoresceins, a 270kDa dextran can be cross linked with 20 fluorescein and 10 streptavidin moieties. Thus, such a fluoresceinated dextran provides a strong fluorescent signal upon excitation and can bind 10 times more biotinylated class I pMHC monomers than conventional tetramers (Siiman et al, 1999). This has enabled the simultaneous use of multiple fluorophores and thereby facilitated multi-parameter screening (Batard et al, 2006). pMHC complexes with quantum dot-coupled streptavidin is another major advance (Iyer et al, 2006; Michalet et al, 2005). Quantum dots are semiconductor fluorescent nanocrystals of cadmium, selenium/ sellurium with a wide fluorescent excitation but very narrow emission spectra (Hadrup et al, 2009). pMHC multimers, built on qdot-

coupled streptavidin allow the simultaneous measurement of multi-parameter T-cell responses in a single sample (Hadrup & Schumacher, 2010; Jaiswal et al, 2003).

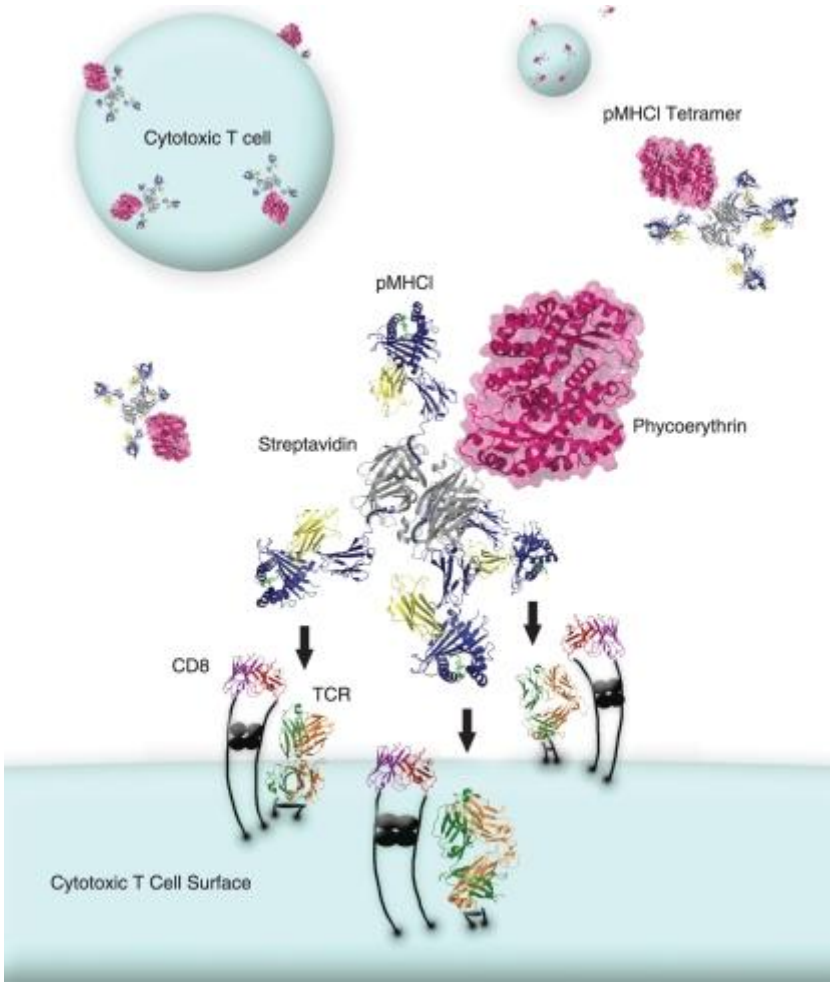

Figure 1-5 Tetrahedral avidin–biotin-based class I pMHC complex binding the TCR

Tetramers engage three T-cell receptors and three CD8 molecules at the cell surface. Adapted from Wooldridge *et al* (Wooldridge et al, 2009)

In intracellular staining (ICS), the cells are rendered nonviable and Elispot and limiting dilution assays (LDA) are cumbersome and time consuming in comparison to pMHC multimer staining for characterisation of antigen-specific T-cells. pMHC multimers provide a relatively straight forward method to analyse and characterise antigen-specific T-cells. Goulder *et al* compared all four methods for HIV derived peptides and found the ratio of

epitope-specific CD8 T-cells detected by tetramer, ICS, Elispot and LDA to that detected by tetramer as 1, 0.76, 0.29 and 0.07 respectively (Goulder et al, 2000). A good correlation was observed between ICS and tetramer staining with ICS, detecting a median of 75% of the response detected with tetramers. However HLA peptide complexes can only be used to detect T-cells specific to a known HLA peptide restriction and thus identification of all T-cells specific to a given antigen or pathogen is not possible. In addition, a substantial number of tetramer-binding T-cells can be functionally inert (Zajac et al, 1998) and closely packed pMHC complexes could induce T-cell death (Cebecauer et al, 2005).

pMHC multimers could play a significant role in cell selection for adoptive immunotherapy to enhance desirable or suppress unwanted responses. They have been used for rapid and efficient *ex vivo* isolation alone or isolation followed by expansion of antigen (tumour or virus) specific T-cells. In either case the antigen-specific T-cells were re-infused to enhance anti-tumour or anti-viral immunity (Barnes et al, 2004; Cobbold et al, 2005; Oelke et al, 2003; Prakken et al, 2000). Studies in mice have shown that MHC dimers inactivate autoreactive T-cells *in vivo* thereby inducing clonal anergy (Casares et al, 2002). This application can be used to delay the onset as well as reduce the severity of type I diabetes (Masteller et al, 2003) and arthritis (Zuo et al, 2002). Isotope-coupled MHC multimers such as ^{225}Ac-labelled MHC tetramers (Yuan et al, 2004), can be used to kill specific T-cell populations. This technique could prove promising in a variety of malignancies and so far seems to be tolerated with reasonable toxicity in mice (McDevitt et al, 2001). Streptamers (Knabel et al, 2002) developed with the intention of enriching antigen-specific T-cells for adoptive transfer according to clinical grade manufacturing guidelines will be discussed in detail in section 5.3.1.

1.4 Adenovirus

Over 50 years ago two research groups simultaneously identified adenoviruses. Rowe *et al* (Rowe et al, 1953) demonstrated a cytopathic agent whilst investigating spontaneously degrading adenoid tissue. Hilleman and Werner on the other hand recovered a new agent responsible for an epidemic of respiratory disease amongst U.S. army recruits (Hilleman & Werner, 1954). They were soon identified as the same agent and named adenovirus after the original tissue in which the prototype strain was discovered (Enders et al, 1956). The Adenoviridae family is divided into 5 genera of which human adenoviruses fall into the Mastadenovirus genus (Davison et al, 2003).

1.4.1 Classification and structure

1.4.1.1 *Classification*

Human adenoviruses were classified originally on the basis of their ability to be neutralised by specific animal antisera. The 52 known human serotypes can be further classified into 6 subgroups or species (A-F) (Benko¨ et al, 2005; Horwitz, 2001; Shenk, 2001) based on genome size, composition and organisation, DNA homology, haemagglutination of erythrocytes and oncogenicity in rodents. Sequence availability has allowed more detailed phylogenetic analysis (Crawford-Miksza & Schnurr, 1996). Species B is further subdivided into B1 and B2 (Segerman et al, 2003a). Serotype 52 has recently been identified after genomic sequencing and phylogenetic analysis of an isolate and potentially constitutes a new species (G) (Jones et al, 2007).

1.4.1.2 *Structure*

Human adenoviruses are non-enveloped DNA viruses with icosahedral symmetry and a size of 60-90nm. Adenovirus structure (reviewed (Russell, 2009)) has been determined to a resolution of 3.5Å by cryoelectron microscopy single particle image reconstruction and X-ray crystallography (Reddy et al, 2010) (Figure 1-8). The DNA is linear, double-stranded and non-segmented, of about 36Kb carrying approximately 40 genes (Medina-Kauwe, 2003). The outer structure of the virus, the protein capsid, is an icosahedron comprising 252 capsomeres (protein subunits) - 240 hexons and 12 pentons. The homotrimeric hexons are on the faces and edges of the capsid whilst 12 pentons are at the vertices of the icosahedron (Valentine & Pereira, 1965) (Figure 1-6).

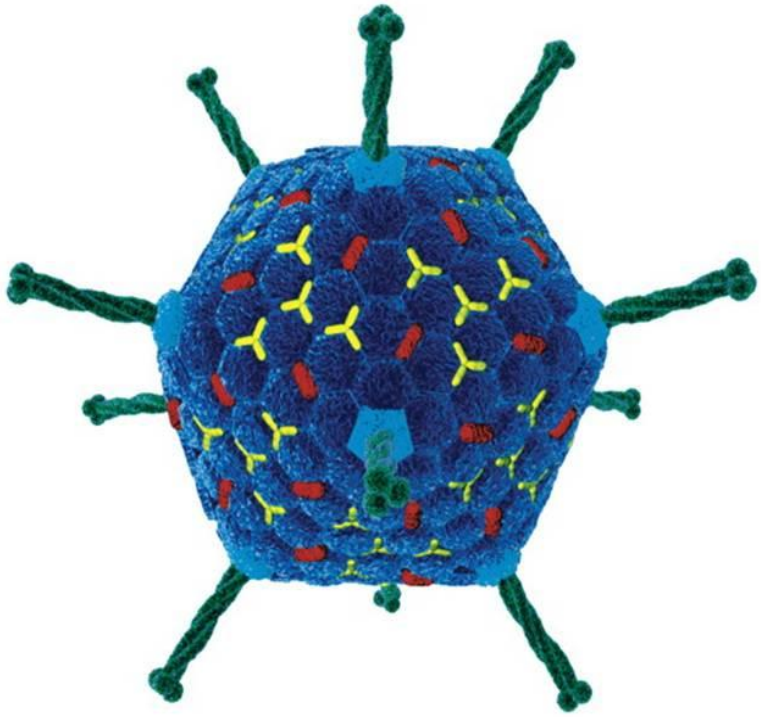

Figure 1-6 Three dimensional icosahedral structure of adenovirus

Three-dimensional representation of the icosahedral virion, showing the major and minor capsid proteins localised in the outer capsid. The three major capsid proteins, hexon, penton base and fibre, are shown in dark blue, light blue and green, respectively. The two minor capsid proteins are superimposed; protein IX is depicted in yellow and protein IIIa in red. Adapted from Vellinga *et al* (Vellinga et al, 2005b)

Hexon quantitatively represents the dominant capsid protein and contains several regions that are conserved among different human serotypes (Ebner et al, 2005; Reddy et al, 2010). It is a pseudo-hexagonal trimer on the facets of the icosahedral capsid. Each hexon molecule contains 2 pedestal regions and 4 loops (Figure 1-7). Each pedestal region has 4 conserved regions, C1 to C4, and 3 variable regions, V1 to V3. Loop 1 (amino acid (aa) 131 to 331) and loop 2 (aa423 to 477) are encoded by the hypervariable regions of the hexon gene and are responsible for the serotype-specific epitopes (Nemerow & Stewart, 2001). This renders the hexon the most important adenovirus protein for the classification and recognition of individual human serotypes, as it shows minimal variability between different human adenovirus serotypes (Crawford-Miksza & Schnurr, 1996). Adenovirus serotype is determined by neutralising serotype-specific antibodies directed against the hypervariable regions of the hexon (Lenaerts et al, 2008a; Smith et al, 2010). Although there is antigenic cross reactivity among members of each species owing to conserved epitopes located on the hexon protein of the virion, there is no known antigen common to all human adenoviruses. Ad epitopes are discussed in (section 1.4.2.1.1).

Each penton consists of a pentameric base and a trimeric fibre. The fibre consists of a tail, shaft and knob domain. The knob interacts with cellular receptors enabling virus cell entry. Adenovirus fibres of serotype and species-specific lengths extend from the penton base and are associated with haemagglutination properties. Other so-called ‘minor’ components, IIIa, VI, VIII and IX, are also associated with the capsid and are involved in cementing the virion structure (Vellinga et al, 2005a; Vellinga et al, 2005b). There are six other structural components situated in the virus core, five are associated with the double stranded DNA genome [V, VII, Mu, IVa2 and the terminal protein (TP)] and the remaining component is the 23K virion protease which plays a vital role in the assembly of the virion. These basic

polypeptides and TP together with the virus DNA make the core of the virion. Whilst polypeptide pVII, the major core protein, binds the viral genome tightly forming a compact nucleoprotein complex (San Martin & Burnett, 2003), protein pV is linked to the nucleoprotein complex as well as the virion shell (Matthews & Russell, 1998) (Figure 1-8).

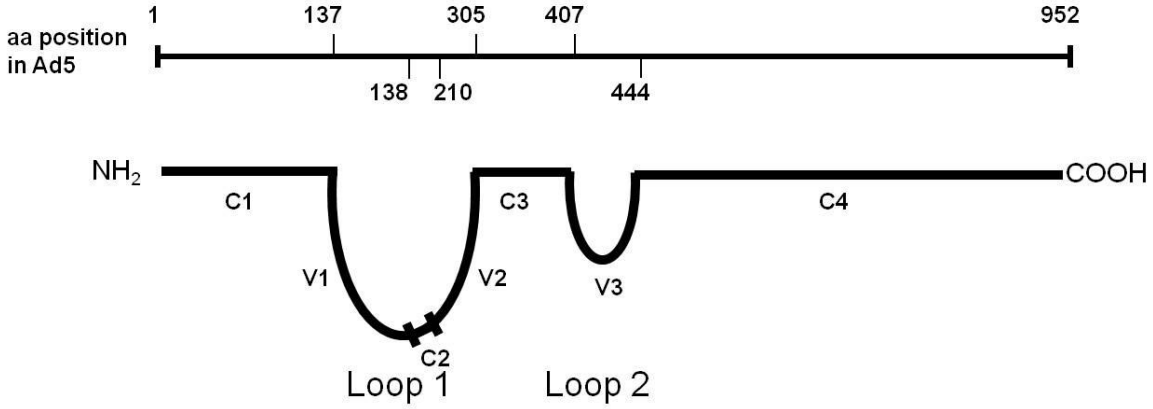

Figure 1-7 Schematic illustration of hexon protein structure

Variable regions are V1 to V3 are located in the loops designated 1 and 2 project away from the virus surface. The indicated aa positions are based on the reference Ad5. Adapted from Ebner (Ebner et al, 2005)

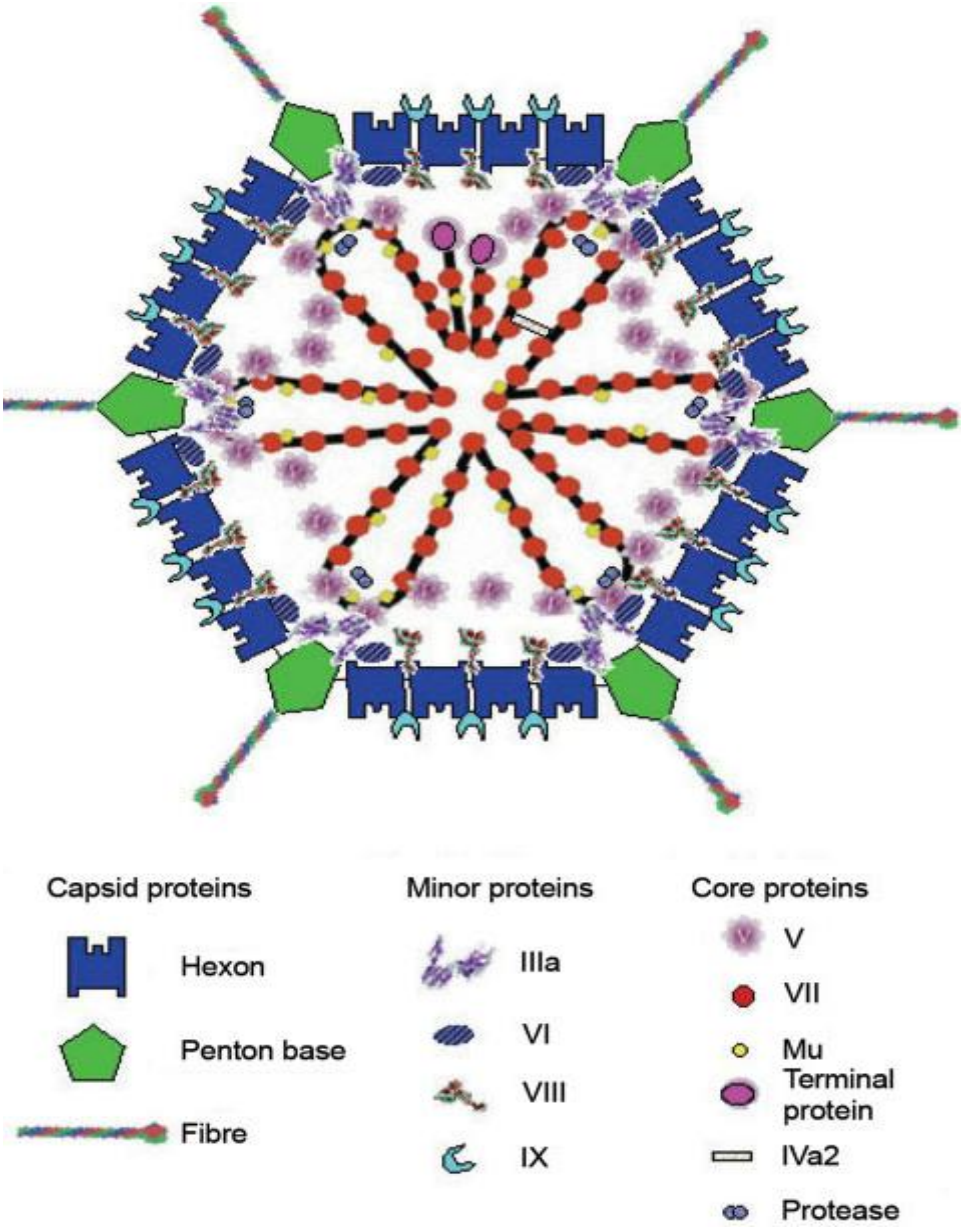

Figure 1-8 Structure of adenovirus

A schematic depiction of the structure based on cryoelectron microscopy and crystallography. The locations of the capsid and minor components are reasonably well defined and are not to scale. The disposition of the core proteins and the virus DNA is largely conjectural. The symbols for IIIa and VIII are based on the structures defined by Saban *et al* (Saban et al, 2006) (Adapted from Russell) (Russell, 2009))

1.4.1.2.1 Viral genome

All human adenovirus genomes that have been examined to date have the same general organisation. The viral genome consists of a linear double stranded DNA molecule that, in the prototypic HAdV-2, is 36Kb in length. Each of the genome termini contains a 93 –371bp nucleotide long inverted terminal repeat (ITR) sequence in which the viral origin of replication is embedded. On the basis of kinetics of viral gene expression, the adenovirus DNA can be divided into 6 early [*E1A, E1B, E2A, E2B, E3, E4*], 2 intermediate and 1 major late transcription units. The transition from early to late gene expression is defined by the onset of viral DNA replication.

The early genes promote cellular entry into S phase (to enhance viral replication), transcriptionally activate the Ad genome, initiate replication and inhibit apoptosis. *E1A* is the first gene to be expressed after the virus reaches the infected cell [reviewed by (Gallimore & Turnell, 2001)]. The E1A proteins are transcriptional transactivators (Shenk & Flint, 1991) and activate other viral and cellular proteins, driving the cell into S phase, thereby initiating virus gene expression and proliferation. E1B proteins inhibit cellular apoptosis initiated as a result of E1A proteins driving cellular replication (Rao et al, 1992). *E2A* (early) is involved in synthesis of DNA binding protein (DBP) whereas *E2B* (late) generates precursor TP and DNA polymerase (Pol). Pol is a DNA polymerase which uses pTP as a protein primer and has both 5'-3' polymerase and 3'-5' exonuclease activities. It has a molecular mass of 140KDa and is well conserved among different serotypes with an overall homology between 70-80% (Liu et al, 2000). DBP unwinds dsDNA and is required for chain elongation and stabilisation of the pTP-DNA interaction. It is an antigenic target for CTLs but is not well conserved amongst serotypes (Joshi et al, 2009). Pol, pTP and DBP proteins play a role in DNA replication, elongation, transcriptional regulation, DNA recombination and virus

assembly (Chase & Williams, 1986). E3 gene products are not essential for virus replication but play a role in the immune escape mechanism of the virus by modulating the host immune responses against virus-infected cells (Fessler et al, 2004). This is discussed in detail in 1.4.3. The E4 transcriptional unit has 7 ORFs (Davison et al, 2003) which encode proteins involved in multiple tasks including transcriptional activation of heterologous promoters, preferential translation of virus mRNA and host cell protein synthesis shutoff (Leppard, 1997).

The intermediate genes encode proteins IVa2 and polypeptide IX. Protein IVa2 (Lutz & Kedinger, 1996) is required for activation of late gene expression from the major late promoter (MLP). Polypeptide IX (Lutz et al, 1997) is a structural protein and has nuclear reorganisational and transcriptional activatory functions. Late gene expression is controlled by the MLP which initiates transcription of the late mRNA concurrent with DNA replication (Nevins & Wilson, 1981). MLP produces one large RNA (~ 29k nucleotides) transcript spliced into 18 mRNAs coding for the structural proteins and proteins involved in virus assembly. The late mRNAs are grouped into 5 families (L1-L5) based on the usage of the common poly (A) sites and encode 18 structural proteins (Nevins & Darnell, 1978). These proteins are not only responsible for virus assembly and replication but also form the structure of the virus capsid (Perez-Romero et al, 2006).

1.4.2 Adaptive immune responses to adenovirus

The relative importance of the humoral and cellular immune response to adenovirus infection is unclear despite detailed molecular study of the virus over the last five decades. Studies in mice with severe combined immune deficiency showed that though cidofovir suppressed mouse adenovirus type 1 replication and thereby delayed disease progression whereas all infected mice succumbed to adenovirus (Lenaerts et al, 2005). This highlights a significant role for the adaptive immune system in virus control and clearance. An age-related decrease

in the adenovirus-specific immunity has been shown in healthy volunteers (Sester et al, 2002). This could be either by immune elimination or from depletion of latent states. This elimination of adenovirus within a lifetime may explain the predominant incidence of adenovirus related complications in children and young adults, compared to older adults, following HSCT. Primary infections occur mainly during infancy and childhood, are normally mild and do not require therapy.

Subsequent to its original isolation from epithelial and fibroblast tissue of tonsils and adenoids (Rowe et al, 1953) persistence of virus after primary infections in the tonsils and prolonged shedding in the urine and stool was repeatedly demonstrated by virus watch programs in the 1970s (Lichtenstein & Wold, 2004; Magwalivha et al, 2010; Mautner, 1989). T-lymphocytes in tonsils and adenoids harbour adenovirus DNA even in asymptomatic infection (Garnett et al, 2007). The virus immune evasion mechanisms (section 1.4.3) play a role in maintaining the virus in a persistent state. On the other hand, PCR studies have demonstrated the absence or low presence of Ad DNA in peripheral blood of healthy volunteers (Flomenberg et al, 1997). Studies have shown that though human lymphoid derived cell lines adsorb the virus without internalisation. Similar mechanisms result in ineffective infection of PBMCs (Silver & Anderson, 1988). Thus the virus may be strictly associated with the mucosa-lymphocyte compartment and rarely found in circulation (Amrolia et al, 2003; Watzinger et al, 2004). It would seem that adenovirus persists in a quiescent form in these cells and is kept under control by cellular immunity. Absence of immune control in the immune deficient favours virus reactivation (Calcedo et al, 2009). Modern immunological techniques are now beginning to reveal the phenotype and functionality of Ad-specific T-cells. Few animal models can reproduce the human adenovirus disease (Ginsberg, 1999). The recently established Syrian hamster, fully permissive immune

competent animal model, is an important tool for studying immune responses to Ad (Thomas et al, 2006).

1.4.2.1 *Cellular immune response*

T-cells provide an effective defence via both CD8 T-cells and CD4 helper T-cells. CD8 T-cells recognise virus derived peptide fragments in a complex with class I proteins of the MHC on the cell surface. Following pMHC TCR engagement, perforin is released, resulting in target cell lysis, thereby eliminating the virus infected cells preventing virus assembly and release. The class I recognition mechanism depends on the availability of the antigen to complex successfully within the ER membrane with an MHC component and then being transported to the plasma membrane. The CD4 helper cells on the other hand are important in mounting a proliferative response to infection. This is mediated in a similar fashion by recognition of a virus target antigen in association with class II MHC. These helper T-cells can also stimulate proliferation of B-cells to provide immunoglobulins for the humoral response.

Despite virus immune evasion strategies (described in 1.4.3.), cellular immune response plays a significant role in virus control and elimination. Cellular immune responses in semi-permissive mouse models primarily involve CD8 T-cells that recognise antigen derived from the early proteins, E1A and E2A (Mullbacher et al, 1989; Rawle et al, 1991), whereas in healthy human donors CD4 T-cells specific for capsid (late proteins) derived antigens pre-dominate (Heemskerk et al, 2003; Leen et al, 2004b; Olive et al, 2002; Sester et al, 2002). Cytotoxicity (Flomenberg et al, 1996; Smith et al, 1996) as well as species and serotype cross reactivity (Heemskerk et al, 2003; Leen et al, 2004a; Leen et al, 2004b; Smith et al, 1996; Smith et al, 1998) of human Ad-specific CD8 and CD4 T-cells has been well demonstrated. The majority of isolated T-cells recognise antigen from capsid components though epitopes

derived from the early regions have also been described (section 1.4.2.1.1). This implies that cellular immune response is mainly directed against the conserved hexon capsid. Ad hexon-specific T-cells were found in 72% of healthy volunteers screened and were predominantly CD4 (Feuchtinger et al, 2005). A recent study demonstrated adenovirus hexon-specific CD4 T-cells (81%) and CD8 T-cells in (38%) healthy donors *ex vivo* (Zandvliet et al, 2010).

Fibre or IIIa structural polypeptides have been implicated as possible antigens initiating a proliferative response in addition to the early proteins and hexon (Souberbielle & Russell, 1995). CD4 proliferative responses to the uncommon Ad35 has been documented in individuals without any serological evidence of previous Ad35 infection (Flomenberg et al, 1995). This indicates the ability of CD4 T-cells to recognise conserved antigens such as hexon (Leen et al, 2008) and suggests that this arm of the immune system plays a role in modulating infection against a wide range of serotypes. It is possible that the adaptive CD4 T-cell responses helps maximise the range of epitopes recognised by CD4 and CD8 effector T-cells (Leen et al, 2008).

Cytotoxic T-cells generated by using adenovirus-pulsed dendritic cells contain a mixture of effector cells that recognise both MHC class I and II antigens (Smith et al, 1996). Additionally CTLs prepared *in vitro* against adenovirus from one of the six species can lyse cells infected with adenovirus from other species (Smith et al, 1998). Adenovirus-specific cord blood CTLs have also been generated successfully and were found to be functionally similar to those generated from the peripheral blood of healthy donors (Hanley et al, 2009).

Flow cytometry based intracellular staining assays have proved to be less sensitive than IFN-γ elispot assays at detecting adenovirus-specific T-cells in the peripheral blood, but have shown that the T-cell response is characterised predominantly by CD4 T-cells that produce IFN-γ, IL-2 and TNF-α in response to Ad antigen but not IL-4, IL-5 or perforin, consistent

with an effector/memory phenotype able to produce Th1 cytokines (Sester et al, 2002). A recent study confirmed this finding and concluded that Ad-specific CD4 T-cells were primarily monofunctional expressing memory markers CD27 and CD45RO. In contrast, Ad-specific CD8 T-cells were polyfunctional, expressing effector-like combinations of IFN-γ, MIP1-α and perforin, and lacked CD27 and CD45RO expression (Hutnick et al, 2010). In contrast to CMV-specific CTLs, the Ad-specific CTLs have higher capacity to produce perforin than IL-2 (Makedonas et al, 2010).

The importance of T-cell immunity in controlling adenovirus replication is highlighted by the higher incidence of infections in HSCT patients who have GvHD as they receive lympholytic and immune suppressive therapies (La Rosa et al, 2001). Recipients of T-cell depleted grafts with longer T-cell recovery period are also at a higher risk of Ad infection (Chakrabarti et al, 2002; Symeonidis et al, 2007). It is also well documented that HSCT recipients can clear Ad infection without intervention, provided they have sufficiently reconstituted their cellular immunity (Ohrmalm et al, 2010; Walls et al, 2005).

Ad-specific T-cells have been detected in blood samples of transplant recipients and healthy individuals (Feuchtinger et al, 2005; Flomenberg et al, 1995). Low lymphocyte count at the time of infection and lack of T-cell reconstitution is associated with increased risk of progression to Ad disease as well as high mortality (Chakrabarti et al, 2002; Kalpoe et al, 2007; Kampmann et al, 2005). Children with fatal Ad disease have higher levels of cytokines like TNF-α, IL-6 and IL-8 (Mistchenko et al, 1994). Reduction in immunesuppression helps in virus clearance and failure to taper immunesuppression (GvHD patients) results in a poor outcome (Chakrabarti et al, 2002; Sivaprakasam et al, 2007).

In a longitudinal study, Feuchtinger *et al* (Feuchtinger et al, 2005) found that Ad-specific T-cells were absent in all patients with Ad disease associated mortality, post SCT. In this study

patients capable of developing an Ad-specific T-cell response comparable to healthy donors had a lower risk of life threatening Ad infections and did not progress to adenovirus disease. Also patients with no Ad infection had lower frequencies of Ad-specific T-cells, presumably because in the absence of Ad antigens there is no need for clonal expansion of specific T-cells *in vivo*. In a recent prospective study, 58% of T-replete HSCT recipients had Ad-specific cellular immune reconstitution (Ohrmalm et al, 2010). This highlights the significance of cellular immunity in the control and clearance of Ad in HSCT recipients.

1.4.2.1.1 Adenovirus derived T-cell epitopes

As discussed (section 1.4.1.2) hexon is the most abundant capsid protein and contains several regions that are conserved among different human serotypes. Most of the adenovirus derived T-cell epitopes identified to date are within the hexon and conserved across serotypes with minor variations in the amino acid sequences (Table 1-4, Table 1-5). Only a few of these have been well characterised with regards to their minimal sequence, HLA type, immunogenicity and cross reactivity. Epitopes will be referred by the first 3 letters of their amino acid sequence hereafter. Ad E1, E3, Pol, DBP and fibre derived peptides are not only recognised by CD4 and CD8 T-cells but also bind to multiple MHC alleles (Haveman et al, 2006; Joshi et al, 2009) Table 1-6.

Ad hexon is now known to contain at least 15 HLA class I restricted peptides, 8 HLA-A, 5 HLA-B and 2 HLA-C. The difference in HLA restriction may be relevant. EBV or HIV-specific CTLs to HLA-B restricted epitopes induce a higher magnitude of response in comparison to HLA-A and HLA-C alleles (Bihl et al, 2006; Frahm et al, 2007). HLA-B alleles were preferentially recognized by polyclonal T-cell lines specific to influenza A or B virus (Boon et al, 2004). Five CD8 T-cell epitopes (TYF, aa36-44; MPN, aa319-328; LPG,

aa 575-583; IPY, aa705-713; YVL, aa916-925) overlap with CD4 T-cell epitopes (FAR, aa 31-55; PNP, aa 321-335; FFA, aa 566-590; LGS, aa 691-715; EVD, aa 906- 930).

Two regions on the hexon; aa 710-724, aa 906-928; have strong immunogenicity. 50% of the cord blood CD8 T-cells generated by Hanley *et al* (Hanley et al, 2009) recognised the hexon region aa 710-724. The cord blood CD8 T-cells were similar to peripheral blood derived CD8 T-cells implying a role in the early cellular immunity. 2 class I (A*02 aa711-721, A*11 aa713-720) and 2 class II (DPB1*0201 and DRB1*1501 aa 710-724) epitopes have been identified in this region. The region aa 906 to 928 also generated CD4 and CD8 responses. Three class I (A*02 aa916-925, B*13/49aa 913-927, Cw*0702 912-920) and three class II epitopes (DPB1*0401/02, DQ*0402 aa 913-921) have been identified in this region so far. The HLA*DP4 restricted epitope, the most common human class II allele (Castelli et al, 2002), is reported to be recognised by 50-75% of the Caucasian population (al-Daccak et al, 1991, Tang, 2004 #138), however Onion *et al* did not find a similar frequency of this epitope (Onion et al, 2007). Hexon region aa906-928 has been recognized after natural infection, whole virus or Ad 5 vector priming, highlighting the importance of this region in antigen recognition (Leen et al, 2008; Olive et al, 2002; Tang et al, 2004; Tang et al, 2006; Veltrop-Duits et al, 2006).

HLA Restriction	Sequence	Ad5 hexon aa position	Reference
A*01	TDLGQNLLY	885-893	(Leen et al, 2004a) (Zhu et al, 2010)
A*02	GLRYRSMLLGNGRY	542-555	(Myers et al, 2007)
A*02	TFYNHTFKKV	710-720	(Leen et al, 2004a)
A*02	LLYANSAHAAL	892-901	(Tang et al, 2006)
A*02	YVLFEVFDVV	916-925	(Tang et al, 2006)
A*03	QSMPNRPNYIA	318-330	(Zhu et al, 2010)
A*11	TFYLNHTFKKV	710-724	(Zhu et al, 2010)
A*24	TYFSLNNKF	36-44	(Leen et al, 2004a)
B*07 **B*0702**	KPYSGTAYNSL	114-128	(Leen et al, 2004a) (Zhu et al, 2010)
B*07/35 **B*35, A*33**	MPNRPNYIAF	319-328	(Leen et al, 2004a) (Zhu et al, 2010)
B*07	FRKDVNMVL	585-593	(Zandvliet et al, 2010)
B*13/49	LFEVFDVRV	918-927	(Tang et al, 2006)
B*35	LPGSYTYEW	575-583	(Leen et al, 2008)
B*35/53	IPYLDGTFY	705-713	(Leen et al, 2008)
B*35	IPFSSNFMSM	873-882	(Zandvliet et al, 2010)
B*52	ETYFSLNNKF	36-45	(Zandvliet et al, 2010)
B*63	YSYKARFTL	78-86	(Zandvliet et al, 2010)
Cw*0401	NFPYPLIGKTA	845-855	(Zhu et al, 2010)
Cw*0702	EPTLLYVLF	912-920	(Zhu et al, 2010)

Table 1-4 Published HLA restricted class I epitopes from human Ad5 hexon

HLA Restriction	Sequence	Ad5 hexon aa position	Reference
DPB1*03/ DQB*04/ DRB1*08	QRLTRLRFIPVD	62-76	(Zhu et al, 2010)
DPB1*03, DQB*04 or DRB1*08	YSYKARFTLAV	74-88	(Zhu et al, 2010)
DPB1*05	RTRYFSMWNQAVD	376-390	(Zhu et al, 2010)
DPB1*0201	AFRGWAFTRLKTKET	674-688	(Zhu et al, 2010)
DPB1*0201 DRB1*15	TFYNHTFKKVAIT	710-724	(Zhu et al, 2010)
DPB1*0401	NFPYPLIGKTA	845-855	(Zhu et al, 2010)
DPB1*04 DQ*0402	DEPTLLYVLFEVFDV	910-924	(Olive et al, 2002) (Zhu et al, 2010)
DQ*05	PNYIAFRDNFIGLMY	320-334	(Zhu et al, 2010)
DQB*06/03	NMGVLAGQASQL	344-358	(Zhu et al, 2010)
DQ*05/03	TKYKDYQQVGILHQH	809-823	(Zhu et al, 2010)
DQ*05	LCDRTLWRIPFSSNF	865-879	(Zhu et al, 2010)
Pan DR	YINLGARWS	513-527	(Haveman et al, 2006)
Pan DR	MDEPTLLYV	906-920	(Haveman et al, 2006)
DR*01/ DR*16 DRB1*01	QWSYMHISGQDASEY	8-22	(Onion et al, 2007),(Serangeli et al, 2010; Zhu et al, 2010)
DR*01	GTAYNALAPKGAPNP	117-131	(Onion et al, 2007; Serangeli et al, 2010)
DR*01	TGNMGVLAGQASQLN	341–355	(Serangeli et al, 2010)
DRB*01	TRYFSMWNQAVD	380-394	(Zhu et al, 2010)
DR*01	TETLTKVKPKTGQEN	422–436	(Serangeli et al, 2010)
DRB*01	VDCYINLGARWSLDY	529-543	(Onion et al, 2007; Serangeli et al, 2010)
DR*03	THDVTTDRSQRLTLR	52-66	(Serangeli et al, 2010)
DR*03	EWNFRKDVNMVLQSS	581–595	(Serangeli et al, 2010)
DR*03	GASIKFDSICLYATF	604-618	(Serangeli et al, 2010)
DR*04	TLRFIPVDREDTAYS	64–78	(Serangeli et al, 2010)
DR*04	SQWYETEINHAAGRV	208–222	(Serangeli et al, 2010)

DRB1*04	MPNPNYIAFRDNFI	324-334	(Zhu et al, 2010)
DR*04	ENGWEKDATEFSDKN	435–449	(Serangeli et al, 2010)
DR*04	GNNFAMEINLNANLW	454–468	(Serangeli et al, 2010)
DR*04	PGSYTYEWNFRKDVN	575–589	(Serangeli et al, 2010)
DR*04/ DR*07	PQKFFAIKNLLLLPG	562–576	(Serangeli et al, 2010)
DR*04	ATFFPMAHNTASTLE	616–630	(Serangeli et al, 2010)
DR*04	GWAFTRLKTKETPSL	676–690	(Serangeli et al, 2010)
DR*07	LMYYNSTGNMGVLAG	335–349	(Serangeli et al, 2010)
DR*07 DRB1*07	DPYYTYSGSIPYLDG	695–709	(Serangeli et al, 2010) (Zhu et al, 2010)
DR*07	FKKVAITFDSSVSWP	717–731	(Serangeli et al, 2010)
DRB1*15/03	EYLSPGLVQFARATE	22-36	(Zhu et al, 2010)
?DRB1*08	PGLVQFARATE	26-36	(Zhu et al, 2010)
DRB1*15/03	TYFSLNNKFRNP	33-48	(Zhu et al, 2010)
Class II	MGVLAGQASQLNA	344-358	(Zhu et al, 2010)
Class II	SIGDRTRYFSM	376-390	(Zhu et al, 2010)
Class II	LCDRTLWRIPFSSNF	865-879	(Zhu et al, 2010)

Table 1-5 Published HLA restricted class II epitopes from human Ad5 hexon

All identified hexon derived epitopes when compared with other Ad hexon sequences show that they fall in conserved regions, implying that they are potentially cross reactive across serotypes and possibly across species. The relevance of epitopes that are recognised by both class I and II HLA molecules is not yet known.

HLA Restriction	Sequence	Ad 5 E1B aa position	Reference
Pan DR	FLAMHLWRA	114-128	**(Haveman et al, 2006)**
HLA Restriction	**Sequence**	**Ad 5 E3A gp aa position**	**Reference**
Pan DR	MFVCLIIMW	39-53	(Haveman et al, 2006)
HLA Restriction	**Sequence**	**Ad 5 Pol aa position**	**Reference**
Pan DR	PTISSNSHA	475-489	(Haveman et al, 2006)
A *02	GLTDASFNV	608-617	(Joshi et al, 2009)
A *02	TLNHRGWRV	779-788	(Joshi et al, 2009)
A *02	VLAWTRAFV	977-986	(Joshi et al, 2009)
HLA Restriction	**Sequence**	**Ad 5 DBP aa position**	**Reference**
A *02	MMGRFLQAYL	206-215	(Joshi et al, 2009)
A *02	KLLPDQVEAL	243-252	(Joshi et al, 2009)
A *02	FLGRQLPKL	407-416	(Joshi et al, 2009)

Table 1-6 Ad E1, E3, Pol and DBP derived epitopes

Pan DR Authors demonstrated ability to bind HLA DRB1 epitopes.

Recently Joshi *et al* demonstrated that Ad-specific CD8 T-cells recognise two early region proteins, DNA polymerase and DNA binding protein (Joshi et al, 2009). They demonstrated CTLs specific to the Pol and DBP derived epitopes in HSCT patients recovering from Ad disease. The Pol-specific CTLs demonstrated higher cytotoxicity and were conserved across serotypes in comparison to DBP-specific CTLs that showed lower frequency, lower cytotoxicity and were not conserved among adenovirus serotypes.

Adenovirus-specific T-cells are predominantly hexon or late gene derived protein specific. This could be due to reduced generation of early protein in comparison to late structural

proteins or T-cells responding to early gene derived proteins are of a lower frequency making them harder to detect. The Syrian hamster model may allow us to study this further though the vastly different MHC systems need to be considered. More insight into nonstructural Ad protein derived epitopes may help target infection prior to the onset of replication.

1.4.2.2 *Humoral immune response*

The humoral response is a major component of the defence strategy of the host and depends on the ability of B-cells, to recognise a specific epitope on a foreign antigen. This recognition initiates a massive proliferation mediated by T-helper cells resulting in the release of specific immunoglobulins of various classes into plasma to interact directly with these antigens. The antigen-specific immunoglobulins can neutralise virus infection very efficiently and may play a role in limiting the effiectiveness of Ad gene therapy (Zaiss et al, 2009).

Ad-neutralising antibodies are directed against epitopes on virus capsid components (Gahery-Segard et al, 1998; Gahery-Segard et al, 1997; Sumida et al, 2004; Willcox & Mautner, 1976a; Willcox & Mautner, 1976b). Ad fibre induces neutralising antibodies, and function by aggregating virus particles and thereby inhibiting adsorption whereas hexon and penton induced antibodies inhibit low pH induced conformational change of the virus or trap the virions in the endosomes (Toogood et al, 1992; Wohlfart, 1988). Type-specific antigens described are in the fibre and associated with the trimeric knob or proximal regions of the stem as well as hexon (Watson et al, 1988; Xu & Erdman, 2001)}.

Infection or vaccination with the common serotypes is known to elicit strong humoral immune responses, with the formation of cross-reactive non-neutralising antibodies and serotype-specific neutralising antibodies which protect against re-infection with the same serotype (Lemckert et al, 2005). HSCT recipients showed an increased level of serotype-specific antibodies when they cleared the infections (Heemskerk et al, 2005). In mice

humoral immunity has been shown to be critical for successful virus clearance by anti adenoviral agents (Lenaerts et al, 2008b). These studies highlight that the humoral immune response plays a significant role in control and clearance of Adenovirus.

1.4.3 Immune evasion strategies

Adenovirus is known to persist in its apparently healthy host for long periods with no outward signs of disease (Fox et al, 1969). In order to persist in secondary lymphoid organs, the virus has to dampen host responses to avoid the harmful effects of immune activation. It is possible that the immune regulatory (E3) proteins reduce the efficacy of host immune control, allowing an extended window of virus production and transmission to new hosts. This has led to the study of immune evasion strategies of the virus. Adenoviruses have mechanisms for evading host immune mechanisms such as inhibition of interferon functions by virally associated RNA and E1A, inhibition of intrinsic cellular apoptosis in infected cells, and the prevention of major HLA class I expression on the cell surface [reviewed by (Mahr & Gooding, 1999; Schagen et al, 2004)]. The immune evasion strategies of the virus are summarised in Table 1-7.

E1A suppresses nitric oxide (NO) production through transcriptional control of the inducible NO synthase (iNOS) gene. As NO is an antiviral effector its inhibition may enable the virus to persist in human tissue. E1A also blocks secretory leukoprotease inhibitor (SLPI) and elafin/skin-derived antileukoproteinase (SKALP) secretion by alveolar epithelial cells which assist in virus clearance (Higashimoto et al, 2006).

Type I IFNs (IFN-α and β) are the earliest host response to viral infection. They induce apoptosis as well as upregulate MHC class I expression enabling efficient viral antigen presentation to the host T-cells (Garofalo et al, 1996). In addition they activate the NK cells causing them to secrete IFN-γ which upregulates MHC class I and II on target cells (Garcia-

Sastre & Biron, 2006). Ad E1A and E1B proteins inhibit the transcriptional activation of the Type I IFN genes thereby preventing efficient viral antigen presentation and elimination (Ackrill et al, 1991). E1A protein also increases the inflammatory response by enhancing intercellular adhesion molecule-1 and interleukin-8 mRNA expression following lipopolysaccharide stimulation (Higashimoto et al, 2006). This has been demonstrated in patients with persistent respiratory tract infections and may indicate a mechanism for virus survival.

The E3 region of the virus can be totally deleted without inhibiting the replication ability in tissue culture [reviewed by (Burgert & Blusch, 2000; Horwitz, 2001)]. It is well conserved amongst species of human Ad (Burgert & Blusch, 2000) and can be regulated in T-cells by the T-cell's activation state, indicating a possible role for the E3 genes in viral persistence in T-cells (Mahr et al, 2003). The E3 proteins can subvert the immune responses to Ad in three different ways (Mahr et al, 2003). E3gp19k is known to bind and retain HLA class I molecule in the endoplasmic reticulum (Burgert & Kvist, 1987; Burgert et al, 1987) and prevent tapasin processing of peptides which bind to HLA class I molecules (Bennett et al, 1999). This provides a mechanism to reduce antigen presentation to CD8 T-cells and may be one explanation for the relatively low frequencies of memory Ad-specific CD8 T-cells in the peripheral blood. A number of E3 proteins interfere with mediators of apoptosis such as TNF-α, TNF related apoptosis-inducing ligand (TRAIL) and Fas ligand (Fas-L). This could help prevent cytotoxic deletion of Ad infected cells by CD4 T-cells, which often use Fas/Fas-L mechanism rather than the granzyme/perforin mechanism utilised by CD8 T-cells. However, interference with this group of apoptotic mediators could also prevent the destruction of Ad infected T-cells by activation-induced cell death and thus provide a mechanism of persistence in T-cells. E4 gene products have also been demonstrated to inhibit

CD8 T-cell cytolysis (Kaplan et al, 1999), by an unknown mechanism though E4ORF has been implicated.

Protein	Function	Mechanism	Reference
Virus associated RNA (160 nucleotides)	Prevents induction of an antiviral state by type I IFNs	Inhibition of the translational initiation of dsRNA dependent protein kinase Antagonises the pro-apoptotic activity of dsRNA dependent protein kinase	(Schmedt et al, 1995)
E1A	Inhibition of inflammatory cytokines	Increased intercellular adhesion molecule-1 and IL-8 with lipopolysaccharide stimulation	(Higashimoto et al, 2006)
	Enables virus invasion of host cell	Inhibit transcriptional activation of the nitric oxide synthase gene	(Higashimoto et al, 2006)
	Inhibits efficient viral antigen presentation to host cell	Inhibit transcriptional activation of Type I IFNs Inhibit the functions of Type I IFNs genes	(Ackrill et al, 1991)
E1B 19k	Inhibit TNF- α mediated lysis	Inhibition of p53 inducible and death promoting bax protein	(Perez & White, 2000)
	Inhibits Fas-L induced lysis.	Inhibits Caspase-8 activation	(Perez & White, 1998)
E3-gp19k	Blocks CD8+ T-cell recognition.	Retains HLA class I molecules in the ER and prevent tapasin processing of peptides.	[(Burgert & Kvist, 1985); (Bennett et al, 1999)}
E3-14.7k	Inhibits TNF-α mediated lysis	Inhibits TNF-α induced synthesis of arachidonic acid	[(Krajcsi et al, 1996)
	Inhibits Fas-L induced lysis.	Binds caspase 8	(Chen et al, 1998)
RIDα/β complex (E3-10.4k/14.5k)	Inhibits TNF-α mediated lysis	Block translocation of phospholipase A2 to the plasma membrane and so inhibits release of arachidonic acid	[(Krajcsi et al, 1996); (Dimitrov et al, 1997)]
	Inhibits Fas-L induced lysis	Internalises Fas- and promotes its degradation in lysosomes	[(McNees et al, 2002; Shisler et al, 1997); (Elsing & Burgert, 1998; Hilgendorf et al, 2003); (Tollefson et al, 1998)]
	Inhibits TRAIL induced lysis.	Internalises TR1 and TR2 (in conjunction with E3-6.7K) and promotes their degradation in lysosomes	[(Tollefson et al, 2001); (Lichtenstein et al, 2004)]

Table 1-7 Immune evasion strategies of adenoviruses

E1 or 3 Early 1 or 3 genes TR1 or 2= TRAIL receptor 1 or 2

1.4.4 Molecular pathogenesis

Adenovirus can be transmitted by respiratory droplets, faeco-oral route and in medical settings by virus contaminated instrument. The incubation period is generally short, ranging from 2 to 14 days [reviewed by (Russell, 2009)]. Transmission is from human to human and there are no animal reservoirs for most human Ads. The fibre is responsible for the initial attachment of virus to the host cell and binds to coxsackie-adenovirus receptor (CAR) cell surface protein with high affinity (Bergelson et al, 1997; Roelvink et al, 1998). Except for species B, all other adenoviruses use the CAR (Einfeld et al, 2001; Roelvink et al, 1998). Species B viruses have been shown to use CD46 as their primary receptor (Gaggar et al, 2003; Segerman et al, 2003b). Sialic acid (Arnberg et al, 2002) and heparan sulphate glycosaminoglycans have been implicated as receptors for Ad 37 and for Ad2 and Ad5 respectively (Dechecchi et al, 2001; Dechecchi et al, 2000).

Subsequent to binding the cellular receptors, virus is internalised and endocytosed to clathrin coated pits (Nemerow et al, 1994; Wickham et al, 1993). A step-wise disassembly of the Ad particle begins at the time of endocytosis with the rapid loss of fibres (Meier & Greber, 2004). Following endosomal release, Ad capsids translocate towards the nucleus and dock at the nuclear pore complex (NPC) (Greber et al, 1997). After complete dismantling, virus DNA and protein VII are extruded through the nuclear pores. Viral capsomeres assemble in the nucleus with the association of hexon trimers with penton and DNA packaging [reviewed by (Ostapchuk & Hearing, 2005)]. The cysteine protease cleaves precursor proteins VI, VII, VIII and terminal proteins to their mature forms yielding infectious virus particles. Virus escape is helped by disruption of cellular intermediate filaments and cytokeratin K18 by Ad death protein and virus protease (Tollefson et al, 1996a; Tollefson et al, 1996b{Chen, 1993 #601)

respectively resulting in cell instability and lysis. The excess fibre produced aids in viral escape as well as spread to neighbouring cells (Walters et al, 2002). Figure 1-9

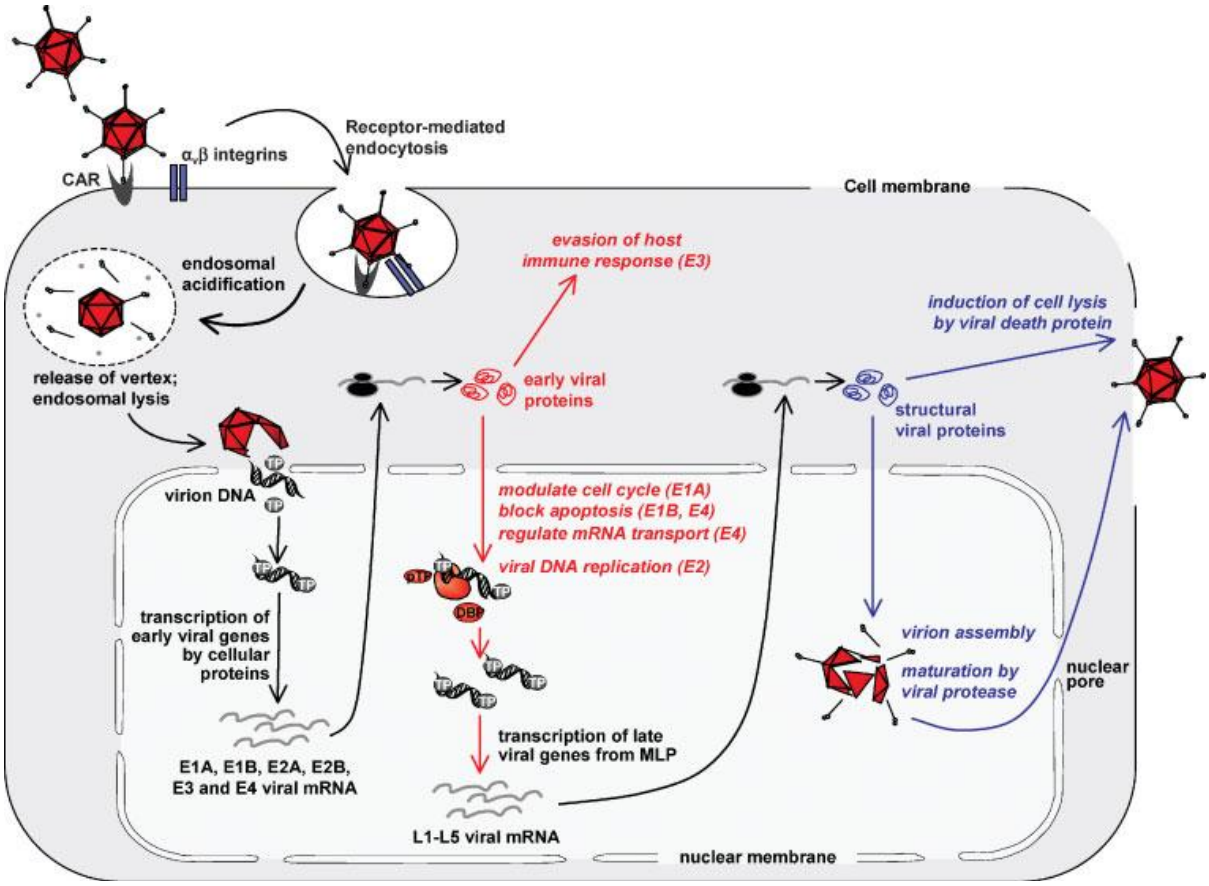

Figure 1-9 Ad infection pathway

CAR, coxsackie-adenovirus receptor; DBP, DNA-binding protein; E, early; L, late; MLP, major late promoter; Lenaerts *et al* (Lenaerts et al, 2008a)

1.4.5 Epidemiology

Adenoviruses are endemic worldwide. They were first associated with conjunctivitis (Mitsui & Jawetz, 1957) and outbreaks of acute respiratory syndrome in groups of U.S. military recruits (Hilleman, 1957). They account for 3% of infections in the civilian population and 7% of all febrile illnesses (Fox et al, 1969). The corresponding figures in young children are 5 and 10% respectively (Kotloff et al, 1989). The majority of infections occur in the first 5

years of life, with a peak incidence during the first 2 years (Pacini et al, 1987). They account for around 5% of acute respiratory illnesses (species C in particular) and are a major cause of viral gastroenteritis (species F in particular) in infants (Brandt et al, 1985; Jeffries et al, 1988). A large scale study demonstrated that civilians mostly encountered adenoviruses from species A and C whilst the most prevalent species found in military trainees were C, B1 and E (Gray et al, 2007). The receptor usage (most used is Coxsackie adenovirus receptor - CAR) is mostly responsible for the tissue tropism of the different species (Mei et al, 2004; Segerman et al, 2006; Shayakhmetov & Lieber, 2000; Xiao et al, 2005)Table 1-8.

As nonenveloped viruses, adenoviruses are highly resistant to physical and chemical agents. They are stable at low pH and are resistant to gastric and biliary secretions, thus allowing the virus to replicate and achieve a high viral load in the gut. Sodium hypochlorite (50 ppm) for 10 min or 70% ethanol for at least 1 minute can be used to inactivate pure virus or virus contaminated surface (Rutala et al, 2006). They remain infectious at room temperature for prolonged periods (up to 3 weeks).

Subgroup	**Serotypes**	**Sites affected**	**Transplant recipients**
A	12, 18, ***31***	Gastrointestinal tract	Pneumonia, Enteritis
B1	***3***, ***7, 16***, 21, 50	Respiratory tract, Ophthalmic	Pneumonia, Enteritis
B2	***11, 14, 34, 35***	Urinary tract	Haemorrhagic cystitis, Acute renal failure
C	***1, 2, 5, 6***	Respiratory tract	Hepatitis, Pneumonia,
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	Gastrointestinal tract, Ophthalmic	
E	4	Respiratory tract, Ophthalmic	
F	***40, 41***	Gastrointestinal tract	Enteritis
G	52	Gastrointestinal tract	

Table 1-8 Classification of Ad serotypes and common sites of infection

The serotypes highlighted in bold and italics are common amongst transplant recipients and the last column highlights the organs mainly affected in this cohort of patients. All serotypes can cause disseminated disease.

In immunecompetant individuals Ad infection is asymptomatic or mild and self-limiting with few long-term consequences (Lichtenstein & Wold, 2004), and therefore does not warrant anti viral therapy. It manifests primarily as upper and lower respiratory tract infection (tonsillitis, otitis media, pharyngitis) and bronchiolitis or bronchitis, gastroenteritis or keratoconjunctivits, haemorrhagic cystitis (Chuang et al, 2003). Fatal outcome is relatively rare and has been reported in the context of pneumonitis (Hong et al, 2001). In rare cases, hepatitis, myocarditis, menigoencephalitis or nephritis is encountered (Straussberg et al, 2001).

1.4.6 Defining adenovirus infection

Ad infections were defined based on propositions by Flomenberg *et al* (Flomenberg et al, 1994). Ad infection was defined as the presence of 1 positive result of tissue culture or PCR assay from blood, stool or urine irrespective of clinical symptoms. Definite disease was defined by either the presence of Ad nuclear inclusions, by a positive result of tissue culture or PCR assay from a sterile site (excluding the gastrointestinal tract), or by a positive immunohistological study with compatible symptoms without other identifiable cause. Probable disease was defined as the presence of 2 positive results of tissue culture or PCR assay from other body sites with compatible symptoms without other identifiable cause.

These definitions rely on the use of biopsies or tissue culture not frequently performed currently. A recent classification by Tebruegge *et al* (Tebruegge & Curtis, 2010) accounts for the changes in current clinical practice with relevance to PCR assays and also includes disseminated disease. The definitions have been described in the following Table 1-9.

Terms	Definitions (Tebruegge & Curtis, 2010)
Adenovirus infection	Detection of adenovirus by immunofluorescence, culture, histology or PCR from any site
Asymptomatic adenovirus infection	Detection of adenovirus from any site in the absence of clinical symptoms
Adenovirus disease or symptomatic adenovirus infection	Detection of adenovirus from any site with anatomically corresponding, compatible symptoms in the absence of any other identifiable cause
Disseminated disease	Adenovirus disease affecting two or more organs

Table 1-9 Definitions of terminology related to adenovirus infection

1.4.7 Adenovirus infections in the immunocompromised

An immunecompromised patient is incapable of developing a normal immune response due to deficient or defective components of the immune system. This could be congenital or acquired due to corticosteroids, cytotoxic agents, radiation, AIDS, malnutrition or severe burns. AIDS patients and HSCT recipients are most vulnerable to Ad in this group (Kojaoghlanian et al, 2003). In immunecompromised individuals, species C, B1 and E are more prevalent (Echavarria, 2008). Coinfection with more than one Ad serotype per clinical event was more frequent in immunecompromised patients (50%) than in immunecompetant patients (5%) (Gray et al, 2007; Suparno et al, 2004). Clinical manifestations in immunocompromised patients include pneumonia, hepatitis, hemorrhagic cystitis, colitis, pancreatitis, meningoencephalitis, and disseminated disease, depending on the underlying disease, affected organ system, patient age, and virus serotype (Echavarria, 2008).

1.4.7.1 *Adenovirus infection in transplant recipients*

Ad has been increasingly recognised as a pathogen causing significant morbidity and mortality in immunocompromised hosts in particular HSCT recipients since the late 1980s. It occurs in 40% of paediatric and 10% of adult HSCT recipients and 5-10% of solid organ

transplant recipients (Tebruegge & Curtis, 2010). Prospective and retrospective studies of Ad infections in HSCT recipients since 1985 are summarised in Table 1-10.

In HSCT recipients, adenovirus mainly affects the gastrointestinal, respiratory, renal or hepatic systems Table 1-8. There are distinct differences in the reported serotypes across the globe, i.e., Species B is commonly isolated in the American studies whereas in UK species C viruses are responsible for respiratory, intestinal or hepatic disease (Suparno et al, 2004). Ad11 causes most cases with haemorrhagic cystitis in Japan (Akiyama et al, 2001) whereas in the UK and USA polyoma virus accounts for most cases (Chakrabarti et al, 2003). In adults post HSCT infections most likely result from reactivation of adenoviruses, in children on the other hand prior lack of exposure result in primary infection (Runde et al, 2001). The sequential emergence of adenoviruses of different serotypes post paediatric HSCT recipients confirms this (Kroes et al, 2007).

The risk factors for virus infections following HSCT have already been discussed (section1.2.4.3). Ad infection occurs <30 days or 90-100 days following a HSCT in a paediatric or adult transplant recipient respectively (Flomenberg et al, 1994). Younger age (<15), allogeneic HSCT and GvHD are most important amongst risk factors for Ad infections (Suparno et al, 2004). There is well documented evidence for an increased incidence of Ad infections in paediatric HSCT as well as in adult allograft recipients (Tebruegge & Curtis, 2010). With regards to GvHD there is still much debate; some studies have identified it as an independent risk factor (La Rosa et al, 2001) whereas others find no correlation (Feuchtinger et al, 2005).

T-cell depleted allogeneic HSCT and lymphocyte count at the time of infection (<300/μl) have been identified as important factors associated with progression of Ad infection to disseminated disease (Chakrabarti et al, 2002). Severity of immune suppression and

continuation of immunesuppression despite Ad infection may also result in Ad disease. Ad disease associated mortality is around 20-50%; higher amongst paediatric transplant recipients[reviewed by (Tebruegge & Curtis, 2010)]. In a recent prospective study, 5/97 SCT patients had Ad infection and none required treatment or progressed to Ad disease (Ohrmalm et al, 2010).This study had no patient who had T depletion which may explain their ability to clear virus without intervention. A recent single institution study discusses the problems faced with patients who have concurrent GvHD. Virus clearance was achieved only in 1/6 (16%) of patients whilst 2 of the other survivors had chronic urinary persistence of virus (Fowler et al, 2010).

Reference	**Prospective/retrospective**	**Total patients / Duration of study**	**% of Ad infection** (No of ad infected patients / total no of patients)	**% of Ad disease** (no of symptomatic patients / no of infected patients)	**% of Ad related mortality** (no of deaths / no of infected patients)	**Comments**
(Shields et al, 1985)	Retro	1051, 6 years	5 (51/1051)	20 (10/51)	12 (6/51)	First study, identified GvHD and disseminated disease as risk factor
(Wasserman et al, 1988)	Retro	96, 7 years	18 (17/96)	ND	6(1/17)	This was a paediatric study autografts included
(Ljungman et al, 1989)	Prosp	78, 1 year	6 (5/78)	ND	20 (1/5)	
(Flomenberg et al, 1994)	Retro	201, 4years	21 (42/201)	31 (13/42)	17 (7/42)	Incidence in adults 14% (16/118), and children 31%(26/83) autografts included
(Blanke et al, 1995)	Retro	74, 2 years	14 (10/74)	10 (1/10)	50(5/10)	Poor response to ribavarin, one responder to DLI
(Hale et al, 1999)	Retro	206, 4 years	6 (13/206)	46 (6/13)	50 (7/13)	TBI and graft type identified as independent risk factor autografts included
(Howard et al, 1999)	Retro	532, 11 years	12 (64/532)	64 (41/64)	17(11/64)	Adults 9(35/405), children 23 (29/127) autografts included allo / auto 16 to 3%

(Baldwin et al, 2000)	Retro	572, 10 year	17(100/572)	NA	6(6/100)	Adults 9% children 21% 50% of patients had other virus infections
(Venard et al, 2000)	Pros	65, 8 months	20 (13/65)	61(8/13)	70(9/13)	autografts included
(Hoffman et al, 2001)	Pros	36, 1 year	47(17/36)	82(14/17)	12(2/17)	Sibling versus alternative donor 27% v 67% Demonstrated a role for cidofovir therapy
(Bordigoni et al, 2001)	Retro	303,14 years	12 (35/303)	60 (21/35)	42(15/35)	GvHD and delay in onset of treatment where risks for progression to ad disease. Cidofovir and DLI were found effective whereas Vidarabine and ribavarin ineffective treatment options.
(Echavarria et al, 2001)	Retro	328, 14years	12 (38/328)	45(17/38)	18 (7/38)	Identified PCR as a tool for early Ad detection
(La Rosa et al, 2001)	Retro	2889, 8 years	3 (85/2889)	89(76/85)	26(22/85)	Allograft recipients with GvHD and on immunesuppression had a higher risk of infection. Mortality was higher in those with pneumonia and disseminated disease (61%)
(Chakrabarti et al, 2002)	Pros	76, 4 years	20 (15/760	40(6/15)	20 (3/15)	Adult allografts, Ad infection developed exclusively in the T-cell depleted cohort given Campath. Lymphocyte count of 300/µl at the time of Ad infection detection improved prognosis

(Lion et al, 2003)	Pros	132, 6 years	27(36/132)	42 (15/132)	22 (8/36)	Paediatric allografts; 82% of patients with detectable AD in blood by PCR died.
(Bruno et al, 2003)	Retro	5233, 15 years	9 (450/5233)	9 (42/450)	NA	Higher incidence after 2nd allograft.
(Leruez-Ville et al, 2004)	Pros	44, 7 months	18 (8/44)	ND	25 (2/8)	Ad viral load measured by PCR correlated to response to treatment (cidofovir)
(Kampmann et al, 2005)	Pros	155, 3 years	17% (26/155)	ND	19 (5/26)	Ad infection developed exclusively in the T-cell depleted cohort (126/155)83 %. Failure of lymphocyte recovery was associated with mortality.
(van Tol et al, 2005)	Retro	328, 14years	11 (37/328)	46 (17/37)	19 (7/37)	Paediatric Allo graft alone. Delayed cellular and humoral immune recovery was associated with poor outcome.
(Yusuf et al, 2006)	Retro	177, 3 years	32(57/177)	14 (8/57)	2 (1/57)	Paediatric Allo graft alone. Cidofovir was successful in 98% of patients.
(Kroes et al, 2007)	Pros	83, 3 years	34 (28/83)	ND	36 (10/28)	Paediatric Allo graft alone. Demonstrated multiple serotypes in same patient
(Kalpoe et al, 2007)	Retro	165, 4 years	A 5(5/107) C 14 (8/58)	A 20 (1/5) C 38 (3/8)	A 20 (1/5) C 38 (3/8)	Different conditioning regimens did not affect outcome. 80% of adults cleared virus without treatment.
(Symeonidis et al, 2007)	Retro	687, 7 years	9 (64/687)	17 (11/64)	7(5/64)	Adult and paediatric allograft recipients included. 73% with Ad diseases were T-cell depleted graft recipients

(Sivaprakasam et al, 2007)	Pros	71, 2 years	11(8/71)	88(7/8)	38(3/8)	All 8 patients had T-cell depletion. 2 patients developed graft failure and were treated with DLI
(Ohrmalm et al, 2010)	Pros	97, 18 months	5 (5/97) A 3 (2/77) C 15(3/20)	0	0	No T depleted HSCT included. No patient needed treatment. They concluded that PCR monitoring for Ad DNA is not warranted.
(Omar et al, 2010)	Pros	344, 4 years	5(13/344) A 5.4 C 3	46 (6/13)	23(3/13)	All children cleared virus. They conclude that patients with sustained Ad viraemia have a poor outcome despite cidofovir or ribavarin.
(Bil-Lula et al, 2010)	Retro	116, 3 years	43 (52/ 116) (C 98/ A18)	37(19/52)	19(10/52)	Older age and MUD associated with increased risk of infections. Low lymphocyte counts observed in patients with Ad infection

Table 1-10 Incidence of Ad infection, disease and mortality in HSCT recipients

A: adult; C: children; NA: Not available; ND: Not defined; MUD: Matched unrelated donor

1.4.7.2 *Diagnostic methods for detection of Adenovirus*

Adenovirus infected cells have large nuclei with basophilic inclusions surrounded by a thin rim of cytoplasm identified on histopathology. Growth of adenoviruses from patient material is best achieved in cells of human origin. Primary human embryonic kidney cells (HEK) are probably the best host for the replication of the human adenoviruses (Krisher & Menegus, 1987). The 293 cell line, a primary HEK transformed to a continuous line by Ad5, retains the E1A and E1B regions of the Ad genome covalently linked to the host DNA (Graham et al, 1977). The A549 cell line derived from a human lung carcinoma is a good host for most but the ocular strains of Ad. Except for serotypes 40 and 41 all human adenoviruses grow well in epithelial cell lines and produce a cytopathic effect characterised by clumping and cell rounding with refractile intranuclear inclusion bodies (Echavarria, 2008). Cytopathic effect is usually visible in 2- 7 days. Unfortunately blood specimens may be unsuitable for virus cultures (probably due to neutralising antibodies), slow (could take up to 28 days) and uninterpretable due to contamination.

Apart from culture other direct methods of Ad detection include antigen detection and genome detection with or without amplification (Hierholzer, 1992). Methods like immunofluorescence, enzyme immunoassay, immunechromatography and latex agglutination enable use of various specimen samples and are very sensitive and rapid. Indirect methods include serology but are limited by lack of sensitivity or inadequate antibody production by the immunocompromised patient (Tebruegge & Curtis, 2010). Molecular methods using DNA amplification by PCR have increased the sensitivity and rapidity of diagnosis (Echavarria et al, 1999). Myers *et al* (Myers et al, 2007) defined a QPCR result of >1500 Ad copies/ml of blood, to diagnose Ad infection (Myers et al, 2007). Virus DNA load monitoring in a high risk patient cohort may be useful for preemptive therapy (Ohrmalm et al, 2010).

1.4.7.3 *Therapeutic options for Adenovirus infection*

1.4.7.3.1 Antiviral agents

To date no randomised control trials of antiviral agents have been conducted in patients with Ad infections. A placebo controlled randomized trial for the treatment of Ad infections would be unethical given the high morbidity and mortality associated with it in the immunecompromised host. Also none of the available antiviral drugs are licensed for use in patients with Ad infections. However existing literature of prospective or retrospective studies provides compelling evidence that cidofovir is the most effective therapeutic option (Lenaerts et al, 2008a).

Adenoviruses encode their own DNA polymerase, single stranded DNA binding protein (DBP) and pre-terminal/ terminal DBP. They do not encode any of the multiple nucleotide pool- modifying enzymes found in the herpes viruses such as dUTPase, thymidine kinase and ribonucleotide reductase and hence are not responsive to anti-herpes virus agents activated by these enzymes (Kinchington et al, 2005). Recently a new compound with antiadenoviral properties has been identified 2-[[2-(benzoylamino) benzoyl] amino]-benzoic acid (Andersson et al, 2010).

1.4.7.3.1.1 Zalcitabine

Zalcitabine (2', 3'-dideoxycytidine) is primarily used in the treatment of HIV infection. Though antiadenoviral activity of this drug has been demonstrated *in vitro* (Mentel et al, 1997; Mentel et al, 2000; Uchio et al, 2007) as well as in an animal model (Mentel & Wegner, 2000), there are no reports of clinical use of this agent for Ad infections. Significant mitochondrial toxicity at adequate serum concentrations needs to be considered as well (Naesens et al, 2005).

1.4.7.3.1.2 Ganciclovir

Most acyclic nucleoside analogues, including acyclovir, penciclovir and foscarnet have no antiadenoviral activity *in vitro*, except for ganciclovir (Naesens et al, 2005) though its mechanism of action in Ad infection is unknown. In a prospective randomized trial in patients who received prophylactic or preemptive ganciclovir for CMV, lower rates of Ad infection were observed (Bruno et al, 2003). Its potential as a therapeutic option has not been tested.

1.4.7.3.1.3 Vidarabine

Vidarabine, (9-β-D-arabinofuranosyladenine) is active *in vitro* against double-stranded DNA viruses, including human Ad. Successful clearance of post transplant Ad associated hemorrhagic cystitis has been reported (Kawakami et al, 1997; Kitabayashi et al, 1994). On treatment most patients (except for one patient in the Kawakami *et al* study) had resolution of symptoms as well as viral clearance. However there are reports of patients who continued to deteriorate clinically despite therapy (Hatakeyama et al, 2003). Also in the study by Bordigoni *et al* none of the 7 patients who received Vidarabine alone or in combination with ribavarin survived (Bordigoni et al, 2001).

1.4.7.3.1.4 Ribavarin

Ribavarin (1-β-D –ribofuranosyl; y-1, 2, 4- triazole -3 carboximide), is a purine nucleoside analogue, that is converted to its triphosphate forms by cellular enzymes (Graci & Cameron, 2006). The principal target for ribavirin (in its 5′-monophosphate form) is Inosine Monophosphate (IMP) dehydrogenase that converts IMP to Xanthosine Monophosphate (XMP), a key step in the de novo biosynthesis of GTP and dGTP. *In vitro* studies have established that ribavarin has activity against all serotypes from species C and certain

serotypes of species A, B and D (Morfin et al, 2009; Naesens et al, 2005). However when tested *in vivo* by monitoring patients infected with species C serotypes post ribavarin therapy, decrease in Ad DNA in blood (by quantitative PCR) could not be demonstrated in any patient (Lankester et al, 2004).

In the 1990's Ribavarin was reported to clear Ad in transplant recipients (Cassano, 1991; Liles et al, 1993; Murphy et al, 1993), HIV (Maslo et al, 1997) and combined immune deficiency patients (Wulffraat et al, 1995) as well as those on immune suppression (Sabroe et al, 1995). However failure of ribavarin therapy in treating Ad disease has been reported for larger patient cohorts (Chakrabarti et al, 1999; Hromas et al, 1994a; Mann et al, 1998). Whilst the study by Howard *et al* showed inconsistent results (Howard et al, 1999), in another study by Bordigoni *et al*, 70% of the patients receiving ribavarin alone as the antiviral agent died (Bordigoni et al, 2001). Also only 2/12 patients in the La Rosa *et al* study recovered post ribavarin therapy (La Rosa et al, 2001). Interestingly patients in whom therapy was unsuccessful were more immune suppressed or had T depleted grafts.

1.4.7.3.1.5 Cidofovir

Cidofovir [(S) - HPMPC; (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine], is an acyclic nucleoside phosphonate (monophosphate nucleoside analogue of cytosine), belonging to a generation of drugs licensed for treatment of DNA viruses and retroviruses (De Clercq, 2007). Following cellular uptake by means of endocytosis, the cellular enzymes convert it to diphosphate form which is used by viral DNA polymerase instead of deoxyribonucleotide triphosphate. Though the precise mechanism is not known, cidofovir inhibits Ad DNA polymerase activity (Lenaerts & Naesens, 2006). *In vitro* data shows that cidofovir has good antiviral activity against all species of Ad as well as herpes simplex virus, CMV, EBV (De

Clercq & Holy, 2005; Safrin et al, 1999). Its ability to abrogate virus replication in Syrian hamster was demonstrated recently (Diaconu et al, 2010).

Cidofovir is the most commonly used antiadenoviral agent in the post transplant setting with reports of inconsistent success. However, its use is limited by the renal toxicity (Vandercam et al, 1999) on the proximal convoluted tubule cells. Concomitant administration of probenecid is thought to reduce this side-effect by competing for cidofovir uptake (Cundy et al, 1996; Masereeuw et al, 2000). For Ad infections cidofovir is administered intravenously at 5mg/kg per week during the first 2 weeks, then 5mg/kg every other week, with sufficient hydration and under cover of probenecid to prevent nephrotoxicity (1997).

Relative success has been demonstrated with the absence of Ad related fatalities in a number of studies (Anderson et al, 2008, Saquib, 2010 #852; Bateman et al, 2006; Hoffman et al, 2001; Leruez-Ville et al, 2006; Muller et al, 2005; Nagafuji et al, 2004). However, therapy related failures have also been reported (Flomenberg et al, 1994; Howard et al, 1999; La Rosa et al, 2001; Shields et al, 1985). 2 large studies in this context (Ljungman, 2004; Yusuf et al, 2006) demonstrate low Ad related mortality and good clearance of virus. In conclusion, available data suggests that cidofovir is currently the most effective antiviral drug for the treatment of Ad infections in HSCT recipients (Lenaerts et al, 2008a) followed by ribavarin. Prompt identification of Ad infection and early cidofovir management is very effective in virus clearance (Legrand et al, 2001; Sivaprakasam et al, 2007). Patients with prolonged viraemia have a poor outcome despite antiviral therapy (Omar et al, 2010). A novel orally bioavailable lipid conjugate of cidofovir, CMX001 was successful in eradiacating disseminated Ad disease in a SCT recipient following failure to respond to cidofovir (Paolino et al, 2011).This product needs to be evaluated in future prospective randomised trials.

1.4.7.3.2 Immunological agents

Apart from withdrawal of immunesuppression if possible, IVIg and adoptive immunotherapy using donor lymphocyte infusions (DLI) are therapeutic options for Ad infections in HSCT recipients. DLI has been discussed (section1.3.1.1)

1.4.7.3.2.1 Intravenous Immunoglobulin (IVIg)

IVIg is pooled immunoglobulin obtained from the plasma of several hundreds of blood donors and is administered intravenously. It is mainly rich in IgG and has a half-life of around 4-6 weeks (Lux et al, 2010). It is used to provide passive immunity as well as for its anti-inflammatory properties. IVIg preparations contain neutralising antibodies against common Ad serotypes. Unfortunately it is unlikely to confer protection against less prevalent serotypes in the general population which are often encountered in immunocompromised patients. Though effective against exogenous or primary Ad infections it is unlikely to provide protection against reactivating virus. Patients with virus reactivation may already have high neutralising antibody titres resulting in incomplete virus clearance. This may explain why it has been used in transplant recipients with inconsistent results (Dagan et al, 1984; Flomenberg et al, 1994; Sabroe et al, 1995; Wigger & Blanc, 1966).

1.4.7.3.2.2 Adoptive cell therapy

Pharmacological agents (section 1.4.7.3.1) widely used for Ad infection or disease have substantial toxicities, but they are not effective against all serotypes and may drive the outgrowth of resistant viral strains (Kinchington et al, 2002). They also do not boost antiviral immunity and hence infections may recur following therapy. The inconsistent responses to anti adenoviral agents are probably secondary to the T-cell immunity at the time of treatment. Patients with partial Ad-specific immunity are able to clear or reduce virus load whereas

those lacking Ad-specific immunity show minimal or no response to cidofovir or ribavarin (Feuchtinger et al, 2005). It is also well documented that HSCT recipients can clear Ad infection without intervention, provided they have sufficiently reconstituted their cellular immunity (Ohrmalm et al, 2010; Walls et al, 2005).

Different methods for enrichment of antigen-specific T-cells are discussed in 1.3.1. There is no standard adoptive transfer protocol for Ad infections. Currently the widely used method is isolation of Ad-specific T-cells based on cytokine secretion selection. The efficacy and safety of this method was established by Feuchtinger *et al* in a phase II trial (Feuchtinger et al, 2006) using species C virus lysate as antigen. They have since demonstrated that Ad hexon can be used as an alternative antigen with no statistically significant difference in outcome (Feuchtinger et al, 2008) confirmed by a French group (Aissi-Rothe et al, 2010). Phase I and II trials have proven safety and efficacy of multivirus (CMV, EBV and Ad) (Comoli et al, 2008; Cruz et al, 2010; Karlsson et al, 2007)-specific CD8 T-cells as prophylaxis for high risk patients. The long duration involved in generation of these CD8 T-cells make it an almost impossible option as therapy. This protocol has since been improved for rapid generation of CD8 T-cells to GMP standards but needs to be tested in a clinical trial (Vera et al, 2010).

Antiviral drugs may play a role primarily in controlling viral replication while clonal expansion of the antigen-specific T-cell is required for adequate control and clearance of virus. This provides a platform for reconstitution of HSCT recipients with antigen-specific T-cells resulting in an effective non toxic strategy to provide both immediate and long term protection (Chatzidimitriou et al, 2010). Adoptive immunotherapy has an established role in the therapeutic and prophylactive management of CMV (Walter et al, 1995) and EBV (Rooney et al, 1998).

1.4.7.3.2.3 Donor lymphocyte infusion (DLI)

DLI has been discussed in (section 1.3.1.1). Successful treatment of Ad disease after stem cell transplantation with unmanipulated donor lymphocyte infusion (DLI) was reported in 7/9 published cases (Bordigoni et al, 2001; Chakrabarti et al, 1999; Chakrabarti et al, 2000; Chakrabarti et al, 2002; Howard et al, 1999; Hromas et al, 1994b; Miyamoto et al, 1998)}. The cell dose ranged from $1x10^5$-$2x10^7$ CD3 T-cells/kg. This treatment option is associated with a considerable risk of severe graft versus host disease and marrow aplasia. The successful outcome in the majority of treated patients highlights the advantages of adoptive transfer of antigen-specific T-cells alone, which will reduce the risk for GvHD and favour antigen-specific immune reconstitution.

1.5 Aims of thesis

Adenovirus infections are a major cause of morbidity and mortality following HSCT. Currently there are no licensed anti adenoviral agents and those used give inconsistent outcomes. The importance of adenovirus-specific immune reconstitution for adenovirus clearance highlights the role of cellular immune response in the control and clearance of Ad in this cohort of patients. DLI has shown success in patients refractory to antiviral therapy but it is associated with the risk of severe GvHD. Adoptive transfer of Ad-specific T-cells to HSCT with adenovirus infections is a good therapeutic strategy in this setting. Virus-specific T-cells facilitate early Ad-specific T-cell reconstitution and reduce the risk of GvHD.

The aim of this thesis is to

1. Establish the pre-clinical criteria for the clinical grade selection of Ad-specific T-cells by cytokine secretion and multimers for purposes of a randomised controlled clinical trial to compare the safety of cells selected by either method.
2. Generate tetramers for Ad class I MHC epitopes
3. Determine frequency of Ad-specific T-cells by tetramer staining and cytokine secretion
4. Characterise the phenotype, proliferative potential, antigen recognition, cytotoxicity and cross reactivity of cells identified by either method.

These parameters allowed understanding of the homing and survival potential of these T-cells as well as their ability to respond to antigen. 3 patients with Ad infections following HSCT were also studied. This has given us valuable information on assessing Ad-specific immune reconstitution following adoptive transfer. Obstacles encountered during the establishment of clinical grade selection and the possible methods to overcome these have also been discussed.

2 Materials and Methods

2.1 Participants in the study

Blood samples were obtained following informed written consent. Donors HLA types were determined by molecular tissue typing for class I and class II HLA molecules, carried out by the tissue typing department at the National Blood Service, Birmingham, UK (Table 2-1). Local ethical approval (07/Q2602/41) was obtained from the South Birmingham Ethics committee for obtaining blood and tissue from healthy volunteers within the School of Cancer Sciences, University of Birmingham, UK.

2.2 Peptides

Ad peptides were synthesised by Severn Biotech, Redditch, UK and dissolved in DMSO. The concentration was checked following solubilisation using biuret reagent (Sigma, UK). 180µl of biuret reagent was added to 20µl of peptide, and to 20µl of two fold serial dilutions of BSA dissolved in DMSO in a 96 well V bottom plate. Plates were incubated for 30 min at room temperature, centrifuged (2600g) and 100µl of each sample was transferred to a 96 well flat bottomed plate. Absorbance λ=540nm was read on a Victor plate reader (Wallac, UK). Peptide concentration was determined by absorbance λ=540nm readings by linear regression from a standard curve generated for known concentrations of BSA dilutions.

Volunteer number	Sex	Class I HLA	
LD1	M	A*01 A*01,B*08 B*57	
LD2	M	A*01 A*11, B*07 B*35	
LD3	M	A*01 A* 24 B*62 B*49	
LD4	F	A*01 A*30 B*08 B*13	
LD5	M	A*01 A*01 B*08 B*62	
LD6	F	A*01 A*01 B*07 B*08	
LD7	M	A*01 A*11 B* 22 B*37	
LD8	F	A*01 A*02B*27 B*62	
LD9	F	A*01 A*02 B*44 B*57	
LD10	F	A*01 A*02 B*61 B*57	
LD11	M	A*01 A*02B*44 B*55	
LD12	M	A*01 A*02 B*16 B*40	
LD13	F	A*01 A*02 B*08 B*40	
LD14	M	A*03 A*23 B*07 B*44	
LD15	F	A*03 A*31 B*07 B*16	
LD16	M	A*02 A*23 B*04 B*44	
LD17	F	A*02 A*11B*60 B*51	
LD18	M	A*02 A*02 B*40 B*44	
LD19	M	A*03 A*29 B*07 B*44	
LD20	M		Not known
LD21	M		Not known
LD22	M		Not known
LD23	M		Not known
LD24	M	A*02 A*24 B*39 B*39	

Table 2-1 Participants in the study

2.3 Adenovirus methods

Ad 3, 5, 12 and 40 were obtained from, D Onion, University of Birmingham, UK, Ad 11 from G. Wilkinson, University of Cardiff, UK and Ad19a from H Burger, University of Warwick, UK.

Unless otherwise stated all viruses were obtained from Dr. V. Mautner, Gene and Immunotherapy group, School for Cancer Sciences, University of Birmingham UK.

2.3.1 Adenovirus mutants

AdGFP an E1 (from nt 358-3329), E3 (from nt 28593-30470) deleted Ad5 expressing enhanced green fluorescent protein under the control of the CMV immediate early promoter inserted in the E1 region was constructed by Drs. V. Mautner and P. Searle(Read et al, 2003).

Adβ-Gal an E1 (from nt 358-3329), E3 (from nt 28593-30470) deleted Ad5 expressing β-galactosidase under the control of the CMV immediate early promoter inserted in the E1 region was constructed by Drs. V. Mautner and P. Searle.

AdΔE3 an E3 deleted (from nt 28593-30471) Ad5 constructed by Dr. S. Dolen and Dr V Mautner.

CTL102 - E1, E3-deleted replication-deficient human Ad serotype 5 vector, containing the *E. coli* nfsB gene under control of the cytomegalovirus immediate early promoter (Palmer et al, 2004)

2.3.2 Propagation and purification of adenoviruses

2.3.2.1 *Virus stocks*

Viruses were propagated in A549 (replication competent virus) or 293 cells (replication deficient virus), both in DME + 15mM HEPES, 2mM glutamine, 2% FCS. Sub-confluent cell monolayers in 150 cm^2 flasks were infected at an MOI of 10 pfu/cell in 5 ml of medium for 90 min, (37°C, 5% CO_2) before the addition of a further 18 ml of medium. Incubation was continued (37°C, 5% CO_2) until extensive cytopathic effect was seen (3-5 days). Cells were harvested and pelleted by centrifugation (538g, 5 min) and re-suspended in 0.5 ml of medium/flask. Virus was released from cells by 3 cycles of freezing (in liquid nitrogen) and thawing (at 37°C), cell debris was pelleted by centrifugation (800g, 10 min) and the supernatant containing free virus particles transferred into a fresh tube. Virus stocks were stored at -80°C until use as a virus infected cell lysate or further purification.

2.3.2.2 *Density gradient purification of adenovirus*

Virus seed stocks were incubated on ice for 1 hour following addition of 1% (v/v) water saturated n-butanol. The cell suspension was centrifuged (840g, 10mins, 4°C) and the supernatant layered on top of a glycerol/CsCl gradient [lower layer 2 ml ρ=1.45 CsCl (in 10mM Tris, pH 7.9) ; middle layer 3 ml ρ=1.32 CsCl (in 10mM Tris, pH 7.9) ; top layer 2 ml 40% glycerol (in 10 mM Tris, pH 7.9)]. Gradients were centrifuged (160,000g, 90 min, 4°C) using a SW40Ti rotor (Beckman Coulter, High Wycombe, UK). The opalescent band corresponding to intact virus particles was removed using a 21gauge needle and 5ml syringe. Virus particles were dialysed using a Slide-A-Lyzer dialysis cassette with 10,000 molecular weight cut off (Thermo Scientific, Waltham, MA, USA) against PBSg, (PBS, 10% glycerol

(v/v), 491µM $MgCl_2$, 898µM $CaCl_2$) overnight (4°C). Viruses were aliquoted and stored at -80°C.

2.3.2.3 *Determination of virus particle number*

The concentration of virus particles in CsCl gradient purified stocks was determined by the use of DNA binding PicoGreen (Molecular Probes, Eugene, USA) (Murakami & McCaman, 1999). 0.1% (w/v) SDS solution was mixed in a 1:1 ratio with a sample of virus and heated (30 min, 56°C) to inactivate the virus and release the viral DNA. DNA standards from serial two fold dilutions of a standard DNA stock solution (phage λ DNA, New England Biolabs (UK) LTD, Hitchin, UK) were serially diluted in $T_{10}E_1$(10mM Tris, 1 mM EDTA, pH 7.5) in triplicate in a 96-well Falcon Microtest assay plate and incubated (15 min, RT). Concentrations ranged from 0-200ng per well in a final volume of 100µl per well. 100µl of virus and DNA standards were added to 100µl of PicoGreen (diluted 1in 200 in $T_{10}E_1$). After incubation (2 min, dark) fluorescence (excitation λ = 485 nm, emission λ = 535 nm) was determined using a Victor plate reader (PerkinElmer, Monza, Italy (Formerly Wallac, Finland)). DNA concentration was calculated from the standard curve generated using serially diluted DNA standards. Adenoviruses have one double stranded DNA genome therefore every single DNA molecule represent one virus particle. Virus particle number was determined by multiplying the calculated DNA concentration, number of bases in viral DNA (for Ad5 35506bp), Avogadros number (6.0221415×10^{23}) and the average weight of bases (660Da/bp)- 1µg of DNA= $2.7x10^{10}$ virus particles.

2.3.2.4 *Determination of infectious virus particles*

A549 cells (to titre wild type viruses) or 293 cells (to titre replication deficient vectors) were seeded at $1x10^5$/dish on 6cm petri dish. After 16 hours when cells are confluent and properly

adherent, cells were incubated with 100µl of virus that had been serially diluted in infection medium. After 90 min incubation (37°C, 5% CO_2) medium was removed and monolayers covered with 4ml overlay agar [DME + 15mM HEPES, 2% FCS, 0.7% Nobles agar (Invitrogen), 100U/ml penicillin, 0.1mg/ml streptomycin, 491µM $MgCl_2$, 898µM $CaCl_2$, 6mM $NaHOH_3$]. Cells were fed by addition of 3 ml of overlay agar every three to four days. By macroscopic inspection, plaques were identified on days 10 and 14 following infection. The mean of triplicate values was taken and used to determine the number of infectious particles present in the original undiluted viral stock or purified virus, referred to as plaque forming units (pfu). The ratio of virus particles to pfu (particle to infectivity ratio) was taken as an indication of the quality of the virus. All viruses used in this study had a particle to infectivity ratio less than 100. The AdGFP used for the majority of experiments had a particle to infectivity ratio of 10. Unless otherwise indicated the multiplicity of infection (MOI) in this study is stated as particles/cell.

A Zeiss Axiovert 25 inverted microscope was used for all phase-contrast and fluorescence microscopy using 5, 10, 20, 40x objectives with 21x, 42x, 84x and 168x overall magnification. Pictures were taken using a Spot camera and processed using Spot Advanced software (Diagnostic Instruments, Michigan, USA). All fluorescence pictures shown have the same exposure and gain settings (unless otherwise stated green colour only, exposure time = 2 sec and gain = 4).

2.3.3 Quantitative PCR for virus and cellular DNA

Real time QPCR was used to quantify the copy number of adenovirus particles per cell in a virus infected population. Cells were infected as above and DNA harvested by the addition of lysis buffer from a QIAMP DNA mini kit (Qiagen, Crawley, UK) and purified following the

manufacturer's instructions. Samples were eluted into a final volume of 100µl, 5µl aliquots were taken for analysis by QPCR. As standards, cells of the same origin in the experiment were counted, washed and harvested as before. A known amount of purified Ad5WT was 'spiked' into the sample; a typical standard consisted of $5x10^6$ cells with $2x10^{10}$ particles of virus. DNA from the standards was prepared as before. For detection of virus genomes BARTS primers and probe set was used and for cellular genomes β_2m primer and probe set was used (Table 2-2).

Amplification of DNA was by real-time monitoring of changes in fluorescence intensity using dual-labeled fluorogenic Taqman probes (PE Applied Biosystems), [hybridizing between the forward and reverse primers labelled at the 5'end with the reporter dye FAM (6-carboxy-fluorescein) or VIC (Applied Biosystems proprietary dye)] and with the quencher dye TAMRA at the 3' end (6-carboxy-tetramethylrhodamine) listed in Table 2-5, in a total reaction volume of 20µl/sample. Primers and probes were prepared in a UV irradiated class I laminar flow hood designated for DNA free preparation of PCR samples with DNAase/RNAase free H_2O (Sigma) and 2x TaqMan master mix (Applied Biosystems, Warrington, UK). In a laminar flow hood designated for addition of DNA to samples 5µl of DNA was added to wells already containing 15µl of primers, probes and TaqMan master mix. Standard DNA was 10 fold serially diluted in DNAase/RNase free H_2O and 5µl samples used. All samples were prepared in triplicate in MicroAmp Fast Optical 96 well reaction plates (Applied Biosystems, Warrington, UK) and covered tightly with EU Opti-Seal optical film (Geneflow, Fradley, UK) before centrifugation at 360g for 5min in a Heraeus Megafuge 2.0R. After activation of the uracil-N-glycosidase, to eliminate contamination from previous PCR products (2 min at 50°C) and denaturation (10 min at 95°C), amplification was for 40 cycles (denaturation for 15 s at 95°C and annealing and extension for 1 min at 60°C). Fluorescent signals were detected by an ABI Prism 7500 Fast Sequence Detection System

(PE Biosystems). Each sample was analysed using both the Barts primers and probe set for Adenovirus hexon and the β_2m primers and probe set for cellular DNA.

FAM and VIC fluorescence intensities were plotted against cycle number and amplification curves used to determine the cycle threshold (Ct) values, defined as the fractional cycle number at which the amplification curve crosses a threshold level (set in the exponential phase of amplification). Because this Ct value is proportional to the initial amount of starting material, the number of hexon or β_2m DNA copies in the test samples could be calculated from standard curves generated from 10 fold serial dilutions of the standard DNA containing known quantities of virus and cellular DNA. Results were calculated for the virus copy number and cellular copy number and expressed as number of virus genomes/cell. Standards, tests, and positive and negative controls (no template) were analyzed in triplicate. Samples were considered negative if the Ct value exceeded 40 cycles. Results of individual experiments are shown.

Name	Target	Primers and Probe	5'-3'sequence	Final concentration	Position on Genome	Reference
Barts primers and probe	Adenovirus hexon gene conserved for species C (Ad1, 2, 5 and 6)	Forward primer	CCACCCTTCTTTATGTTTTGTTTGA	300nM	21623-21647	(Bruton et al, 2007; Haanen et al, 1999)
		Reverse primer	GCAGGTACACGGTCTCGATGA	300nM	21689-21709	
		Probe	**(FAM)**TCTTTGACGTGGTCCGTGTGCACC (**TAMRA)**	200nM	21650-21673	
CMV – Nr primers and probe	Region between CMV promoter and nitroreductase gene	Forward primer	TGGCTTATCGAAATTAATACGACTCA	300nM	981-1006	(Patel et al, 2009)
		Reverse primer	GACGCTTTAAGGCGACAGAAA	300nM	1068-1048	
		Probe	**(FAM)**TTGCCGCCAGCCATGGATATCA(**TAMRA)**	200nM	1026-1047	
β_2m primers and probe	Cellular DNA	Forward primer	GGAATTGATTTGGGAGAGCATC	300nM		(Heid et al, 1996)
		Reverse primer	CAGGTCCTGGCTCTACAATTTACTAA	300nM		
		Probe	(**VIC)**AGTGTGACTGGGCAGATCATCCACCTTC (**TAMRA)**	50nM		

Table 2-2 Primers and probes for Q-PCR.

The reporter and quencher dyes are shown in bold

2.4 Tissue culture

2.4.1 Generation of human cell lines

All cells were obtained from consenting healthy laboratory volunteers by myself or trained personnel with local ethical approval.

Cell Line	Origin	Culture Medium	Reference
PBMCs	Whole blood	RPMI1640 containing 2mM glutamine, 10% FCS, 100U/ml penicillin and 0.1mg/ml streptomycin.	
Monocyte Derived Dendritic Cells	PBMCs	RPMI1640 containing 2mM glutamine, 10%FCS, 100U/ml penicillin and 0.1mg/ml streptomycin, 1000U/ml IL-4 (Peprotech, London, UK) and 50ng/ml GM-CSF (Schering Plough, Welwyn Garden, UK)	(Sallusto & Lanzavecchia, 1994)
Lymphoblastoid Cell Lines	PBMCs	RPMI1640 containing 2mM glutamine, 10% FCS, 100U/ml penicillin and 0.1mg/ml streptomycin	(Allday et al, 1995)
Primary Human Fibroblasts	Dermal skin biopsy	DME + 15mM HEPES, 2mM glutamine, 10% FCS, 100U/ml penicillin and 0.1mg/ml streptomycin.	

Table 2-3 Human Donor derived cell lines and growth medium

RPMI (Roswell Park Memorial Institute); DME (Dulbecco's Modified Eagle's); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

2.4.1.1 *Peripheral blood mononuclear cells and plasma*

Whole blood was obtained from consenting donors into syringes containing sodium heparin (10 U/ml) and processed as soon as possible. Buffy coats were obtained from National Blood

Service (NBS, Birmingham, UK). Both were diluted by the addition of an equal volume of RPMI 1640 and layered on top of 15 ml of Lymphoprep (Nycomed, Torshov, Norway) before centrifugation (800g, 30 min, RT). Peripheral blood mononuclear cells (PBMCs) were removed from the sample/medium interface with a Pasteur pipette. Plasma was removed from above the lymphoprep and stored at -20°C for future use. PBMCs were washed twice by the addition of RPMI 1640 and pelleting cells by centrifugation (840g, 10 min, RT). Cells were re-suspended in medium and counted as described in section 2.4.3.1.

2.4.1.2 *Human monocyte derived dendritic cells*

Freshly isolated PBMCs were plated out at 10^7cells/ml in medium in 25cm^2 flask and incubated for 2 hr (37°C, 5% CO_2). Non-adherent cells were removed by two washes with 5 ml of PBMC medium before removal and addition of dendritic cell medium. Cells were fed by half medium change on days 3 and 6. After 6-7 days culture cells were termed immature DCs and phenotyped by measuring cell surface antigens (CD1a, CD14, CD80, CD86, class I and class II MHC) using monoclonal antibodies and flow cytometry (Table 2-5). Batches containing more than 10% of cells expressing the monocyte marker CD14 were not used. Immature DCs were matured by addition of TNF-α (50ng/ml) to medium and cultured for two further days (Day 9). Cells were phenotyped by measuring cell surface antigen using monoclonal antibodies and flow cytometry. Cells not up-regulating expression of CD1a, CD80, CD86, HLA class I or HLA class II from their immature phenotype were not used.

2.4.1.3 *B95-8 transformed lymphoblastoid cell lines (LCLs)*

EBV-transformed lymphoblastoid cell line (LCLs) was prepared with prototype 1 strain B95-8 were used as alternative antigen presenting cells (Miller & Lipman, 1973). 5x10^6 PBMCs were exposed to 10 ml of culture supernatant from the prototype B95-8 EBV cell lysate

(provided by Alison Leese, T-cell group, School for Cancer Sciences, University of Birmingham, UK) in the presence of cyclosporine A (1µg/ml, 2 hr) before washing and re-suspending in 2ml of medium containing cyclosporine A (1µg/ml). B95-8 strain, originally produced by infecting with the Hawley strain of EBV (Miller et al, 1974), stably releases B95-8 strain of EBV in the presence of cyclosporine A (1µg/ml). Cells were fed weekly with half medium change and after 2 weeks cell density maintained between $10^5$and 10^6cell/ml.

2.4.1.4 *Primary human fibroblasts*

Primary human fibroblasts from donors in the School for Cancer Sciences, Birmingham, UK were established from skin punch biopsies. Skin biopsies were transferred to a 100mm Petri dish and minced finely. Tissue pieces were distributed into a 12-well tissue culture plate. One millilitre of medium was added to each well and the plate incubated (37°C, 5% CO_2). When fibroblast outgrowth was apparent at 1 to 2 weeks, 0.5 to 1.0 ml medium was added. Cells were fed by half medium change until cells were nearly confluent; each well was then trypsinised and seeded into a 25 cm^2 flask and after 1 to 2 weeks into 75 cm^2 flasks.

2.4.2 Transformed human cell lines

Cell line	Origin	Medium	Reference
293	Human embryonic kidney transformed with a left end fragment of Ad5 DNA	DME + HEPES, 2mM glutamine, 10% FCS	(Graham et al, 1983)
A549	Human lung carcinoma cell line with properties of Type II alveolar epithelial cells	DME + HEPES, 2mM glutamine, 10% FCS	Lieber et al, 1976(Lieber et al, 1976)
911	Human embryonic retinoblasts integrated with a plasmid containing base pairs 79-5789 of the Ad5 genome	DME + HEPES, 2mM glutamine, 10% FCS	(Fallaux et al, 1998; Fallaux et al, 1996)

Table 2-4 Transformed Human Cell Lines and Growth Medium

RPMI (Roswell Park Memorial Institute); DME (Dulbecco's Modified Eagle's); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

2.4.3 Maintenance and passage of human cells in culture

Adherent cells were routinely grown in 25, 75 and 150 cm^2 flasks (Iwaki, Japan) containing 5, 18 and 25 ml medium (RPMI 1640, 10% FCS, 2 mM glutamine, 100U/ml penicillin and 0.1 mg/ml streptomycin) respectively. At approximately 80% confluence, cells were passaged by removing the medium, washing with PBS and incubating in PBS containing 0.05% trypsin, 0.02% EDTA until cells detached. Cells were collected in 10ml of medium to inhibit trypsin and seeded into fresh flasks for routine growth in a Galaxy R 37°C humidified incubator with 5% CO_2 (RS Biotech, Irvine, UK).

2.4.3.1 *Cell counting*

Cells were counted using a FAST-READ 102® disposable counting slide (ISL, Paignton, UK). 25µl of cell suspension was pipetted into a single counting chamber and cell number counted from 4 individual large grid squares.

Cell count/ml = Average cell count from 4 large squares x 10^4

2.4.3.2 *Cryopreservation of cell lines*

Frozen stocks of cell lines were kept in liquid nitrogen. To prepare cells for storage, trypsinised cells were centrifuged (840g, 10min, RT) and re-suspended in FCS containing 10% DMSO before freezing slowly to -80°C in an isopropanol containing cryofreezing container (Nalgene, Hereford, UK). Cells were transferred the next day to liquid nitrogen.

Recovery of frozen cells was by rapid defrosting at 37°C in a water bath followed by the drop-wise addition of 10ml warm medium. Cells were then pelleted by centrifugation (302g, 10min, RT followed by 540g, 10min, RT) and resuspended in pre-warmed culture medium.

2.5 T-cell assays

2.5.1 Flow cytometry

Flow cytometry was used to determine expression of surface antigens

2.5.1.1 *Sample preparation*

Freshly prepared PBMCs or polyclonal T-cell lines (section 2.6.1) or T-cell clones were counted and washed (840g, 10 min, 4°C). Supernatants were discarded and the appropriate antibodies were added to each sample (20 min, 4°C). The cells were washed again (840g, 10 min, 4°C) and resuspended in 500µl of MACS Buffer (PBS pH 7.2, 0.05% BSA and 2mM EDTA). The optimal concentration for each antibody was determined by titration. For every primary antibody species/fluorophore and isotype combination an appropriate isotype control was used to stain a replicate set of cells, as a negative control.

For tetramer staining, cells were washed and pelleted by centrifugation (840g, 10 min, 4°C) before being re-suspended in 50µl of MACS buffer containing 1, 2 or 5µg of tetramer, (37°C, 15 min). Cells were washed and stained with appropriate antibodies as above.

2.5.1.2 *Antibodies for flow cytometry*

The following antibodies (Table 2-5) were routinely used to phenotype cells and measure expression levels on the surface of cells. Table 2-6 lists the isotype controls used for these antibodies

Antibody	Fluorophore	Isotype	Clone	Source
CD1a	PE	$IgG_1\kappa$	HI149	BD Pharmingen
CD3	FITC/Pacific Blue	$IgG_1\kappa$	UCHT1	BD Pharmingen
CD4	PE/FITC	$IgG_1\kappa$	RPA T4	BD Pharmingen
CD4	Pacific Orange	$IgG_{2a}\kappa$	S3.5	Invitrogen
CD8	PECy5	$IgG_1\kappa$	H1T8a	BD Pharmingen
CD8	APC-H7	$IgG_1\kappa$	SK1	BD Pharmingen
CD11a (LFA-1)	FITC	$IgG_{2a}\kappa$	G43-25b	BD Pharmingen
CD14	FITC	$IgG_{2b}\kappa$	MφP9	BD Pharmingen
CD27	FITC	$IgG_1\kappa$	M-T271	BD Pharmingen
CD28	ECD	$IgG_1\kappa$	CD28.2	Beckman Coulter
CD45RA	ECD	$IgG_1\kappa$	2H4LDH11LDB9	Beckman Coulter
CD45RO	AF-700	$IgG_{2a}\kappa$	UCHL1	BioLegend, UK
CD57	FITC	$IgG_1\kappa$	HNK-1	BD Pharmingen
CD62L	APC	$IgG_1\kappa$	Dreg56	BD Pharmingen
CD80	PerCP	$IgG_{2b}\kappa$	DCN46	BD Pharmingen
CD86	PE	$IgG_1\kappa$	FUN-1	BD Pharmingen
CD197(CCR7)	PE-Cy7	$IgG_{2a}\kappa$	3D12	BD Pharmingen
CD209(DC-SIGN)	APC	$IgG_1\kappa$	HB15e	BD Pharmingen
HLA-DR, DP, DQ	FITC	$IgG_{2a}\kappa$	TÜ39	BD Pharmingen
HLA- A, B, C	APC	$IgG_1\kappa$	G46-2.6	BD Pharmingen

Table 2-5 Antibodies used in this project

Fluorophore	Isotype	Clone	Source
PE/FITC/APC/APC-H7	$IgG_1\kappa$	X40	BD Pharmingen
PECy5	$IgG_1\kappa$	MOPC-21	BioLegend
Pacific Blue	$IgG_1\kappa$	MOPC-21	BioLegend
ECD	$IgG_1\kappa$	2T82F5	Beckman Coulter
PE /FITC	$IgG_{2a}\kappa$	X39	BD Pharmingen,
PE-Cy7	$IgG_{2a}\kappa$	MOPC-173	BD Pharmingen,
Pacific Orange	$IgG_{2a}\kappa$	MG2a30	Invitrogen
AF-700	$IgG_{2a}\kappa$	MOPC-173	BioLegend
FITC	$IgG_{2b}\kappa$	MPC-11	BioLegend
PerCP	$IgG_{2b}\kappa$	MPC-11	BioLegend

Table 2-6 Isotype controls used in this project

2.5.1.3 *Analysis of cells by flow cytometry*

Cells were analysed using a 4 colour Beckman Coulter XL flow cytometer using Coulter System II software (Beckman Coulter, High Wycombe, UK) or 6 colour DakoCyAn ADP flow cytometer using Summit software (BD, Oxford, UK) or 11 colour BD^{TM} LSR II and FACS DIVA software (BD, Oxford, UK) for data acquisition. FlowJo 7.5.3 software (Tree Star Inc.Ashland, OR, USA) was used for data analysis and presentation.

Unstained or mock infected cells were first analysed followed by cells stained with isotype control. The desired cell population was gated using forward scatter and side scatter properties. Voltages were set appropriately using the negative controls. 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. At least $4x10^5$ events were analysed per sample. Data was analysed as the percentage of cells expressing the respective antibody. Percentage positive

refers to the percentage of gated cells fluorescing above the level of the negative control (unstained or isotype control). Negative control is measured to include less than 2% of the negative control cells. Unless otherwise stated flow cytometry data is presented from individual experiments.

2.5.1.4 *Measurement of lymphocyte proliferation using CFSE labelling*

CFSE (Carboxy fluorescein siacetate succinimidyl ester) is a fluorescein dye consisting of a fluorescent molecule containing a succinimidyl ester functional group and two acetate moieties. It 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 the replicating cells. The number of cell divisions can therefore be determined according to the number of equally spaced peaks of CFSE fluorescence, typically up to 6 divisions can be discerned.

PBMCs were washed 3 times with PBS, centrifuged (540g, 10min, RT) and resuspended at $2x10^7$cell / ml in PBS. 10mM stock CFSE (Molecular Probes, UK) in DMSO was diluted in PBS to achieve a 5µM stock. Diluted CFSE was added to the cells at a ratio of 1:1 and vortexed gently. The cell suspension was then incubated (15 min, 37°C) with periodic agitation. An equal volume of complete RPMI culture medium was added to the cells for 1 minute at RT to stop the labelling process. The cells were washed 3 times with medium and resuspended in culture medium. PBMCs were counted and analysed at different time points by flow cytometry. The % of $CFSE^{dim}$ cells was then determined. The calculated number of proliferating cells was divided by the starting number of cells to determine fold proliferation.

2.5.2 Antigen-specific T-cell detection and enrichment

2.5.2.1 *IFN-γ cytokine secretion selection (CSS)*

This assay was performed using a MACS IFN-γ cytokine secretion selection detection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The kit contained IFN-γ catch reagent, anti IFN-γ mAb (PE labelled). PBMCs were seeded at a cell density of 10^7/ml in RPMI 1640, 5% foetal calf serum (FCS), 2mM glutamine, 100U/ml penicillin and 0.1 mg/ml streptomycin. An aliquot of cells was used for controls and baseline flow cytometry. Cells were uninfected (negative control) or pulsed with 10^3particles/cell of heat inactivated (56°C) CTL102 /Ad 5 WT and incubated overnight (16hr, 37°C, 5% CO_2). If peptide was used as antigen, cells were loaded with peptide (10µg/ml) and incubated (3-6hr, 37°C, 5% CO_2). Dimethyl sulfoxide (DMSO) (equivalent to the dilution of peptide) was used as negative control for peptide loaded cells. Staphylococcus enterotoxin (1µg/ml) (SEB, Sigma, Poole, UK) was used as a positive control (3hrs, 37°C, 5% CO_2).

Samples were washed (840g, 10 min, 4°C) in cold MACS buffer (PBS pH 7.2, 0.05% BSA and 2mM EDTA), resuspended and incubated with 20µl of catch reagent (5 min on ice). Then T-cell medium was added. Cells were incubated under constant rotation (37°C, 45 min). Samples were washed in cold MACS buffer (840g, 10 min, 4°C) and 20µl of anti-IFN-γ detection antibody was added (10 min on ice). Cells were washed in cold MACS buffer (840g, 10 min, 4°C) and stained with appropriate fluorescent labeled antibodies (4°C, 20 min). Cells were washed again (840g, 10 min, 4°C) and resuspended in 300-500µl MACS buffer for analysis on the flow cytometer. Propidium iodide (PI; 1µg/ml, Sigma) was added immediately before each sample was analysed to allow exclusion of dead cells.

2.5.2.2 *Enrichment of IFN-γ cytokine secreting T-cells*

Cell selection was performed using MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany) for positive cell selection (Figure 2-1). MACS Technology is based on the use of MACS magnetic microbeads [nano-sized (50nm) super paramagnetic particles coupled to specific antibodies], patented MACS Columns, and MACS Separators which are strong permanent magnets. Cells were labelled with the magnetic microbeads and passed through MACS columns placed in a MACS separator. Magnetically labeled cells are retained within the column whilst the unlabelled cells (negative fraction) are collected. MACS columns are then removed from the magnetic field and labeled cells are eluted.

CSS enrichment and detection assay was performed using a MACS IFN-γ CSS assay-cell enrichment and detection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions using MACS technology for positive cell selection. The kit contained IFN-γ catch reagent, anti IFN-γ mAb (PE labelled) and anti-PE microbeads for binding and selection using magnetic cell sorting. The steps for cytokine secretion detection as described in section 2.5.2.1 were followed till the labeling of anti- IFN-γ detection antibody. Cells were washed in cold MACS Buffer (840g, 10min, 4°C) and aliquots taken for pre-sort analysis by flow cytometry. Samples were washed in cold MACS Buffer (840g, 10min, 4°C) and 20µl of anti-PE microbeads added (15 min, 4°C). Samples were washed in cold MACS Buffer (840g, 10min, 4°C) and resuspended in 500µl

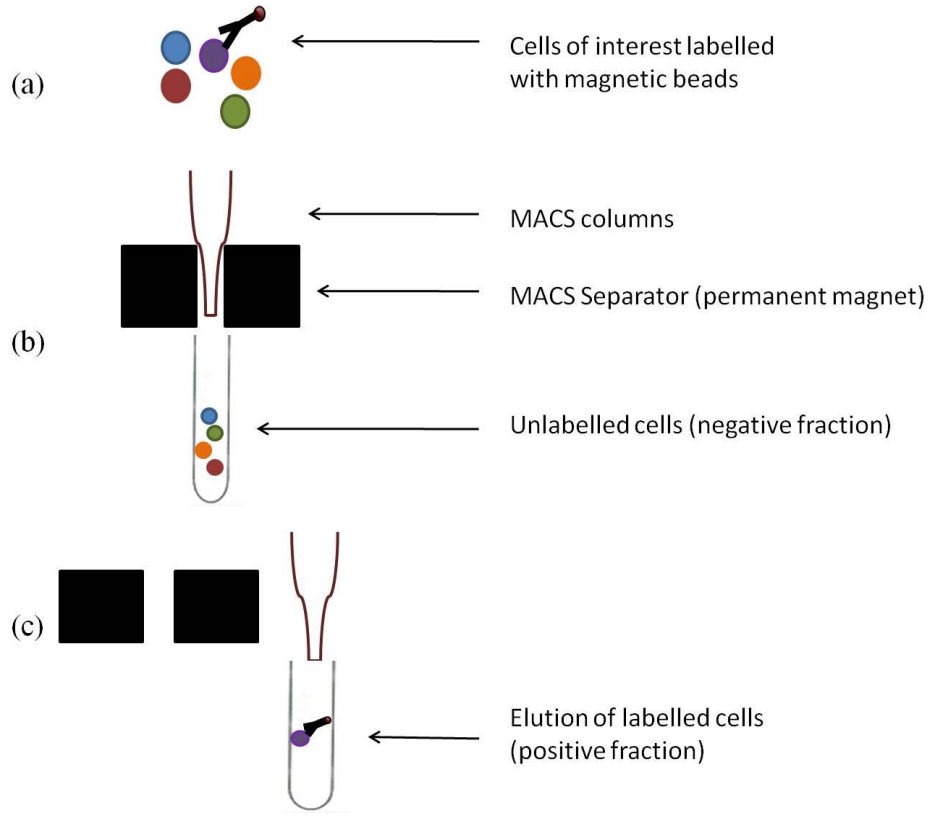

Figure 2-1 MACS technology

(a) Cells of interest are initially labelled with the magnetic microbeads. (b)They are then passed through MACS columns placed in a MACS separator. Magnetically labelled cells are retained within the column whilst the unlabelled cells (negative fraction) are collected. (c)MACS columns are then removed from the magnetic field and labelled cells (positive fraction) are eluted.

The samples were hereafter enriched by auto-magnetically activated cell sorting (MACSQuant® analyzer) separator or MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) (Radbruch et al, 1994).The magnetically labelled cells were placed in an ice rack in the AutoMACS separator and the separation program 'posseld' was chosen. Both positive (pos sort) and negative (neg sort) fractions were collected. For MS column enrichment, 2 columns were placed in the magnetic field of a MACS separator for each enriched sample. Each column was rinsed with 500µl cold MACS buffer prior to applying the magnetically labeled cells. Cells were applied on to the column and washed 3 times with 500µl cold MACS buffer. The effluent was the negative or unlabelled (negative fraction) cells. After

removing the column from the magnetic field, cells in the first column were eluted onto the second column with 500µl cold MACS buffer. The unlabelled fraction was collected. After 3 washes the second column was removed from the magnetic field and 500µl cold MACS buffer added. Magnetically labeled cells (positive fraction) were collected by flushing out the remaining cells with a plunger. Both positive (pos sort) and negative (neg sort) fractions were collected, washed (840g, 10 min, 4°C). All samples (pre, pos and neg sort) were stained with appropriate antibodies (4°C, 20 min). They were then washed (840g, 10 min, 4°C) again, re-suspended in 300-500µl of cold MACS buffer and just prior to flow cytometry propidium iodide (PI; 1µg/ml, Sigma) was added to allow exclusion of dead cells.

2.5.2.3 *Enrichment of pMHC tetramer stained T-cells*

PBMC were pelleted after washing in cold MACS buffer (PBS pH 7.2, 0.05% BSA and 2mM EDTA). An aliquot of cells was used for controls and baseline flow cytometry. Cells were seeded at a density of $1x10^7$cells/ml and stained with the appropriate PE labeled tetramer (50µg, 15 min, 37°C, 5% CO_2). An unlabeled or HLA mismatched tetramer (if available) was used as negative control. The cells were washed (840g, 10min, 4°C) and labeled with anti-PE microbeads (15 min, 4°C). Aliquots were taken for pre-sort analysis by flow cytometry. Magnetically labeled cells were then enriched by auto MACS separator or MS columns as described in section 2.5.2.2. Both positive (pos sort) and negative (neg sort) fractions were collected, washed (840g, 10 min, 4°C). All samples (pre, pos and neg sort) were stained with appropriate antibodies (4°C, 20 min). They were then washed (840g, 10min, 4°C) again, re-suspended in 300-500µl of cold MACS buffer and just prior to flow cytometry propidium iodide (PI; 1µg/ml, Sigma) was added to allow exclusion of dead cells.

2.5.3 IFN-γ Elispot assay

IFN-γ Elispot assay was performed using a kit containing capture and biotinylated detection antibodies and streptavidin bound alkaline phosphatase (AP) (Mabtech, Nacka Strand, Sweden). MAIP N45 96 well plates (Millipore, (U.K.) LTD, Watford, UK) were coated with 50µl of anti human IFN-γ monoclonal antibody (1-D1K) diluted to 15µg/ml in PBS for 3 hr (RT) or overnight (4°C). Antibody was flicked off and each well washed six times with 200µl of RPMI 1640, 10% FCS or HS, 2mM glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin (T-cell medium) and then incubated with 200µl T-cell medium for 1hr (37°C). Medium was flicked off the plate and cells along with antigen added in a total volume of 100µl in T-cell medium. Cells were at a density between $5x10^4$-$5x10^5$cells/well. Plates were incubated for 16 hr (37°C, 5% CO_2) and wells washed 6 times with PBS Tween (v/v 0.05% Tween 20). 50µl of biotinylated anti-IFN-γ antibody (7-B6-1) diluted to 1µg/ml (in PBS) was added to each well and incubated for 3-4 hrs (RT). Antibody was flicked off, wells washed 6 times with PBS Tween and 50µl of streptavidin-AP (diluted 1 in 1000 in PBS) added to each well. Plates were incubated for 1 hr (RT), streptavidin-AP flicked off and wells washed six times with PBS Tween. 50µl of development reagent (1000 fold dilution of AP colour reagent A and B (BioRad) in 1x AP colour development buffer (BioRad) was added to each well. Plates were incubated for 10-20 mins (RT) or until colour had developed and the reaction stopped by washing thoroughly with tap water. Plates were left to air dry overnight before counting the number of spots formed in individual wells.

In addition to visual inspection under a dissection microscope (all data in Chapter 3), spots were counted by an AID automated elispot reader using AID 3.1 software (AID, Strassberg, Germany). Plates originally counted under the dissection microscope were examined using the automated counter and no significant differences were observed. Results are typically

represented as the mean (of three replicate wells) spot forming cells (SFCs)/10^6 input cells. Where negative controls are not shown results are represented as SFCs/10^6 cells in the test wells - SFCs/10^6 cells in the appropriate negative control. Unless otherwise stated results from individual donors are from an individual experiment.

2.5.3.1 *Preparing cells for the IFN-γ Elispot assay*

Freshly prepared PBMCs were stimulated by addition of purified virus (10^3 particles/cell unless otherwise stated), uninfected A549 lysate (60µg/10^6 cells) purified hexon or hexon, penton base and fibre protein (5µg/10^6cells), Ad-specific peptides (10µg/ml) dissolved in DMSO, DMSO alone (equivalent concentration as present in peptides; 5µg/ml) or cells were mock infected (medium only) for 1.5 hr (37°C, 5% CO_2) in 200µl of PBMC medium. Cells were washed with 10 ml of warm medium and re-suspended in PBMC medium before addition to the elispot plate. The mitogen phytohaemagglutinin (PHA) was used as a positive control; while non-quantitative it confirms the presence of similar numbers of cells plated and that the development of the assay has been completed efficiently. PHA (10µg/ml) was added directly to unstimulated cells on the elispot plate.

2.5.4 IFN-γ ELISA assay

This protocol relied on the availability of autologous antigen presenting cells (APCs) that could be infected/ loaded with viral antigen. 3 types of APCs were either generated or readily available within the School for Cancer Sciences, University of Birmingham, UK; dendritic cells (section 2.4.1.2), LCLs (section 2.2.3.3) and primary human fibroblast (section 2.2.3.4). The ability of fibroblasts to present antigen was increased by the addition of 200 U/ml IFN-γ to their medium 3 days prior to use. Autologous APCs were infected with purified virus (3000 particles/cell) and/or Ad peptide (10µg/ml) or un-infected to act as negative controls

and cultured overnight T-cell medium (37°C, 5% CO_2). 50µl of APCs were plated/well of 96 well plates with two wells containing Ad stimulated APCs and two wells negative control APCs/T-cell clone to be screened. T-cells were washed and re-suspended in 200µl of T-cell medium. 50µl of T-cells/well were plated into the 96 well U bottom plates already containing the APCs and incubated for 16 hr (37°C, 5% CO_2).

On the same day ELISA plates (Maxisorp, F96, Nunc, Roskilde, Denmark) were coated with anti human IFN-γ mAb (Pierce, Cramlington, UK) diluted to 0.75µg/ml in coating buffer (0.1M Na_2HPO_4 adjusted to pH 9 with 0.1M NaH_2PO_4) and incubated overnight (4°C). Antibody was flicked off the ELISA plate and blotted on absorbent tissue. 200µl of blocking buffer [PBS, 1% BSA, 0.05% Tween20 (v/v)] was added and incubated for 1 hr (RT). ELISA plates were washed 5 times with PBS Tween (v/v 0.05% Tween 20) and were ready for the addition of supernatants. 96 well U bottom plates had been centrifuged (256 g, 3 min) and 50µl supernatant from each well was transferred to ELISA plates. 2 fold serial dilutions of IFN-γ (Sigma) from 2ng/ml to 31.25pg/ml were prepared in RPMI 1640, 10% FCS, 2mM glutamine and 100 U/ml penicillin and 0.1 mg/ml streptomycin and 50µl of each dilution transferred to ELISA plates in triplicate. Following incubation (3hr, RT) supernatants were removed and plates washed 6 times with PBS Tween. 50µl of biotinylated anti-IFN-γ (Pierce, Cramlington, UK) diluted to 0.375µg/ml in blocking buffer was added to each well. Plates were incubated for 1 hr (RT) before being washed 6 times with PBS Tween. 50µl of extravidin (Sigma) diluted 1000 x with blocking buffer was added to each well and incubated (30 min, RT).

Plates were washed 8 times with PBS Tween prior to the addition of 100µl TMB (Tebu-Bio, Peterborough, UK) to each well and plates incubated (20min, RT, in the dark). Reactions were terminated by adding 100µl of 4M H_2SO_4 (Fisher, Loughborough, UK) /well.

Absorbance at λ = 450 nm was measured using a Victor plate reader (Wallac, Finland). IFN-γ concentration was determined in each well from the value of absorbance at λ = 450 nm by linear regression from a standard curve generated from the values of absorbance at λ = 450nm of known concentrations of IFN-γ dilutions.

2.5.5 Chromium release assay

To determine whether T-cell clones were capable of lysing targets loaded with antigen chromium release assays were used. IFN-γ treated (300 U/ml) autologous fibroblasts were infected with WT Ad from serotypes 3, 5, 11, 19a, 40 or mock infected for 1.5 hr, and 100pfu/cell, washed and incubated at 37°C overnight before γ-irradiation. Alternatively γ-irradiated IFN-γ treated autologous fibroblasts were loaded with relevant or irrelevant peptide (5μg/ml) or DMSO alone (5μg/ml) for 2hr. Fibroblasts were loaded with sodium chromate by adding 15μCi (20μl) to cells in a total of 50μl, incubated for 90min (37°C, 5%CO_2, with agitation), washed twice with PBMC medium and re-suspended in medium (25000 cells/ml) and 100μl aliquots were added to individual wells of a 96 well V bottom plate. T-cells were counted, washed and re-suspended at the correct concentration before addition to 96 well V bottom wells (100μl/well). T-cell clones (effector) were used at a range of effector to target ratios (E: T Ratio) all in triplicate. As a positive control, targets were lysed with 1% SDS; the negative control was target cells only. Effectors and targets were incubated for 4 or 16 hr (37°C, 5% CO_2). Supernatants were sampled and γ-emission quantified using Packard Cobra gamma counter (Global Medical Instrumentation, Minnesota, USA). Percentage lysis was calculated using the following equation:

$$\%\ \text{Specific Lysis} = \left(\frac{\text{Experimental gamma release} - \text{Spontaneous gamma release}}{\text{Max gamma release} - \text{Spontaneous gamma release}} \right) \times 100$$

Results shown represent the average percentage lysis of triplicate samples.

2.6 Production and characterisation of T-cell clones

The isolation of T-cell clones that recognised Ad or Ad-specific peptides as an antigen utilised a two step approach. Firstly a polyclonal T-cell line was established following stimulation with Ad antigen and subsequently this T-cell line was sampled and cells cloned by limiting dilution. Once isolated, T-cell clones were subjected to procedures to amplify their numbers so that they were amenable for characterisation.

2.6.1 Generation of polyclonal T-cell Lines

Freshly prepared PBMCs were stimulated by addition of purified virus (10^3 particles/cell unless otherwise stated), Ad-specific peptides (10µg/ml) or mock. Cells were maintained in RPMI 1640, 10% FCS, 2mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (T-cell medium) and 50IU/ml of recombinant IL-2 (Chiron, Amsterdam, Netherlands). Cells were fed twice weekly by half medium change and restimulated on day 14.

2.6.2 Cloning T-cells by limiting dilution

Polyclonal T-cell lines were sampled 14, 21 and 28 days after original stimulation to be tested in a limiting dilution assay. Cells were washed, counted and plated out in 96 well U bottom plates at 0.3, 3 and 30 cells/ well in one of three different cloning media. Γ-irradiated PBMCs from buffy coats were used as feeder cells with differing cytokine mixes to stimulate T-cell growth.

Protocol 1: Cloning medium was composed of T-cell medium with 0.5µg/ml PHA, 1% human serum (HS) (HD Supplies, TCS Biosciences, UK) and 10^6/ml γ-irradiated PBMC feeder cells. 100µl of cloning medium and T-cells was plated out/well of 96 well U bottom plates. After 3 or 4 days 100µl/well of T-cell medium and 60% MLA (supernatant from the

MLA-144 cell line provided by Alison Leese, ATCC # TIB 201), 50 U/ml IL-2 and 1% HS was added.

Protocol 2: Cloning medium was composed of T-cell medium with 1µg/ml PHA, 200U/ml IL-2, 1% HS, γ-irradiated PBMC feeder cells 10^6/ml. 200µl of cloning medium and T-cells were plated out/well of 96 well U bottom plates.

Protocol 3: Cloning medium was composed of T-cell medium with 1µg/ml PHA, 200U/ml IL-2, OKT3 30ng/ml, 1% HS, γ-irradiated PBMC feeder cells 10^6/ml. 200µl of cloning medium and T-cells were plated out/well of 96 well U bottom plates.

After 2-3 weeks growth, cells were screened by IFN-γ ELISA (section 2.5.4) and those secreting IFN-γ in response to antigen loaded APCs were further expanded by buffy boost protocol (section 2.6.3.1). If few cells grew from the cloning plates then cells were expanded by buffy boost protocol prior to screening by IFN-γ ELISA. Clones were named alphabetically and then numerically in order of isolation.

2.6.3 Expansion and maintenance of T-cell clones

The first expansion of T-cells from the 96 well cloning plate was achieved using a protocol named buffy boost whereby clones reach 10^6 cells or more. From this point onwards T-cell clones were maintained with a regime of fortnightly stimulation with antigen loaded APCs and also expanded further using a rapid expansion protocol (REP) or by further buffy boosting. At the earliest possible time cells were cryopreserved for future use.

2.6.3.1 *Buffy boost protocol for expansion of T-cell clones*

Approximately 10^5 T-cells (or one well from the 96 well cloning plates) were placed into one well of a 24 well plate containing 2 ml of buffy boost mix. Buffy boost mix was composed of

10^6/ml pooled PBMCs (from three different buffy coats) that had been γ-irradiated in PBMC medium supplemented with either 30% MLA supernatant, 0.5μg/ml PHA and 100 U/ml IL-2 or 1μg/ml PHA and 200 U/ml IL-2 depending on whether clones had been generated in the presence of MLA supernatant or not. Cells were fed by half medium change twice weekly with PBMC medium supplemented with IL-2 (100 or 200U/ml) for two weeks. If cell number increase to greater than 10^6/ml they were split into further well.

2.6.3.2 *Rapid expansion protocol for T-cell clones*

Once T-cells were of sufficient number they were maintained in 2 ml wells of 24 well plates in PBMC medium supplemented with 50U/ml IL-2. Cells were fed by half medium change twice weekly. Cells were re-stimulated fortnightly by the addition of irradiated autologous APCs loaded with Ad antigen (10^5 LCLs or $5x10^3$ γ-treated fibroblasts).

2.6.3.3 *Determination of antigen specificity by IFN-γ ELISA*

To determine which antigens individual T-clones recognise a modification of the above IFN-γ ELISA was employed. All conditions detailed in section 2.5.4 were kept constant except for the antigens used and cell numbers of T-cells detailed below. To determine if the antigen recognised by the T-cell was conserved between different virus serotypes, APCs were infected with WT Ad 3, 5, 11, 19a, 40 (100pfu/cell) or Ad-specific peptides (10μg/ml) and incubated overnight (37°C, 5% CO_2) before being used as APCs in the IFN-γ ELISA. To determine whether Ad 5 hexon or fibre protein were recognised APCs were loaded with 5μg/10^6cells of protein for 1.5 hr before use. To determine whether T-cells recognised epitopes present in synthetic peptides, peptides dissolved in DMSO were loaded onto APCs (10μg/ml) in RPMI1640 before being washed and used in the IFN-γ ELISA. As a negative control APCs were loaded with the same amount of DMSO present in the peptide being

tested (if multiple peptides were being tested then the highest amount of DMSO was used). Typically $1x10^3$-$5x10^3$ T-cells were used/well. In most cases a 1 in 10 dilution of the supernatant was also quantified to allow detection of [IFN-γ] between 2 and 20ng/ml.

2.7 Generation of tetramers

Class I HLA tetramers incorporating Ad epitopes were synthesised using standard methods (Altman et al, 1996; Garboczi et al, 1992).

2.7.1 Generation of class I heavy chain and β_2m plasmids

2.7.1.1 *Preparation of competent cell*

Competent cells were prepared using the 'Simple Efficient Method' (Inoue et al, 1990). LB medium (10mg/ml Tryptone, 5mg/ml Yeast extract, 10mg/ml NaCl) was inoculated with either XL-1 blue MRF (Stratagene, CA) or BL-21 DE3 pLysS (Novagen, West Sussex UK) strains of *E. coli*. The cells were incubated (37˚C, shaking, 16 hrs). Cells from the overnight culture were streaked onto an LB agar plate containing no antibiotics and incubated until colonies had reached a diameter of about 5mm. Five or six colonies were used to inoculate 125ml of SOB medium (20mg/ml Tryptone, 2.5mg/ml Yeast extract, 10mM NaCl, 3mM KCl supplemented with 10mM $MgCl_2$ and 10mM $MgSO_4$). The cultures were grown at 18˚C, shaking until the OD 600nm reached approximately 0.6. Cells were harvested by centrifugation (2500g, 4˚C, 20min). Pellets were resuspended in cold SEM medium (15mM Pipes, 10mM $CaCl_2$, 150mM KCl, 30mM $MnCl_2$) gently and spun (2500g, 4˚C, 20min) twice. Cells were then resuspended in SEM medium containing 7% DMSO, aliquoted and snap frozen immediately in liquid nitrogen.

2.7.1.2 *Transformation of competent cell*

Plasmids (courtesy D. Millar, School for Cancer Sciences, University of Birmingham, UK) encoding the class 1 heavy chains (modified by substitution of the transmembrane and cytoplasmic domains by a Bir A target sequence) and beta 2 microglobulin (β_2m) were used to transform BL-21 (DE3) pLysS and λ A90 strains of *E.coli* respectively. Briefly plasmid was added to competent cells of the appropriate *E.coli* strain and incubated (2˚C, 45min) followed by a heat shock (42˚C, 1min) and a brief chill (2˚C, 1min). Cells following incubation in LB medium (37˚C, 5% CO_2, 45min) were streaked onto a Petri-dish containing LB agar medium supplemented with 100µg/ml of ampicillin (37˚C, 5% CO_2 16 hrs). The presence of white plaques in a lawn of bacteria was indicative of successful transformation with recombinant DNA. Plasmids were then stored as glycerol stocks at -80˚C.

2.7.2 Expression of class I heavy chain or β_2m protein

2.7.2.1 *Induction of protein expression*

Bacteria, from glycerol stocks of E.coli transformed with expression plasmids for class I heavy chain or β_2m, was streaked on an LB agar plate (containing 100µg/ml ampicillin) and incubated (37˚C shaking, 8 hrs). Colonies were picked to inoculate LB medium (containing 100µg/ml ampicillin) and cultured (37˚C, 5% CO_2 16 hrs). The following day the overnight cultures were added to LB medium and prepared in bulk (37˚C, 5% CO_2). OD_{600} [optical density] has measured using a spectrophotometer periodically until an OD_{600} of 0.3-0.4 was reached. At this stage, protein expression was induced by isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5mM. After 4hr the cultures were centrifuged (2500g, 4˚C, 20min) in cold PBS. The pellets were resuspended in ice cold PBS

and stored at -80˚C. Small aliquots were taken from the cultures before and after IPTG addition to ascertain the degree of protein induction by IPTG on SDS-PAGE gels.

2.7.2.1.1 Sodium –dodecyl-sulphate-polyacrylamide gel electrophoresis

SDS PAGE was carried out using the BioRad minigel system. Glass plates were first wiped clean in ethanol and then with sterile water. Spacers were attached to the sides and the plates were clamped together and attached to a stand. 2 gel solutions were prepared; a separating gel (12% acrylamide, 0.375M Tris HCl pH8.8 and 0.1% SDS) and a stacking gel ((12% acrylamide, 0.125M Tris HCl pH6.8 and 0.1% SDS). 10µl of TEMED and 50µl of 15% ammonium persulphate (APS) was added to the separating gel which was mixed and then injected into the glass plates quickly. Once the separating gel set, 5µl of TEMED and 25µl APS was added to the stacking gel and the mixture is then injected onto the top of the separating gel and combs inserted. On removal of the combs after the gels are set, wells were washed with running buffer (25mM Tris HCl pH8.3, 250mM Glycine, 0.1% SDS) to flush out any unpolymerised acrylamide. Each sample is mixed with an equal volume of loading dye (0.0625M TrisHClpH6.75, 2%SDS, 10% glycerol, 5%mercapto-ethanol and 0.001%bromophenol blue) and heated (90˚C, 10 min) for protein denaturation. The samples were then loaded onto the gel and run (20min at 200V). Size determination was aided by also running a protein molecular weight marker on each gel. Gels were stained with Coomassie blue solution (25% methanol, 7% acetic acid, 0.25% Coomassie blue) for 30min and then destained (30% methanol, 10% acetic acid) overnight before drying.

2.7.2.2 *Separation of bacterial inclusion bodies*

To release the inclusion bodies containing the protein of interest, class I heavy chain or β_2m, the bacterial cells had to be sonicated. Pellets were removed from the freezer and thawed at

room temperature. A flat titanium horn was used to sonicate (8 bursts of 45 sec, followed by 1 minute between bursts on ice) resulting in cell membrane rupture. The sonicated sample was spun (25000g, 4˚C, 10 mins) and the pellet resuspended in Triton wash (50mM Tris. HCl pH 8.0, 0.5% Triton X-100, 100mM NaCl, 1mM EDTA, 1mM DTT, 0.1% sodium azide and 0.1mM PMSF) at 4ml /litre of LB medium. The mixture was then homogenised to completely resuspend in Triton wash. Following homogenisation the sample was spun (25000g, 4˚C, 10 mins). The detergent wash, homogenization and subsequent spin were repeated twice followed by a wash in resuspension buffer (50mM Tris. HCl pH 8.0, 100mM NaCl, 1mM EDTA and 1mM DTT) at 2ml/ l.

2.7.2.3 *Urea solubilisation*

The homogenized solution was spun again at (25000g, 4˚C, 10 min) and resuspended in freshly prepared urea solubilisation buffer (8M Urea, 50mM Mes pH 6.5, 0.1mM EDTA and 0.1mM DTT) at 5ml/l of LB medium. This sample was incubated (4˚C, 16hrs) on a rotator and spun (25000g, 4˚C, 10mins) to remove any insoluble material. The supernatant was dispensed into 10mg/ml aliquots and stored at -80˚C. Following purification the protein expression and purity was monitored using SDS-PAGE. The appearance of the appropriate sized band at either 35kDa (HLA heavy chain) or 12kDa (β_2m) was used to validate this process. The concentration of protein was determined by using spectrophotometry at OD_{595} on the basis that the absorption of 1µg protein at this wavelength is 0.05.

2.7.3 Generation of class I Major Histocompatibility Complex (MHC)

I am grateful to D. Millar and E Manoli (Moss group, School for Cancer Sciences, University of Birmingham, UK) for their help in refolding the tetramers.

2.7.3.1 *Refolding class I MHC complex*

Heavy chain and β_2m were refolded around the appropriate peptide to form HLA monomers. 500ml refold buffer (400mM L-Arginine, 100mM Tris HCl pH 8.0, 2mM EDTA, 5mM reduced glutathione and 0.5mM oxidised glutathione) was made and allowed to cool (4˚C). 0.1mM PMSF was added prior to adding peptide (5mg/ 500ml buffer). 12.5mg of β_2m and 30mg of class I heavy chain was thawed and diluted to a concentration of 1mg/ml of protein in 8M urea. The diluted protein, ~5mg at a time, was pulsed in a drop wise manner to the refold buffer containing the peptide, first β_2m then class 1 heavy chain over a 48 hour period. This mixture was left stirring at 4˚C for a further 2 days.

2.7.3.2 *Concentration and buffer exchange of refolded complexes*

The refolded complex was concentrated using a Millipore Pellicon XL to approximately 7ml. The concentrated sample was then buffer exchanged using PD-10 columns (Amersham Pharmacia, Bucks UK). The columns were washed 3 times with the Bir A reaction buffer (100mM Tris. HCl pH8.0, 5mM $MgCl_2$, 20mM NaCl, 0.1mM PMSF); the concentrated sample was applied to the column and eluted using the BirA buffer.

2.7.3.3 *Biotinylation and purification of MHC class I complexes*

The final sample was biotinylated overnight at room temperature by the addition of 100mM ATP, 100mM d-biotin, 200µg protease inhibitor and the 7µg BirA enzyme in a final volume of 7ml. Refolded complexes were purified by fast protein liquid chromatography (FPLC) on a G75 Superdex column using gel filtration followed by ion exchange columns on a BioCAD (Amersham Pharmacia, Bucks UK) with an FPLC buffer (20mM Tris pH 8.0, 50mM NaCl). The size of the refolded complex is in the region of 41-42kDa and normally elutes between

145-155ml. The concentration and biotinylation of the monomer was determined using a Bradford assay and biotinylation ELISA.

2.7.3.3.1 Bradford assay

The quantity of refolded protein was determined by performing a simple protein assay using known amounts of bovine serum albumin (BSA) as standards. Dilutions of BSA (5µg/ml to 400µg/ml) were added in triplicate in a 96 well flat bottomed plate. 100µl of 1 in 5 dilution of BioRad protein assay reagent (Bio-Rad) was added (RT, 10 min). Absorbances at 595nm were read using a Victor plate reader (PerkinElmer, Monza, Italy (Formerly Wallac, Finland)) and average values were calculated to derive a standard curve. Dilutions of the refold fractions were simultaneously tested and the standard curve was used to calculate the protein concentration.

2.7.3.3.2 Biotinylation ELISA

Biotinylation was confirmed by a qualitative ELISA. Serial dilutions of each fraction were aliquoted on to a 96 well plate and left (37˚C, 1hr) to allow the proteins to adhere to the well. Each well was washed [4 times x PBS (0.05%) Tween and 2 xs with PBS alone]. 100µl of a 1/1000 dilution of extravidin- peroxidase conjugate (Sigma, UK) in PBS with 0.1% BSA was added to each well. After 15min incubation at room temperature, wells were washed again [4 times x PBS (0.05%) Tween and 2 xs with PBS alone]. 100µl of TMB substrate solution (3.3'-5.5' tetramethylbenzidine liquid substrate system, Sigma, UK) was added to each well. The plate was left to allow for colour development (15min, RT). The appearance of blue colour was indicative of biotinylated protein. The reaction was stopped with 100µl of H_2SO_4 and absorbance at 595nm read using a Victor plate reader (PerkinElmer, Monza, Italy (Formerly Wallac, Finland)).

2.7.4 Tetramerisation

Tetrameric complexes were made by addition of PE- conjugated streptavidin (Molecular Probes) in a molar ratio of 1:4 to the biotinylated monomer over 2-3 days. This is equivalent to adding 0.312µg of streptavidin PE to 1µg of monomer. Each tetramer was hereby designated according to the HLA type and the first 3 letters of the presented peptide. For example, the tetramer composed of HLA*0101, β_2m and TDLGQNLLY peptide is referred to as A1-TDL tetramer. Finally the tetramers were tested on a PBMC sample from a HLA matched donor and titrated for optimal staining.

3 Isolation and enrichment of Ad-specific T-cells

3.1 Introduction

Allogeneic haemopoietic stem cell transplantation (HSCT) is the only curative option for many haematological malignancies. Adenovirus infection in HSCT recipients is a major cause of morbidity and mortality in these patients. There are currently no therapeutic options that have been assessed in randomised controlled trials for the treatment of Ad infection. Several groups have demonstrated ineffective or inconsistent evidence of adenoviral elimination using cidofovir or ribovarin in the SCT setting (La Rosa et al, 2001; Walls et al, 2005). Donor lymphocyte infusion (DLI) has been successfully used to clear Ad infection in patients failing to respond to antiviral agents (Chakrabarti et al, 2002; Hromas et al, 1994b) but comes with a high risk of GvHD. These observations indicate that Ad infection may be treated by Ad-specific cellular immune reconstitution attained through adoptive T-cell therapy.

HLA class I peptide-tetramer complexes specific for CMV $CD8^+$ epitopes have been developed and demonstrated to bind stably and specifically to appropriate T-cell receptors. This technology has been successfully combined with magnetic cell sorting to isolate antigen-specific T-cells for adoptive transfer to SCT recipients without the need for *ex vivo* expansion (Keenan et al, 2001). In 2005, this group completed a phase I study demonstrating the safety, and potential efficacy of adoptive transfer of pMHC tetramer selected donor CMV-specific CD8 T-cells to patients with CMV viral reactivation (Cobbold et al, 2005).

In 2002, the Gene and Immunotherapy group at the School for Cancer Sciences, Birmingham, UK used CSS (section 2.5.2.1) to isolate and infuse Ad-specific T-cells into two SCT recipients who had severe Ad disease refractory to antiviral therapy. Ad-specific T-cells were selected by means of IFN-γ secretion following *in vitro* stimulation (section 2.5.2) with CTL102, an E1, E3-deleted replication-deficient human Ad serotype 5 vector (see section

3.1.2.1). Adoptive transfer was instituted late in the disease on compassionate grounds, due to extremely poor prognosis, and both patients succumbed to Ad disease despite therapy (D.Onion, M.Cobbold, personal communication, School for Cancer Sciences, University of Birmingham). Despite failure to clear the infection this highlighted the potential of cellular therapy to treat patients in a similar setting. A phase I clinical trial demonstrated durable clearance/decrease of viral copies following adoptive transfer of Ad-specific T-cells in 5 out of 6 (83%) patients (Feuchtinger et al, 2006). Ad-specific T-cells were enriched by IFN-γ secretion method and 1.2-50 $x10^3$T-cells/kg infused with minimal side effects (grade 2 GVHD in one patient). Sustained *in vivo* expansion of the T-cells was independent of the dose of infused T-cells.

12 Ad-specific class I epitopes have been identified to date (Table 1-4). Our experience in using pMHC multimers for clinical grade selection, as well as treating patients with Ad infections with Ad-specific T-cells selected by cytokine capture, meant that we were in a position to potentially deliver a study comparing the two selection methods in order to determine the most efficacious method of selection.

3.1.1 pMHC multimers for Ad-specific T-cell isolation and enrichment

The characterisation of HLA class I and II restricted Ad-specific epitopes has been discussed previously (section 1.4.2.1.1). pMHC multimers (section 1.3.1.3.2.1) containing Ad epitopes can aid in the rapid detection and enrichment of Ad-specific T-cells (Figure 3-1). Ad-specific T-cells that recognise hexon-specific epitopes have been shown to be capable of lysing cells infected with adenoviruses of multiple serotypes (Veltrop-Duits et al, 2006). To date, although different groups have used pMHC multimers to determine Ad-specific responses, they have not been used as a tool for adoptive transfer (Chatziandreou et al, 2007; Micklethwaite et al, 2010). Identification of adenovirus derived epitopes will allow the

generation of pMHC multimers, thereby enabling isolation and enrichment of CD8 and CD4 T-cells capable of recognising multiple Ad serotypes without in vitro stimulation. Not only is this a rapid and direct method for selecting antigen-specific T-cells, but also the isolated cells are not activated and hence possibly maintain their functional and proliferative potential. pMHC class II tetramers were not studied due to the commercial and academic unavailability of the class II pMHC molecules.

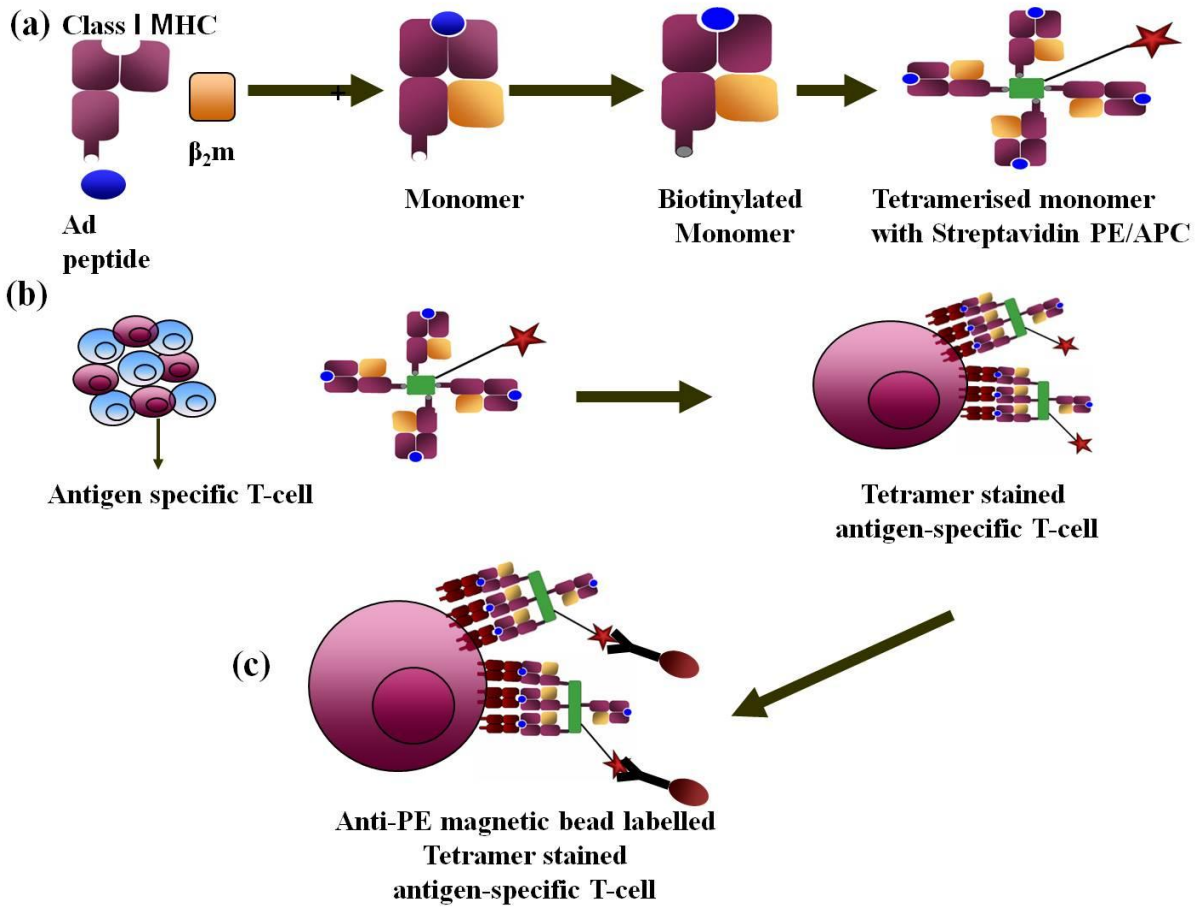

Figure 3-1 Schematic representation of generation of tetramers and enrichment of antigen-specific T-cells

(a) Class I MHC and β_2m protein is refolded with the relevant (adenovirus) peptide. The monomer is concentrated, biotinylated and further purified by FPLC and anion exchange chromatography. It is then tetramerised using of a streptavidin labelled fluorophore. (b) T-cells are stained with flourochrome labelled tetramers to detect antigen-specific T-cells. The tetramer stained antigen-specific T-cells can be (c) enriched by anti-fluorochrome labelled magnetic beads using MACS technology (Figure 2-1).

3.1.2 Cytokine secretion selection (CSS)

The cytokine secretion selection system is used to analyse and enrich live cytokine secreting T-cells. Cells producing cytokines in response to antigenic stimuli can be detected using fluorochrome-conjugated cytokine-specific antibodies and enriched with magnetic microbeads. CSS allows the detection of antigen-specific responses at very low frequencies and makes characterisation of rare cells feasible (Campbell, 2003). Different groups have demonstrated the specificity of this method (Assenmacher et al, 2002; Waldrop et al, 1997). As the cytokine is detected upon release, there is no need to permeabilise the cell for detection. This method thus retains cell viability, enabling enrichment of antigen-specific T-cells for adoptive immunotherapeutic purposes.

At the beginning of my project, there was the possibility that a replication deficient adenovirus generated to Good Manufacturing Practice (GMP) guidelines, CTL102 (section 3.1.2.1) surplus to a previous gene therapy clinical trial (Patel et al, 2009) was available for a future trial of adoptive immunotherapy for Ad infection. Secretion of different cytokines such as TNF-α, IFN-γ, and CD137 can be used to detect antigen-specific T-cells. IFN-γ is a sensitive marker (Feuchtinger et al, 2004) and has already shown potential for adoptive immunotherapy of Ad infection. As a consequence I decided to use the IFN-γ CSS to select Ad-specific T-cells with heat inactivated CTL102 as antigen (Figure 3-2).

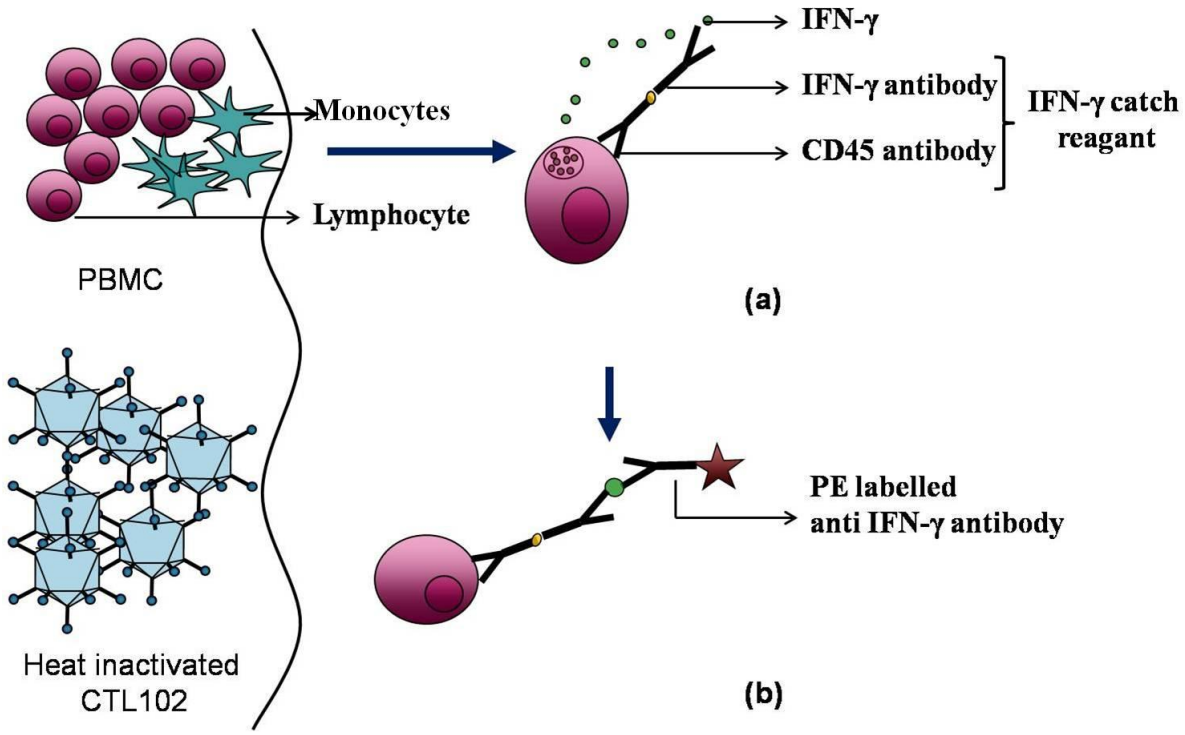

Figure 3-2 Schematic representation of the cytokine capture selection assay

PBMCs are incubated for 16 hours with heat inactivated CTL102 or Staphylococcal enterotoxin B (a) A bi-specific antibody (catch reagent) binds CD45 on the T-cells while the other end is free to bind cytokines (IFN-γ) released by antigen-specific T-cells. (b) Antigen-specific T-cells are then identified by flow cytometry after labelling the cells with a flourochrome labelled (phycoerythrin-PE) anti-cytokine (IFN-γ) antibody.

3.1.2.1 *CTL102 (Vectura)*

CTL102 (Cobra Therapeutics Limited) virus is an E1, E3-deleted replication-deficient human Ad serotype 5 vector, containing the *E. coli* nfsB gene under control of the cytomegalovirus immediate early promoter. This gene encodes the enzyme nitroreductase, which can convert the weak monofunctional alkylating agent CB1954 [5-(aziridin-1-yl)-2, 4-dinitrobenzamide] to a highly potent bifunctional alkylating agent. Live CTL102 had safely been injected into gene therapy recipients in a phase I/II clinical trial at up to 1x 10^{12} particles/patient without serious adverse events (Palmer et al, 2004; Patel et al, 2009). Heat inactivation is known to reduce infectivity of the virus by at least 8 logs and would not only increase the safety profile but would reduce the chance of gene expression from the viral vector (Maheshwari et al,

2004). Thus heat inactivated CTL102 is a clinical grade antigen which is potentially safe for use in the clinical grade CSS.

3.2 Aims of the chapter

The aim of the work in this chapter was to develop and characterise Ad-specific T-cells detected by tetramer selection and CSS, to establish the frequency of Ad specific T-cells detected in the healthy adult UK population by both methods and to assess the suitability of using these methods to enrich Ad-specific T-cells for the purpose of adoptive transfer.

3.3 Results

3.3.1 Isolation and enrichment of Ad-specific T-cells using pMHC tetramers

3.3.1.1 *pMHC tetramers for Ad class I HLA epitopes*

Ad-specific T-cell epitopes identified to date have been discussed in detail (section 1.4.2.1.1). During this thesis I developed tetramers for 8 class I epitopes - TDL (HLA A*01); YVL, TFY, LLY, GLR (HLA A*02), TYF (HLA A*24) and MPN, KPY (HLA B*07) (Table 1-4). β_2m and the respective HLA protein were expressed in E.coli (section 2.7.2). Following purification, pMHC complexes were generated by refolding the proteins with the peptide (section 2.7.3). The resulting monomer was purified on the basis of size and charge and tetramerised with streptavidin conjugated with PE (section 2.7.4). An example of generation of TDL peptide pMHC tetramer is shown Figure 3-3. Tetramers for the other epitopes were generated in a similar manner. TDL tetramer obtained from M. Cobbold, University of Birmingham, UK, was used in initial experiments.

Class I MHC restricted epitopes are mostly 9aa long peptides (Kubo et al, 1994; Peaper & Cresswell, 2008) and two different peptide binding motifs for HLA-A*01 have been defined. These are characterised by a Y (Tyrosine) residue at the carboxyl-amino terminal of a nonamer or decamer and either a small polar or hydrophobic M(methionine), T(Threonine) or S(Serine) residue at position 2 (P2), or a negatively charged E(glutamic acid)/D(aspartic acid) residue at position 3 (P3). TDLGQNLLY fits this canonical motif best, if it is N-terminally extended to position the T at P2 and D at P3 (DiBrino et al, 1994; Kubo et al, 1994). Hence a leucine residue was added to the N-terminus (LTDLGQNLLY) to increase the probability of refolding. (M.Cobbold, School for Cancer Sciences, University of Birmingham, personal communication). For the purposes of this manuscript the tetramer, though generated by

refolding LTDLGQNLLY peptide, will be referred to as TDL tetramer. For all experiments TDLGQNLLY peptide was the antigen.

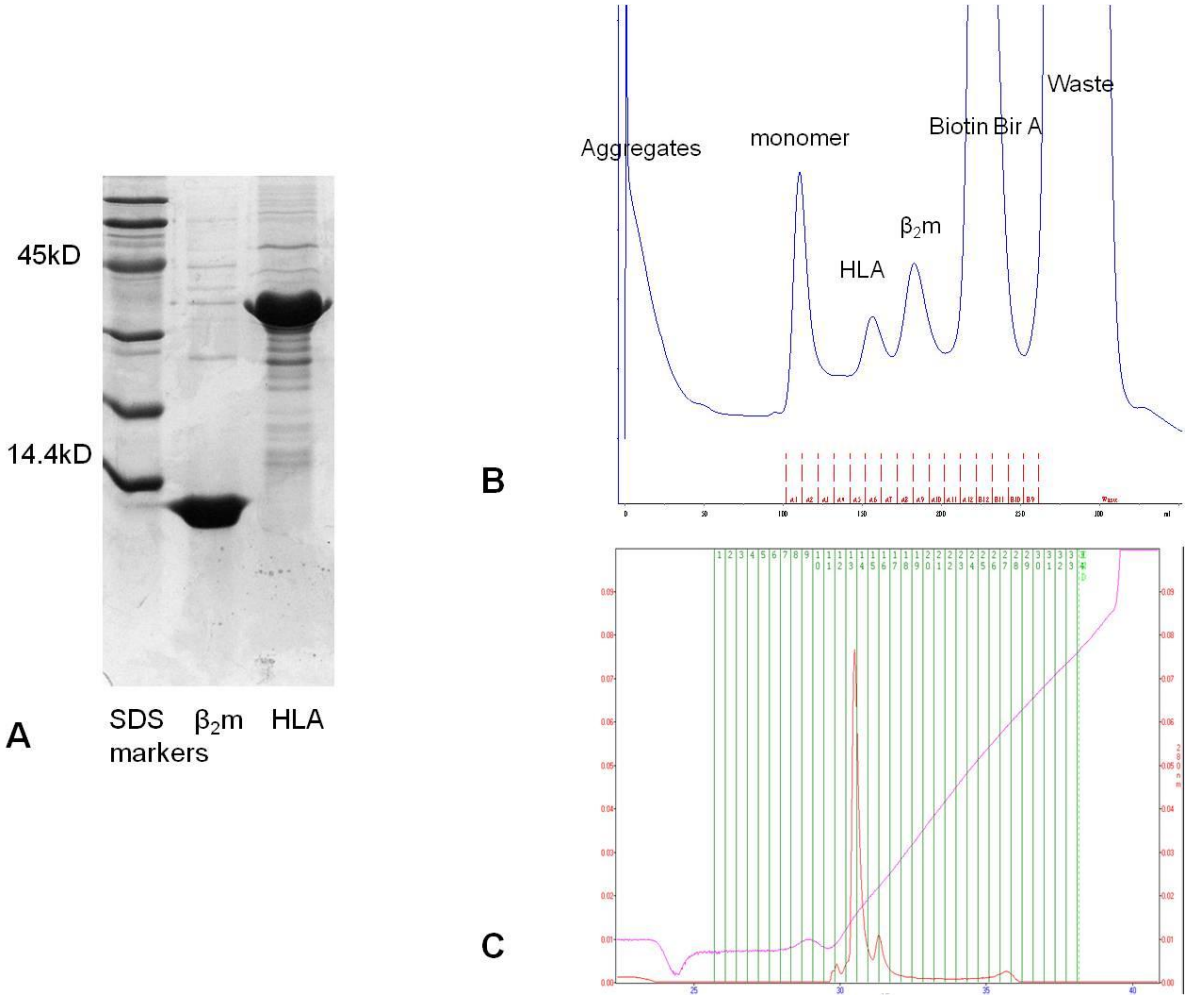

Figure 3-3 Generation of TDL HLA A*01 tetramer

Protein expression was induced in *E.coli* as described in 2.7.2.1 using IPTG induction. 4 hours post induction cells were harvested and lysed by sonication. Proteins were washed and purified as inclusion bodies and solubilised in 8M Urea. A. SDS-PAGE analysis of 1µg purified empty HLA-A*02 and β_2m proteins. 30mg HLA protein, 20mg β_2m and 5mg (LTDLGQNLLY) peptide were refolded in 1L of buffer for 24 hours at 4°C. The refolded proteins were concentrated and then buffer exchanged. After overnight biotinylation the monomer (pMHC complex) was purified by size (**B**) using FPLC and then by charge using anion exchange chromatography (**C**) to separate the biotinylated and non-biotinylated protein.

3.3.1.2 *Determination of the frequency of the HLA A*01 TDL- specific T-cells*

TDL tetramer (5µg) was incubated with 10^6 PBMCs from healthy volunteers (37°C, 5% CO_2, for 15 min) and $4x10^5$ cells were analysed by flow cytometry (Coulter EPICs, Beckman Coulter). The amount of tetramer was finalized after titration experiments with 1, 5 and 10µg (data not shown). The gating strategy followed is shown in Figure 3-4. Having identified the optimal tetramer concentration and flow cytometry gating strategy, 10^6 PBMCs from HLA*01 donors (n=13; LD1-LD13) (age range 18-65) were incubated with or without TDL tetramer. Cells were washed, labelled with anti-CD3 FITC and anti-CD8 PE-Cy5 antibodies and PI and analysed by flow cytometry. Examples of two donors and the collated data are shown in Figure 3-5 and Figure 3-7 respectively.

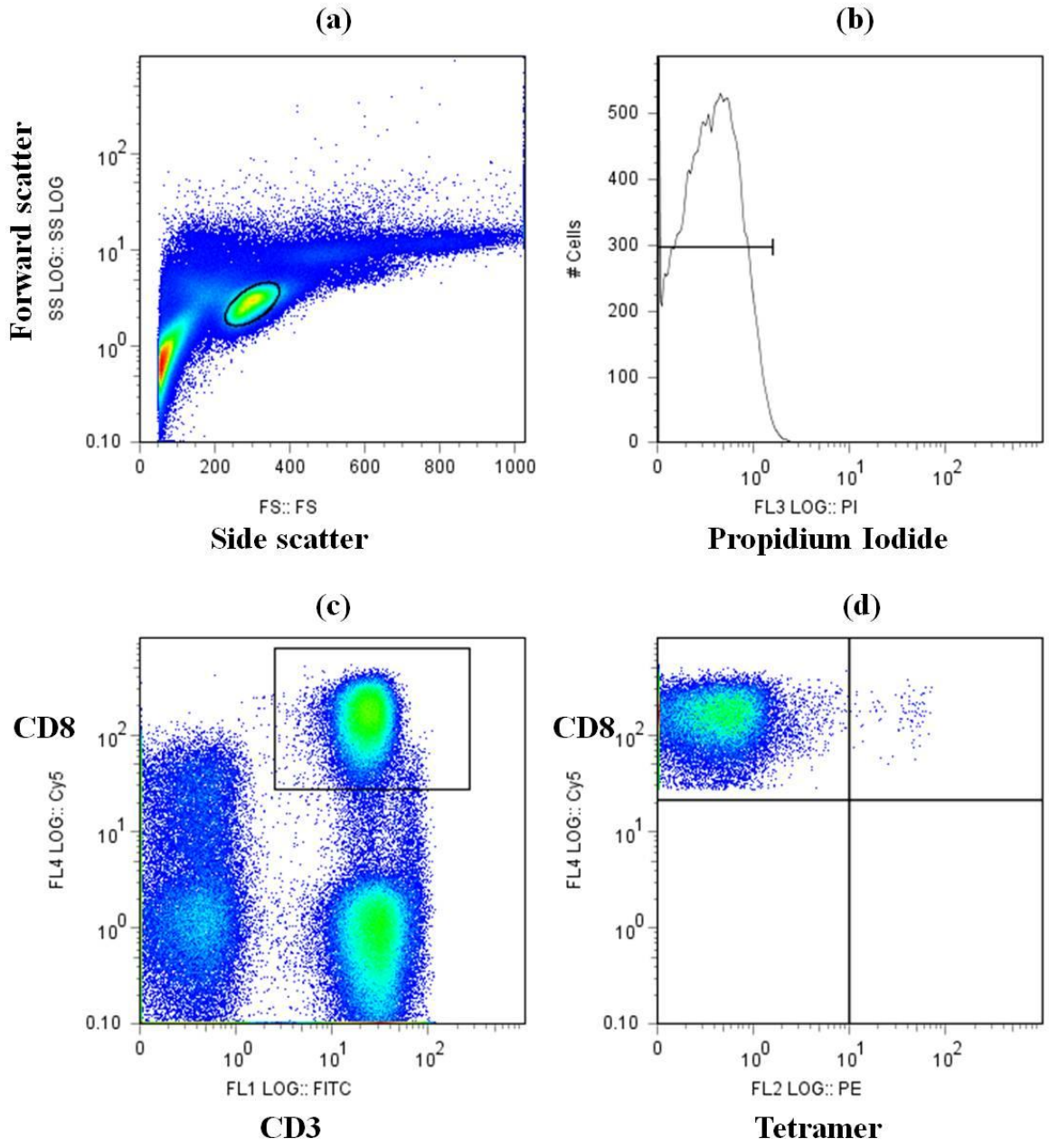

Figure 3-4 Gating strategy for tetramer staining

(a) Forward and side scatter plots were used to gate the lymphocyte subset (b)The propidium iodide negative lymphocytes were determined as alive (c) [(a) and (b) gated cells were then gated for CD3 and CD8 (d) Tetramer-specific CD8 T-cells were then identified on the CD3 and CD8 positive T-cells. The percentage of tetramer-specific T-cells was determined (number tetramer stained CD8 T-cell/Total number of CD8 T-cell) x 100 shown in the top right hand corner. $4x10^5$ lymphocytes were analysed for each donor. FACS plots of LD9 shown.

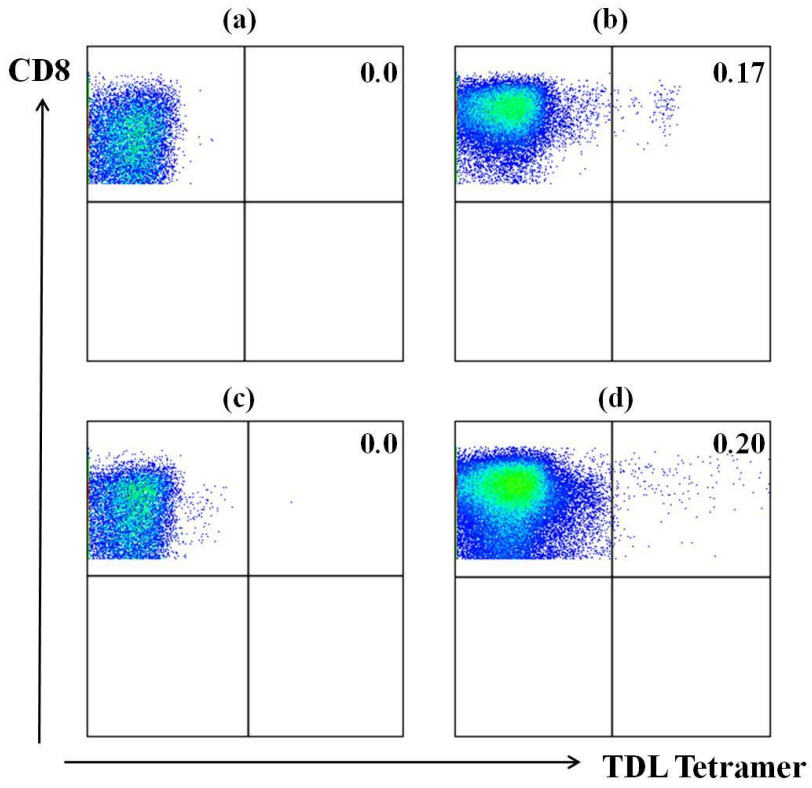

Figure 3-5 TDL-specific T-cells in LD4 and LD9

Donor PBMCs from HLA A*01 healthy volunteers were stained with no (negative control) or TDL-tetramer. Cells were labelled with antibodies to CD3 and CD8 and PI. $4x10^5$ cells were analysed by flow cytometry. The figure shows FACS plots of (a) and (c) negative controls and (b) and (d) tetramer stained T-cells of LD4 and LD9 respectively. The number of TDL-specific CD8 T-cells is expressed as a percentage of the total population of CD8 T-cells.

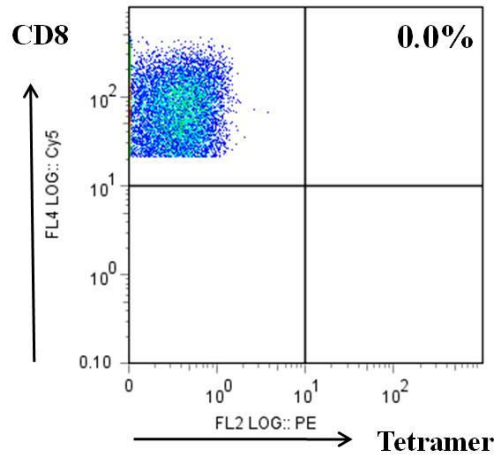

Figure 3-6 TDL tetramer staining on PBMCs of HLA mismatched donor

Donor PBMCs from HLA A*01mismatched healthy volunteers were stained with TDL-tetramer. Cells were labelled with antibodies to CD3 and CD8 and PI. $4x10^5$ cells were analysed by flow cytometry. The figure shows tetramer stained T-cells of LD16.

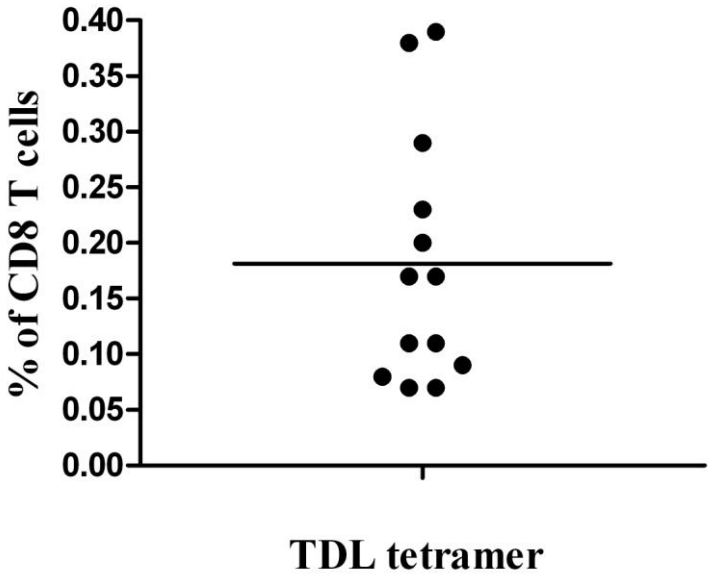

Figure 3-7 Frequency of TDL-specific T-cells in healthy volunteers

Donor PBMCs (n=13) from HLA A*01 healthy volunteers were stained with TDL tetramer and $4x10^5$ cells were analysed by flow cytometry. The line shows the mean value.

For all 13 healthy volunteers screened, a population of TDL-specific T-cells was detectable. The mean frequency for the TDL staining T-cells expressed as a percentage of the total CD8 T-cell population was 0.18% (range 0.07%-0.39%, SD ±0.11%, SEM ±0.03%). The control cells showed less than 0.01% staining in all 13 donors. Two tetramer staining patterns were observed. Tetramer-specific T-cells either appeared as a small cluster in nine donors (as in Figure 3-5 (b)) or were spread out in four donors (as in Figure 3-5(d)). The difference in staining could be a result of variation in affinity of the T-cells to the pMHC complex or due to nonspecific binding of the tetramer.

3.3.1.3 *Enrichment of TDL-tetramer stained T-cells*

To determine whether the TDL-specific T-cells could be isolated, the tetramer stained T-cells were enriched using AutoMACS technology (Miltenyi Biotech, Bergish Gladbach, Germany). PBMCs of healthy volunteers (n=10; LD1-12 excluding LD6 & 11) were subsequent to tetramer staining. 10^7 PBMCs were incubated with TDL tetramer (50µg) followed by anti-PE magnetic bead labelling (4˚C, 20 min). The cells were then enriched by AutoMACS technology (section 2.5.2.3) and analysed by flow cytometry (Beckman Coulter). An example of enrichment (LD4) and collated data for all donors is shown in Figure 3-8 (a) and (b). The mean purity of the sorted T-cells was 86.64% of CD8 T-cells (range 74.70%-95.05%, SD ±6.3%, SEM ±2.0%). The remaining non-tetramer staining T-cells were CD3+ve and at a mean of 14%. The monocytes in the enriched population were not quantified. It is possible that the non tetramer staining CD8 T-cells are capable of allo-reactivity but from a 500ml blood donation, they would account for only $4x10^2$cells following clinical grade selection. The non eluted cells or negative sort were also analysed by flow cytometry in a similar manner for TDL-specific T-cells. The mean frequency of TDL-specific T-cells expressed as a percentage of the total CD8 T-cell population in the negative sort on all donors was 1% (range 0.15%-2.8%; SD±0.87%; SEM±0.27%) (Figure 3-8 (c)) This shows that TDL-specific T-cells were successfully enriched with minimal loss.

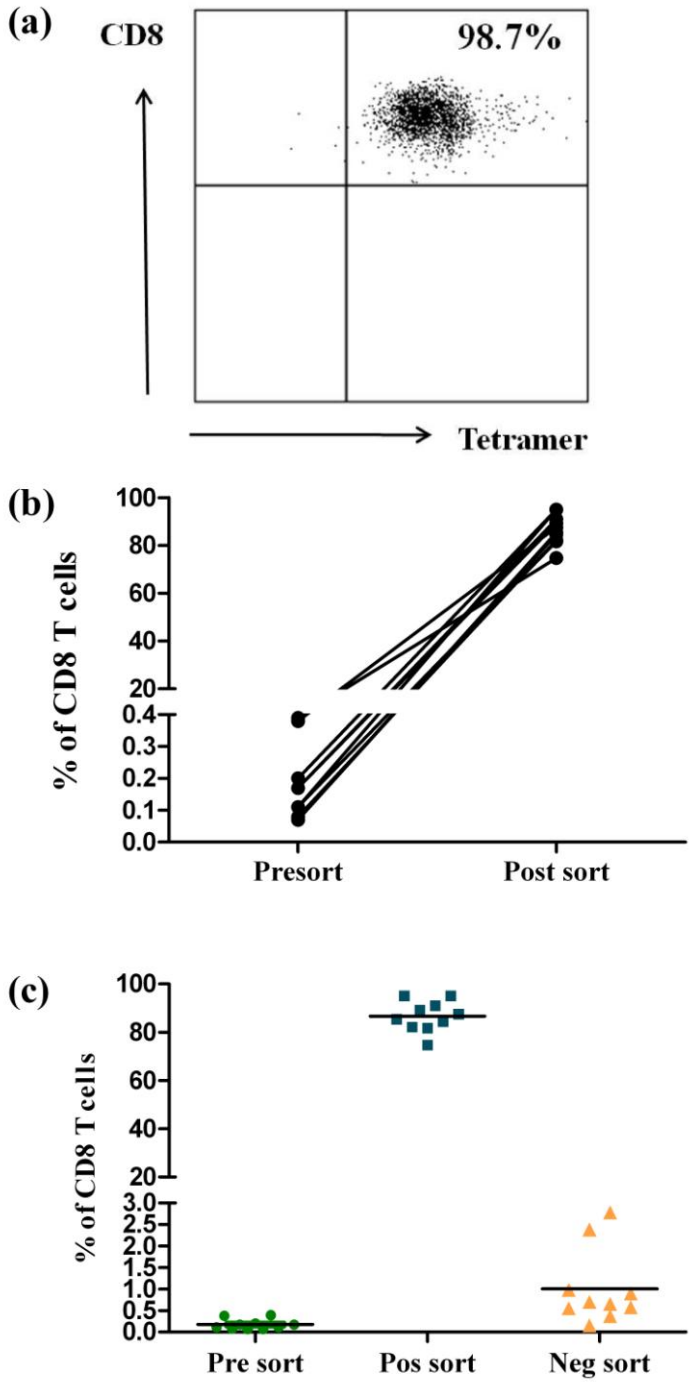

Figure 3-8 Enrichment of TDL tetramer stained T-cells

PBMCs (10^7) (HLA A*01; n=10) were incubated with TDL tetramer (50µg, 37˚C, 15 min) followed by magnetic bead labelling (4˚C, 20 min). All the eluted cells (positively sort) and unselected (negative sort) cells were analysed. (a) FACS plot on LD4 (b) comparison of the number of TDL tetramer + cells as a percentage of the total CD8 T-cell population before and after enrichment (c) comparison of the number of TDL-specific T-cells; before (pre-sort) and after (pos sort) enrichment as well as in the negative sort, as a percentage of the total CD8 T-cell population. The lines indicate the mean values.

3.3.1.4 *Adenovirus epitope-specific HLA A*02 T-cells*

PBMCs from HLA-A*02 healthy volunteers (n=5; LD 9, 10, 11, 12 and 13) were stained with tetramers containing Ad HLA- A*02 peptides YVL, LLY, TFY and GLR. The mean frequency of YVL-specific CD8 T-cells expressed as a percentage of total CD8 T-cell population was 0.03% (range 0.00%-0.09%; SD ±0.04%; SEM ±0.02%) and LLY-specific CD8 T-cells was 0.08% (range 0.02%-0.18%; SD±0.07%; SEM ±0.03%). Figure 3-9 shows the collated data of 5 donors. The control cells showed less than 0.01% staining. TFY and GLR-specific T-cells could not be demonstrated by their respective tetramers in all 5 donors.

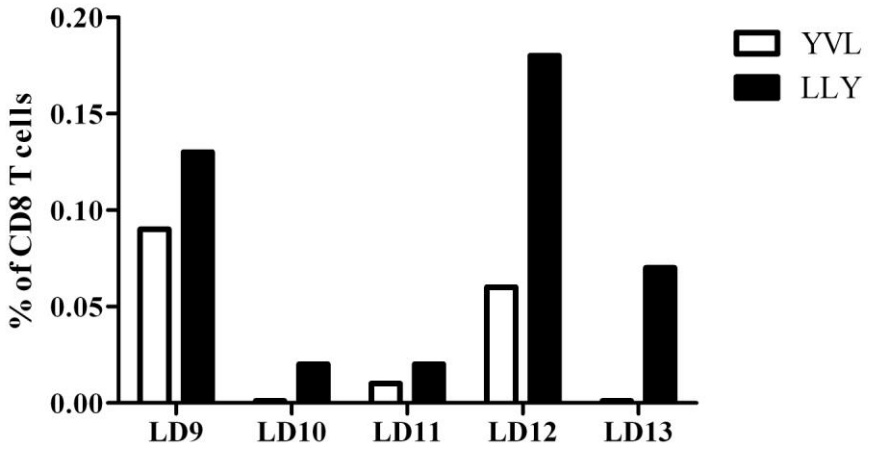

Figure 3-9 Frequency of YVL and LLY tetramer stained T-cells

PBMCs (10^6) were incubated with YVL or LLY tetramer and $4x10^5$ cells were analysed by flow cytometry. Frequency of YVL or LLY tetramer stained T-cells in 5 donors (LD 9, 10, 11, 12, 13) is expressed as a percentage of the total CD8 T-cell population. The line indicates the mean value.

Though YVL and LLY-specific T-cells could be identified by tetramer staining, these T-cells could not be enriched on repeated attempts by the AutoMACS technology. An example of the YVL tetramer on LD9 before and after enrichment is shown Figure 3-10.

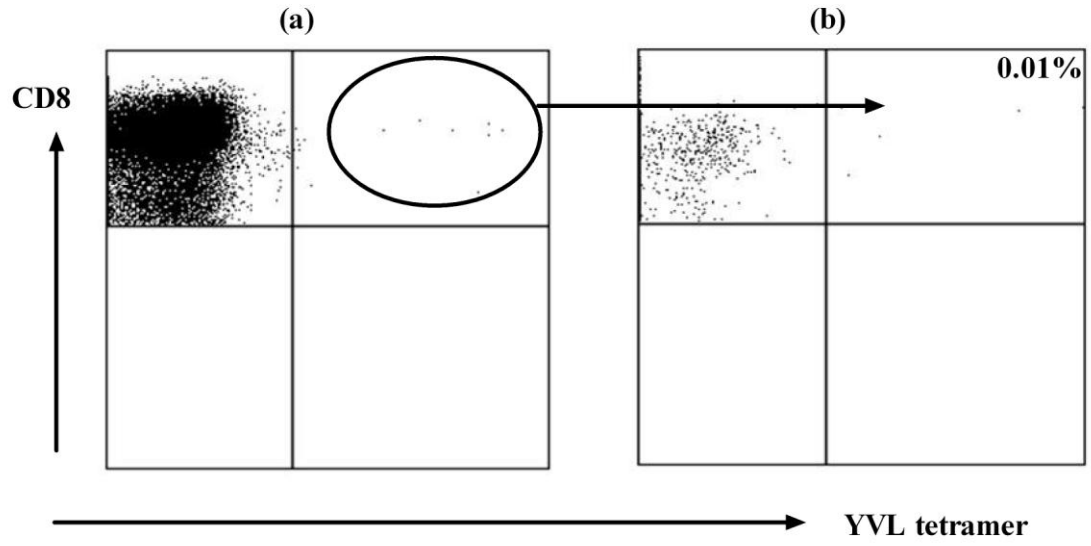

Figure 3-10 YVL tetramer-specific T-cell frequency and enrichment

PBMCs were incubated with YVL tetramer, labelled with anti-PE magnetic beads and enriched by AutoMACS. FACS plots demonstrating the frequency of YVL tetramer stained T-cells as a percentage of the total CD8 T-cell population, prior to [0.08%] (a) and post enrichment (b) in LD 9 is shown.

10^6 PBMCs of these donors were stimulated with the respective peptides at 10μg/ml and kept in culture in T-cell medium and IL-2 50 IU/ml. Staining with the respective pMHC multimer failed to demonstrate peptide-specific T-cells on days 7, 14 and 21 for all 4 (YVL, LLY, GLR and TFY) peptides in all 5 donors (data not shown) on repeated attempts. These results were subsequently supported by IFN-γ Elispot assays (Figure 3-13) where no response was identified above background in all the donors. This indicates that these T-cells are either present at extremely low frequencies or may be detected only following an acute adenovirus infection when the frequencies increase.

3.3.1.5 *Adenovirus epitope-specific HLA B*07 T-cells*

PBMCs from HLA B*07 donors (n=4; LD2, LD6, LD14 and LD15) were stained with pMHC tetramers KPY and MPN (Figure 3-11). The number of KPY-specific T-cells was mean 0.14% (range 0.06%-0.21%; SD ± 0.06%, SEM ± 0.03%) and for MPN mean 0.24% (range 0.06%-0.21%; SD ± 0.11%, SEM ± 0.06%). No response was seen in the negative controls in all 4 donors.

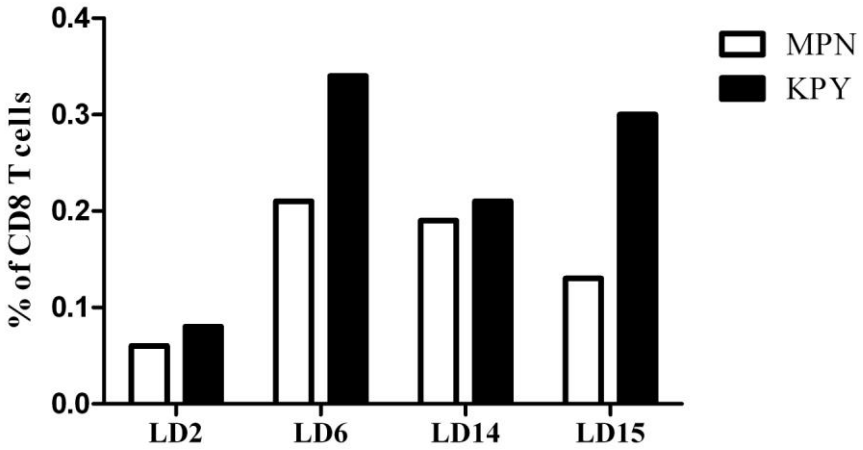

Figure 3-11 Frequency KPY and MPN tetramer stained T-cells

PBMCs of HLA B*07 donors (n=4; LD2, LD6, LD14 and LD15) were incubated with KPY and MPN tetramer and analysed by flow cytometry. The frequency of KPY and MPN-specific T-cells was determined as a percentage of the total CD8 T-cell population.

PBMCs were enriched using an AutoMACS. 3/4 donors enriched tetramer-specific cells for both epitopes (Figure 3-12). LD14 showed tetramer staining to MPN and KPY, which could be enriched (Figure 3-12 (a) & (b)) whereas LD6 and LD15 enriched KPY and MPN staining T-cells respectively. LD2 showed the lowest frequency of MPN and KPY staining T-cells.10^6 PBMCs of these donors were stimulated with the MPN and KPY peptides (10µg/ml) and kept in culture in T-cell medium and IL-2 50 IU/ml. Staining with the respective pMHC multimer

failed to demonstrate an increasing number of peptide-specific T-cells on days 7, 14 and 21 for both peptides in all 4 donors on repeated attempts.

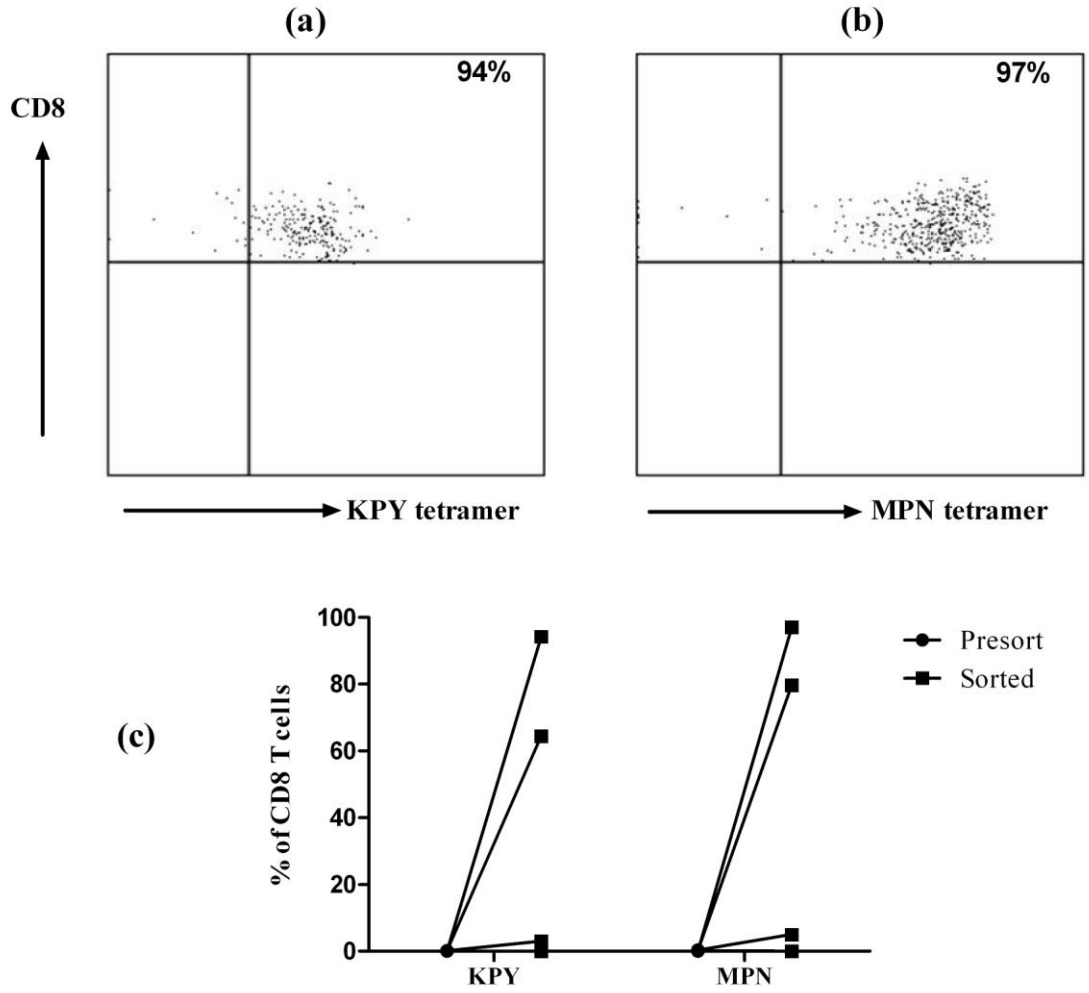

Figure 3-12 Enrichment of adenovirus derived class I B*07 epitope-specific T-cells

PBMCs from HLA B *07 donors were stained with Ad-specific epitope containing tetramers; KPY and MPN. The cells were then labelled with anti-PE magnetic beads and enriched using MACS technology. Enrichment of KPY (a) and MPN (b) specific T-cells in LD 14 are shown in the figure. Frequencies before (pre-sort) and after (sorted) enrichment are summarised in (c) Enrichment was observed in LD 14(both), LD 6 (KPY) and LD15 (MPN).

3.3.2 Detection of adenovirus epitope-specific T-cells by Elispot assay

Elispot assays were performed to further investigate the low frequency of the Ad-derived epitope containing tetramer staining T-cells obtained by flow cytometry. IFN-γ Elispot assays were performed on HLA A*01 (n=7; LD 2, 3, 4, 9, 11, 12), HLA A*02 (n=4; LD 9, 11, 12, 13) and HLA B*07 (n=2; LD2, LD14) donors as described (section 2.5.3). 4 x 10^5 PBMCs were loaded with 10μg/ml peptides or DMSO as negative control or 10μg/ml PHA as positive control. Cells were washed and an Elispot was used to quantify IFN-γ secreting cells. Figure 3-13 shows the collated data on all donors. The mean spot forming units (SFU)/ 10^6 PBMCs for the peptides is shown in Figure 3-13 with the results of statistical analysis in Table 3-1. All donors showed a response to PHA (data not shown).

The SFU/10^6 PBMCs documented for each peptide was very low. Only 3 donors showed responses to the TDL peptide by Elispot assay but tetramer staining showed TDL peptide-specific T-cells in all 7 donors (Figure 3-7). Responses above background to the HLA A*02 Ad epitopes were negligible. TDL multimer has been used by other groups to demonstrate antigen-specific T-cells on peptide stimulation (Chatziandreou et al, 2007; Micklethwaite et al, 2010). Hence I expected a higher response at least to TDL peptide in the donors.

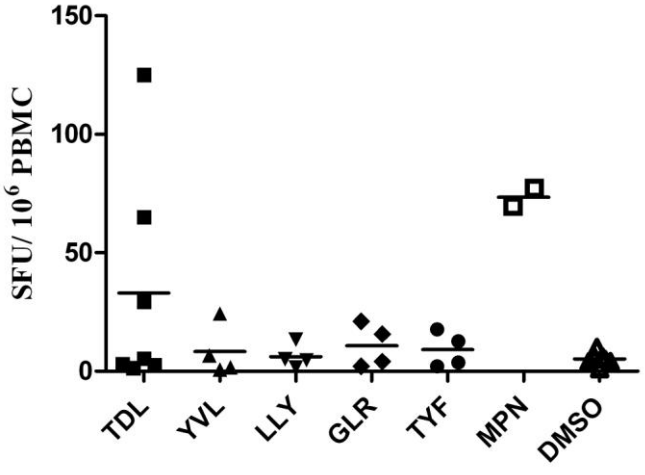

Figure 3-13 IFN-γ elispot assay for adenovirus class I epitope-specific T-cells

IFN-γ Elispot assays were performed on HLA A*01 (n=7; LD 2, 3, 4, 9, 11, 12), HLA A*02 (n=4; LD 9, 11, 12, 13) and HLA B*07 (n=2; LD2, LD14) donors with the respective peptide as antigen. PBMCs were loaded with peptides or DMSO as negative control and PHA as positive control. Cells were washed and an elispot was used to quantify IFN-γ secreting cells. Each value is the mean of 3 replicate wells with $4x10^5$cells/well. The lines indicate the mean values for each cohort. PHA data not shown.

Peptide	Mean	Range	SD	SEM
TDL	33.1	1.3-125	46.7	17.7
YVL	8.3	0.7-24.3	10.9	5.5
LLY	6.2	1.7-13.3	5.1	2.5
GLR	10.7	2.0-21.0	9.5	4.6
TYF	9.1	2.0-17.7	7.4	3.7
MPN	73.4	69.7-77.2	5.3	3.8
DMSO	5.1	1-10	2.7	1.0

Table 3-1 Results of statistical analysis of SFU per 10^6 cells by Elispot assay

3.3.3 The use of cytokine secretion selection (CSS) to determine the frequency and enrichment of Ad-specific T-cells

3.3.3.1 *Development of CCS to determine Ad-specific T-cell frequency*

Previous work in the Gene and Immunotherapy group, School for Cancer Sciences, Birmingham had shown that human serum has high levels of Ad binding antibodies (D Onion, personal communication) which may interfere with the cytokine capture (positively or negatively). Also as the enriched cells were to be re-infused into a recipient the impact of allogeneic serum (especially the risks of acquiring an unidentified infectious disease) was unknown. Thus autologous serum was considered optimal for CSS than commercially available human serum (HS, HD Supplies, TCS Biosciences, UK). Initial experiments using autologous serum from the supernatant of clotted blood that was heat inactivated (56°C, 30mins) and filtered (0.2μm) resulted in cells clumping together, this was not observed with FCS or HS.

After a number of modifications, a method for obtaining serum was finalised: 50 ml of whole blood was clotted (2.5 hrs RT). The sample was centrifuged (2500g, 10mins). Serum (supernatant) was separated and centrifuged again (2500g, 10mins) followed by another spin (16000g, 10mins). Serum was then heat inactivated (56°C, 1 hr) and centrifuged (3500g, 10mins) to remove any aggregates. RPMI with 5% autologous serum, glutamine (2mM), penicillin (100 units /ml) and streptomycin (0.1mg /ml) was prepared and filtered (0.2μm). For all experiments to determine the frequency of Ad-specific T-cells I used the above method to prepare autologous serum from donors.

To determine the optimal antigen titre at which T-cell response is maximum, CSS was performed with 10^6 PBMCs (LD 5) incubated with 10^9 - 10^{11} heat inactivated CTL102 particles (16hr, 37°C, 5%CO_2). PBMCs labelled according to protocol 2.5.2 were then

analysed by flow cytometry. For each sample, $4x10^5$ cells were analysed (Figure 3-14). This was repeated with the same donor on two further occasions and on an alternative donor (LD 18) with similar results. A maximum response was observed at 2.5 $x10^4$ particles/cell on all 3 occasions this was the ratio used for all subsequent experiments. It is possible that the virus though heat inactivated may be toxic at higher concentrations or T-cells may be cross priming.

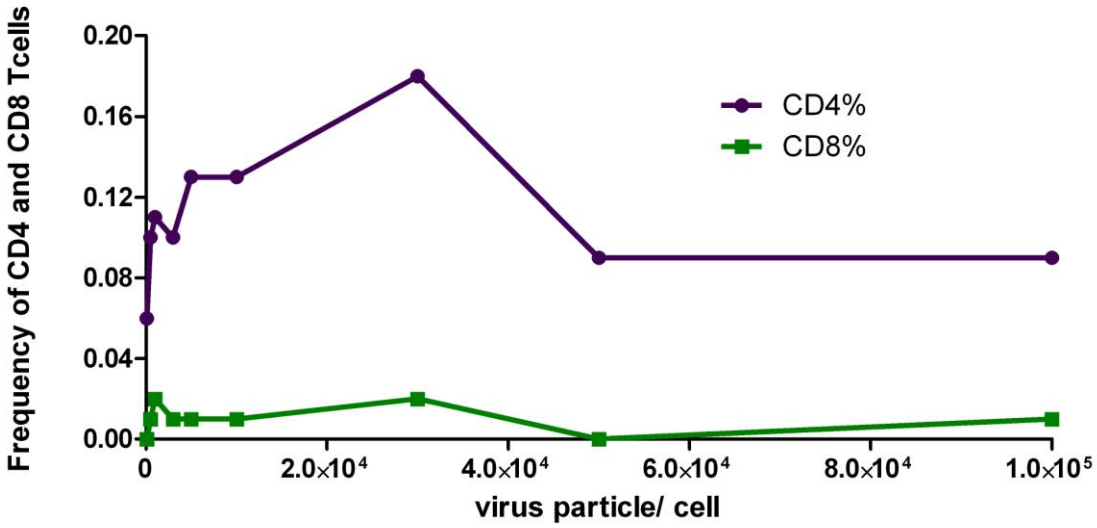

Figure 3-14 Virus titrations by IFN- γ release (cytokine capture selection).

Cytokine capture selection was performed after stimulation with heat inactivated CTL102 (10^3-10^5particle cell). % of CD4 and CD8 T-cells producing IFN- γ was determined by flow cytometry. The results shown are for LD 5

3.3.3.1 *Determination of Ad-specific T-cell frequency by CSS*

The frequency of Ad-specific T-cells following viral antigen stimulation in 12 healthy donors (LD1-12, age 18-65) was determined by CSS. PBMCs were counted and 10^6 cells were incubated with mock (no antigen) or heat inactivated CTL102 (2.5 $x10^4$ particles/cell) overnight (16 hr at 37˚C, 5% CO_2). 10^6 cells were stimulated with SEB (Staphylococcal enterotoxin B) (10μg/ml, 3 hr at 37˚C, 5% CO_2) as positive control, the next day. The CSS

protocol (section 2.5.2) was followed and the cells were washed and labelled with anti- CD4 FITC and anti- CD8 PECy5 antibodies. $4x10^5$ lymphocytes were analysed/sample on Coulter EPICs flow cytometer. The gating strategy is illustrated in Figure 3-15 followed by an example of one donor (LD 5) in Figure 3-16.

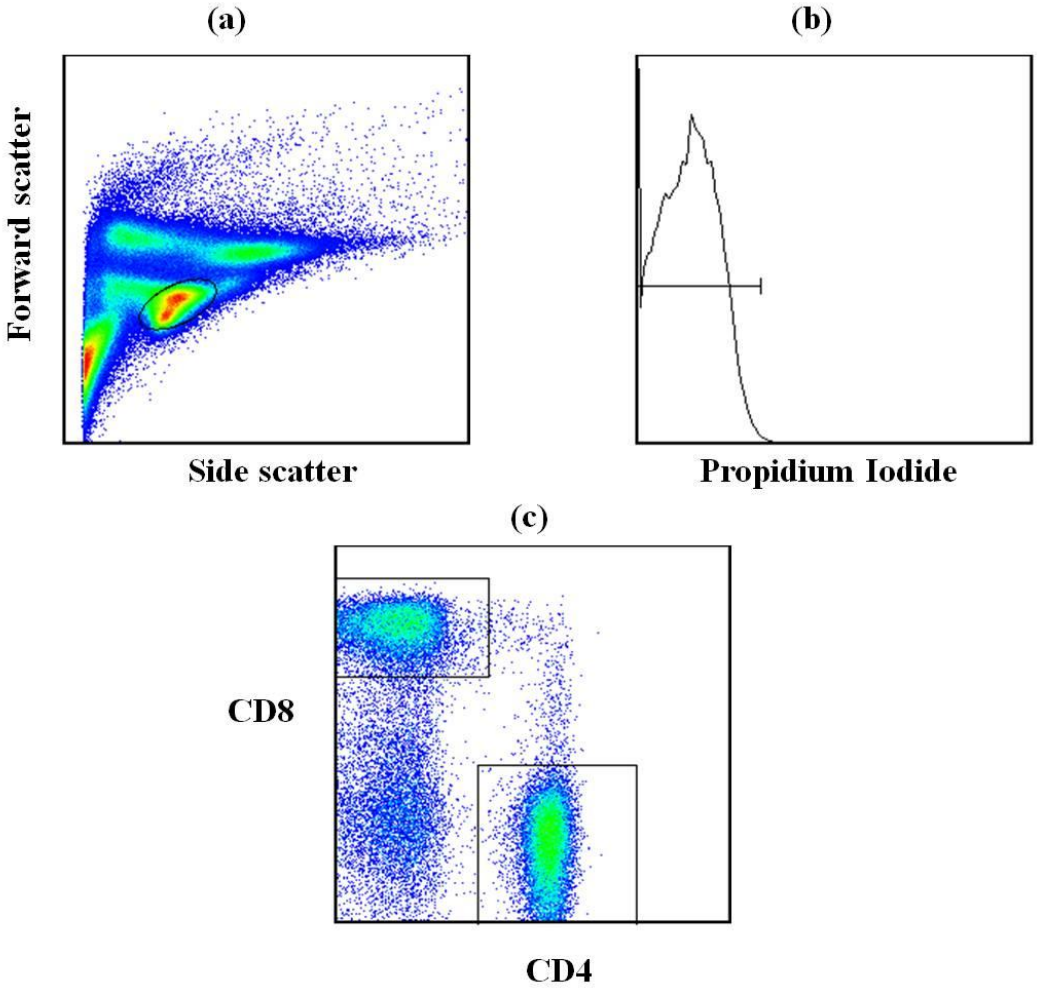

Figure 3-15 Gating strategy for CCS

(a) Forward and side scatter plots were used to determine the lymphocyte subset (b) The lymphocytes (a) were then gated for Propidium Iodide (PI) and the PI negative cells were determined as alive (c) (a) and (b) gated cells were then gated for CD4 and CD8. Ad-specific CD4/8 T-cells were then identified as the IFN-γ positive CD4/8 T- The percentage was then determined as the [(number Ad-specific CD (4 OR 8) T-cell/Total number of CD (4/8) T-cell)] x 100.

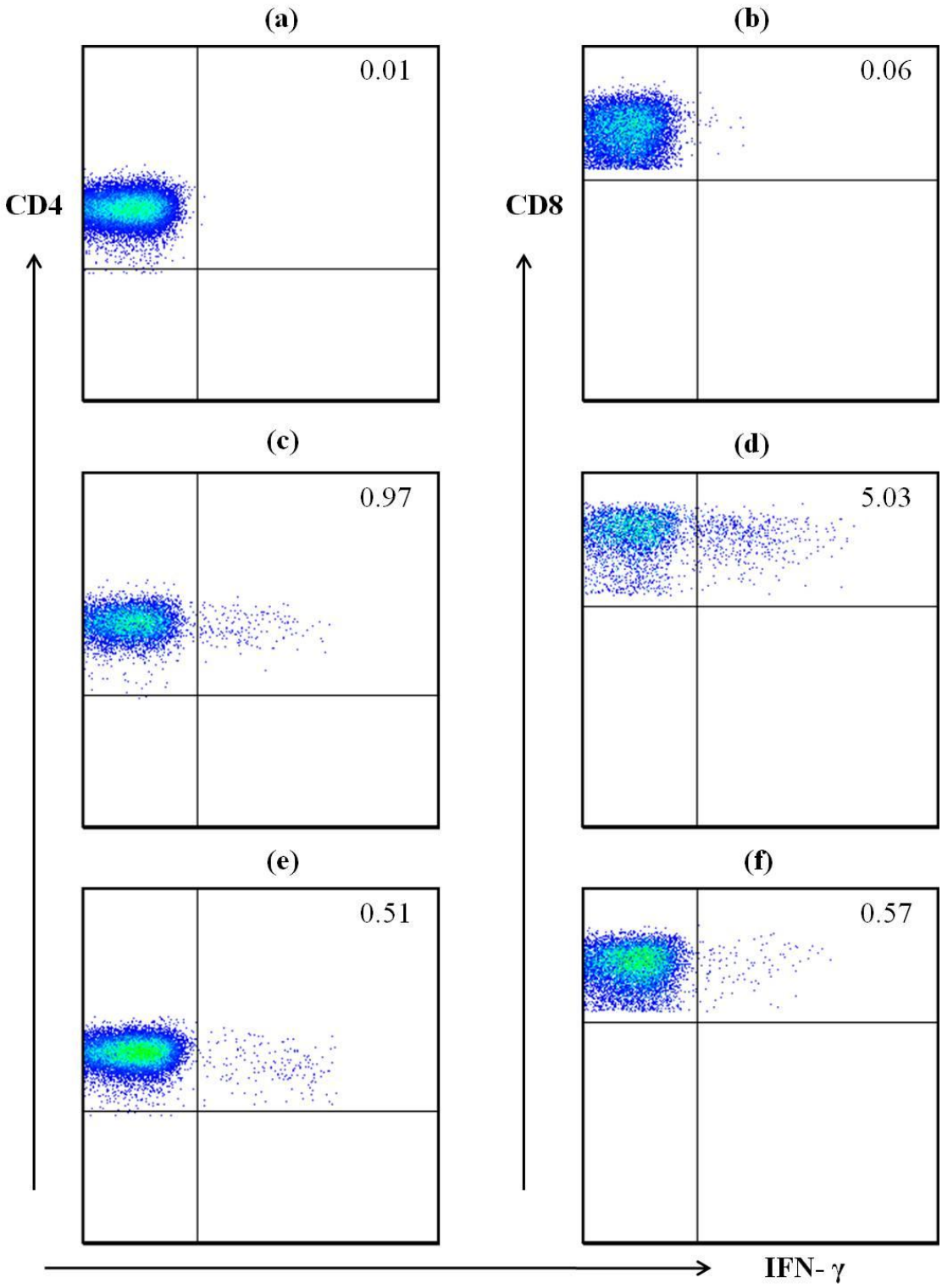

Figure 3-16 Determination of adenovirus-specific T-cell frequency by CSS

Ad-specific CD4 and CD8 T-cells after no stimulation (a) and (b); stimulation with Staphylococcus enterotoxin B (c) and (d) heat inactivated CTL102 (replication defective Adenovirus 5) (e) and (f). The figure shows the results on LD5 and the Ad-specific CD4 (e) and CD8 (f) T-cell frequency in this donor. The numbers represent the frequency of T-cells calculated as percentage of total CD4 or CD8 T-cells as shown (Figure 3-15).

The frequency of Ad-specific T-cells in 12 healthy volunteers (LD1-LD12) was determined by CCS as described above. The frequency of Ad-specific T-cells in each donor was calculated after subtracting the background frequency for the unstimulated T-cells. The CD4/8 T-cell frequency following CTL102 stimulation on all donors is shown in Figure 3-17. All donors responded strongly to the positive control indicating the technical validity of the test. The mean, range, SD and SEM of the frequencies of the unstimulated and CTL102 stimulated CD4/8 T-cells are shown in Table 3-2.

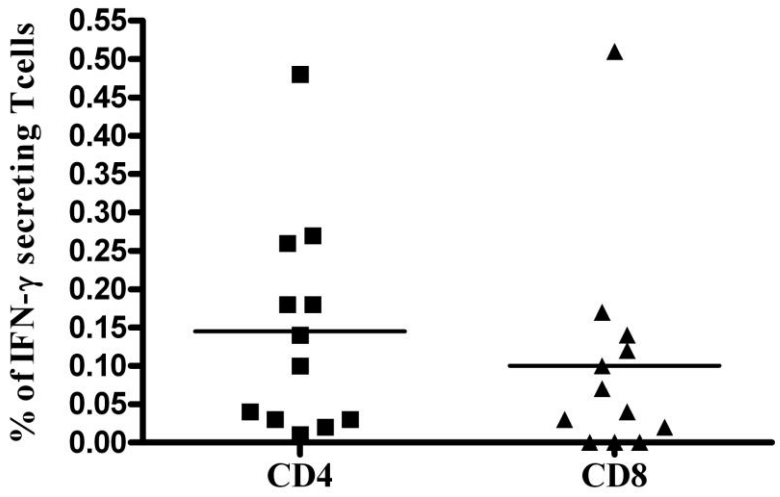

Figure 3-17 Ad-specific T-cells frequency determined by CCS following CTL102 stimulation

Percentages of Ad-specific CD4 and CD8 T-cells producing IFN-γ in response to heat inactivated replication defective Ad (CTL102) (n= 12 donors) after subtracting the unstimulated responses for each donor. Each dot represents an individual donor and the line indicates the mean frequency.

		Mean	Range	SD	SEM
CD4 T- cells	Mock	0.02	0-0.04	0.01	0.00
	CTL102	0.15	0.01-0.46	0.14	0.04
CD8 T-cells	Mock	0.06	0.05-0.09	0.03	0.01
	CTL102	0.1	0.00-0.51	0.14	0.04

Table 3-2 Frequency of Ad-specific T-cells determined by CSS

There was the possibility that permission to use CTL102 for the purposes of a clinical trial would not be granted, hence alternative sources of antigen were explored. Capsid proteins are the antigens recognised by most Ad-specific T-cells characterised to date (Hamel et al, 2002; Leen et al, 2004b; Molinier-Frenkel et al, 2000). Hexon (951aa) is highly conserved across all human Ad species (Davison et al, 2003)(section1.4.1.2). It can be presented by APC in the context of both HLA class I and class II without the need for transcription, i.e. from input virions (Heemskerk et al, 2003; Molinier-Frenkel et al, 2000).

The hexon-specific T-cell responses in 9 donors were compared to their corresponding virus-specific T-cell responses. For 9 out of the 12 donors (LD 1, 2, 4, 7, 8, 9, 10 ,11and 12) purified hexon (courtesy D Onion, University of Birmingham, UK) was used as antigen (10μg/ml, 3hr, 37˚C, 5% CO_2) to determine the frequency of hexon-specific T-cells simultaneously with CTL102 (Figure 3-18 and Table 3-3). By t-test analysis, the CD4 T-cell responses to CTL102 and hexon were significantly different ($P<0.05$). No statistically significant difference was found in the CD8 T-cell responses. Though hexon-specific T-cells are of a lower frequency than Ad-specific T-cells, these results suggest that hexon can be used as an alternative antigen to virus.

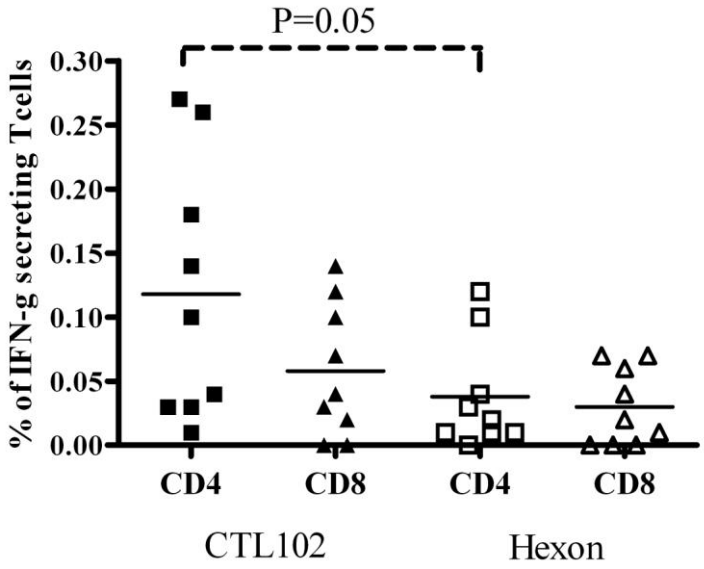

Figure 3-18 Ad-specific T-cells determined by CSS following CTL102 and hexon stimulation

Percentages of Ad-specific CD4 and CD8 T-cells producing IFN-γ in response to heat inactivated replication defective Ad (CTL102) and hexon (n=9 donors) after subtracting the equivalent unstimulated responses. Each dot represents an individual donor and the line the mean frequency.

		Mean	**Range**	**SD**	**SEM**
CD4 T- cells	Mock	0.02	0.01-0.04	0.01	0.0
	CTL102	0.07	0.03-0.09	0.03	0.01
	Hexon	0.04	0.00-0.12	0.04	0.01
CD8 T-cells	Mock	0.03	0.00-0.07	0.03	0.01
	CTL102	0.12	0.01-0.27	0.1	0.03
	Hexon	0.06	0.00-0.14	0.05	0.02

Table 3-3 Ad-specific T-cell frequencies following CCS assay by hexon or CTL102 stimulation

PBMCs from healthy volunteers (n=7; 7 donors LD 2, 3, 4, 9, 11, 12, 13) were either not infected (mock) or infected with Ad5 wild type (10^3 particles/cell, 1.5hr), or incubated with combined hexon, penton base and fibre (10μg/ml), hexon (10μg/ml) or PHA (positive control) (10μg/ml). Cells were entered into an overnight elispot assay to quantify IFN-γ secreting cells, with $5x10^5$cells/well in 3 replicate wells as per protocol (section 2.5.3). The mean value of 3 wells for all donors and mock sample is shown in Figure 3-19 and Table 3-4. Comparing complete data sets using 2-way ANOVA, a significant difference ($P<0.05$) was observed. However Bonferroni post test correction showed no significant difference between virus and combined hexon, penton base and fibre or virus and hexon. The significance by ANOVA can be attributed to high responses in the antigen stimulations in comparison to mock, thereby confirming the validity of the test. The frequency of Ad-specific T-cells determined by whole virus stimulation, combined hexon, penton base and fibre and hexon are 0.26% (range; 0.08-0.47%), 0.21% (range 0.04-0.47%) and 0.11% (range; 0.01-0.31) respectively (Table 3-4). These results are comparable to the frequency determined by Onion *et al*. who reported that the mean frequency of CD4 T-cells by whole virus stimulation was 0.26% (range 0.04-0.71%) and on hexon stimulation was 0.09 % (range 0.03–0.21 %) (Onion et al, 2007).

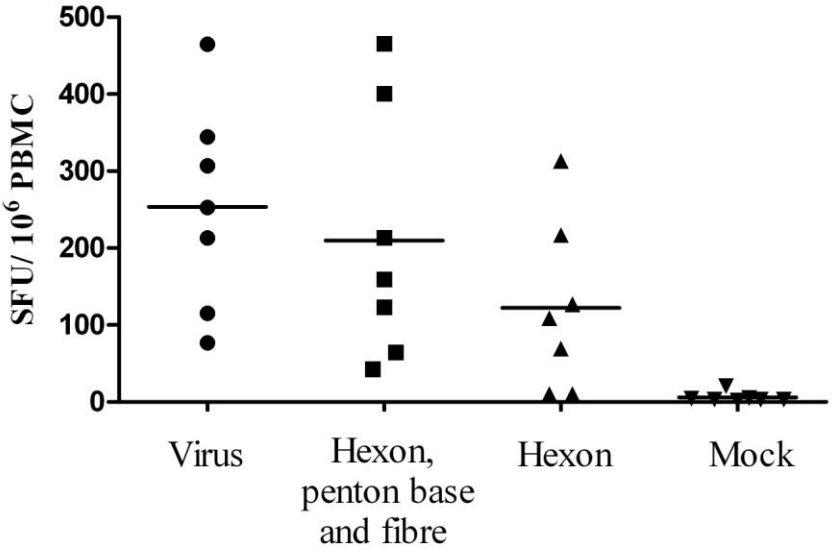

Figure 3-19 Ad-specific T-cells determined by IFN-γ Elispot assay

PBMCs (n=7, LD 2, 3, 4, 9, 11, 12, 13) were exposed to mock or Ad5 Wild type (10^3particles/cell) for 1.5hr or combined hexon, penton base and fibre (10μg/ml) or hexon (10μg/ml) or PHA (10μg/ml). Cells were washed and an overnight elispot was used to quantify IFN-γ secreting cells. Each value is the mean of 3 replicate wells with $5x10^5$cells/well. Each dot represents an individual donor and the line the mean. PHA data not shown.

	Mean	**Frequency**	**Range**	**SD**	**SEM**
Virus	256	0.26	76-465	134.3	50.67
Hexon, penton base and fibre	209.9	0.21	42-465	163.9	61.93
Hexon	122.2	0.11	-313	110.7	41.86
Mock	5.95	0.01	1.67-20.83	6.63	2.5

Table 3-4 SFU per 10^6 cells following Elispot assay

3.3.3.1 *Enrichment of Ad-specific T-cells by CSS*

CCS is an established system for enriching antigen-specific T-cells (Campbell, 2003) as described in section 1.1.1. The cytokine secreting cells are magnetically labelled and applied to an autoMACS Pro Separator (Miltenyi Biotech, Bergish Gladbach, Germany) for enrichment Figure 3-20. The program 'Posseld' which uses a double positive selection was used. The eluted cells will be referred to as positive sort and the remaining cells as the negative sort.

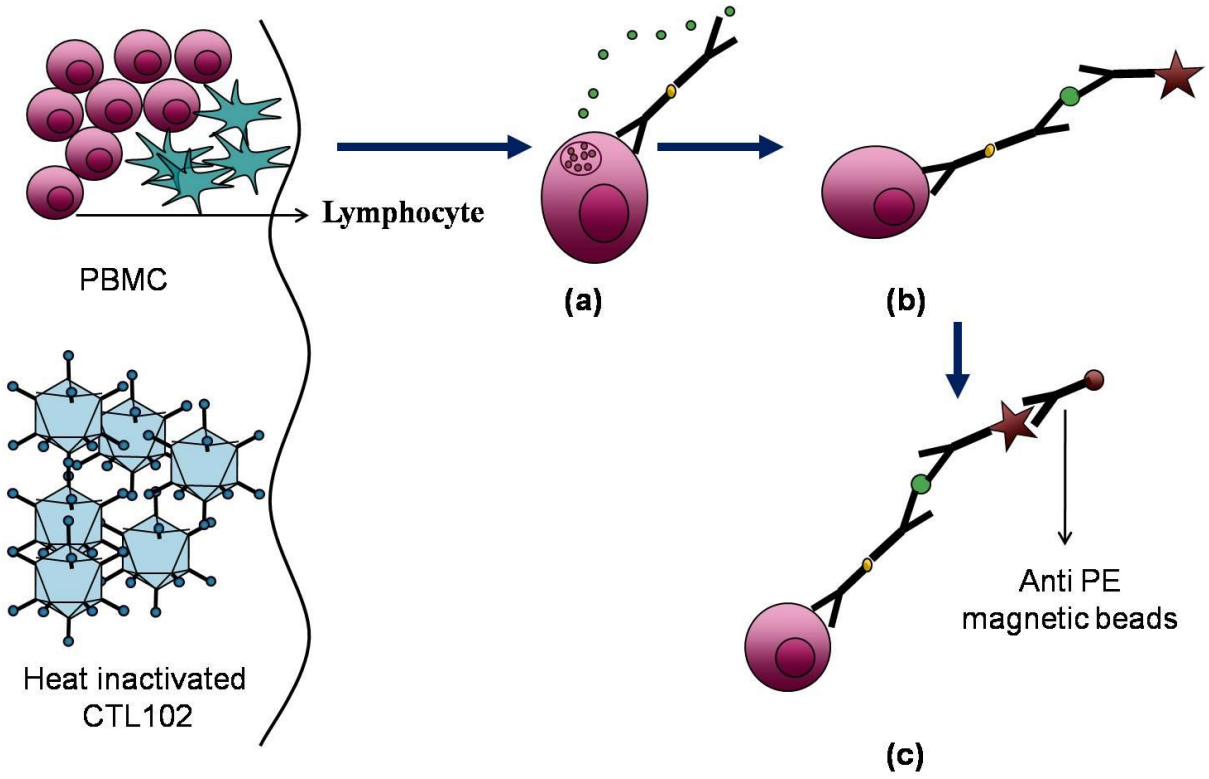

Figure 3-20 Schematic for cytokine secretion selection (CSS) system

(a) PBMCs are incubated for 16 hours with heat inactivated CTL102 and the catch reagent binds to cytokine secreting antigen-specific T-cells (b) Antigen-specific T-cells are then labelled with a flourochrome labelled anti-cytokine antibody and these are in turn labelled with anti Fluorochrome labelled microbeads which when passed through an autoMACS can be magnetically selected.

A CSS was performed on PBMCs of healthy volunteers (n=10; LD1-8, 10 & 11) and their respective frequency of Ad-specific T-cells was calculated as per protocol described in section 2.5.2. 10^7 PBMCs were incubated with heat inactivated CTL102 ($2.5x10^4$particles/cell, 16 hr at 37˚C, 5% CO_2), washed, labelled with anti-PE magnetic beads and enriched (section 2.5.2.3.). Eluted cells were analysed on Coulter EPICs flow cytometer. An example for LD6 is shown in Figure 3-21.

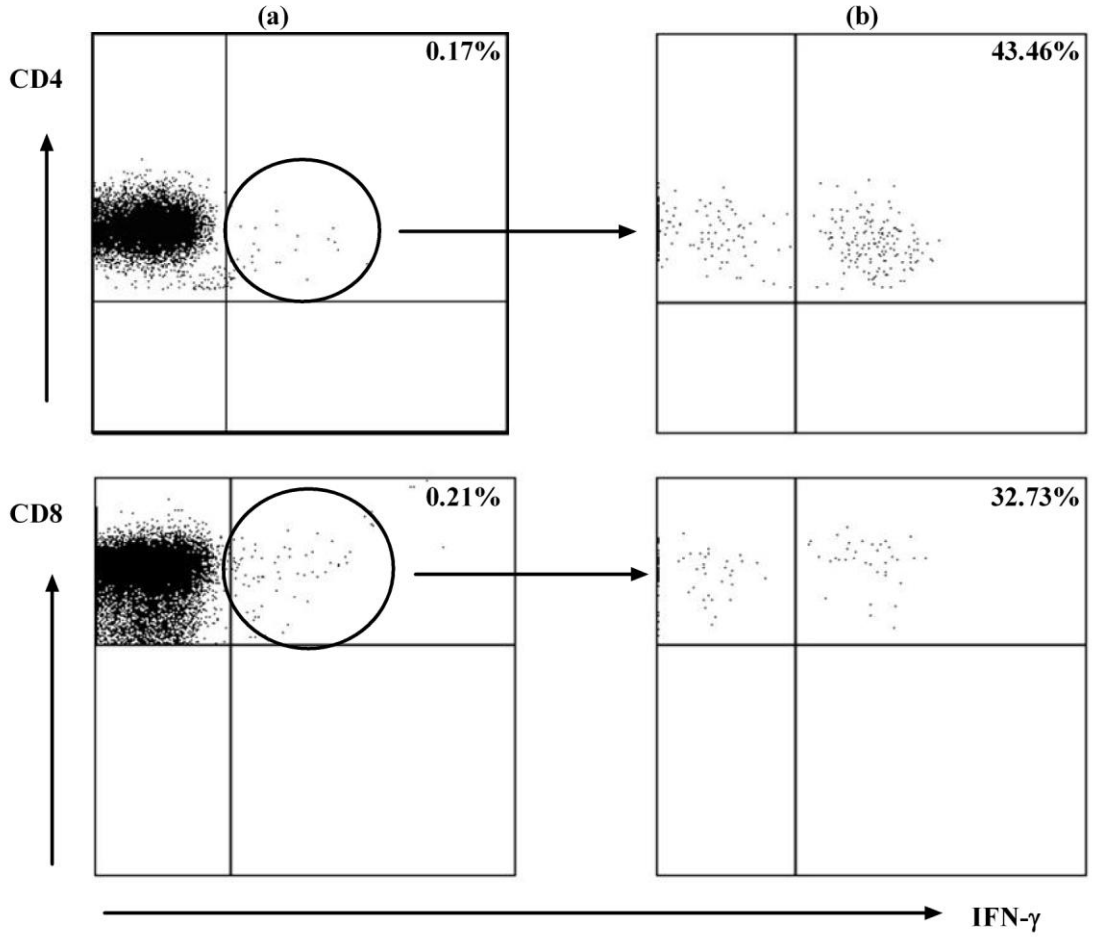

Figure 3-21 Enrichment of Ad-specific T-cells using CSS following CTL102 stimulation

PBMCs (10^7) (LD6) were incubated for 16 hours with heat inactivated CTL102 ($2.5x10^4$particles/cell) and stained with IFN-γ capture antibody, PE labelled anti IFN-γ and anti PE beads as per protocol. (a) CD4 and CD8 Ad-specific IFN-γ producing T-cells prior to sorting; $4x10^5$ cells were analysed for each sample (b) Enriched CD4 and CD8 IFN-γ producing T-cells. The frequency of the cells in the samples is expressed as % of total CD4 or CD8 T-cells

The purity of the positive sorted cells was 39.58% ±18.06%SD for CD4 T-cells and 26% ±11.9 %SD for CD8 T-cells (Figure 3-22). There was minimal loss of Ad-specific T-cells in the negatively sorted samples; CD4 (0.03 %±0.06%), CD8 (0.56%±0.04%). The cell viability was 97.63%±2.02%. This data is comparable with Feuchtinger *et al* (Feuchtinger et al, 2004) where the mean purity of positively sorted cell for 10 selections was 49.7%±20% and 32.2%±26% for CD4 and CD8 T-cells respectively. They demonstrated that 85.1%±12% were CD3 T-cells. The cell viability was higher in my experience in comparison to 91.4%± 4.5% (Feuchtinger group).

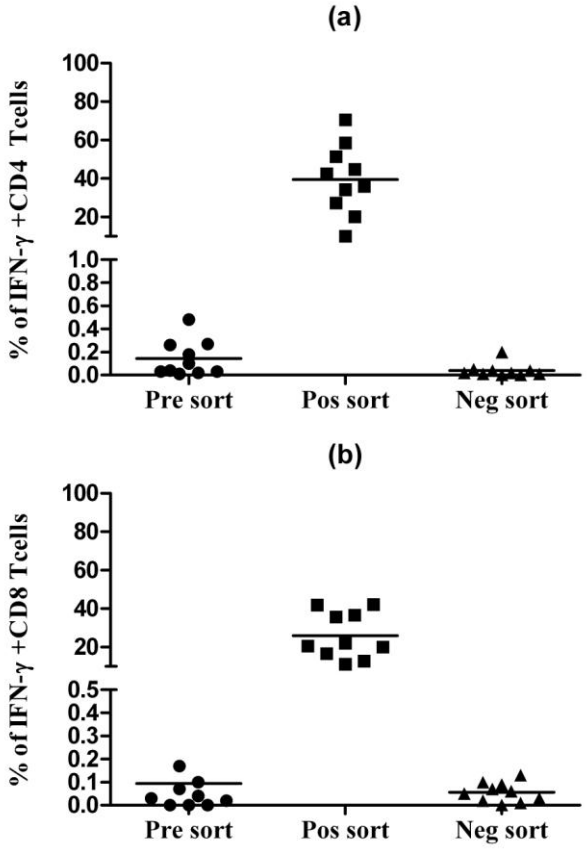

Figure 3-22 Ad-specific T-cell frequencies before and after enrichment by CCS

10^7 PBMCs (n=10) were incubated for 16 hours with heat inactivated CTL102 ($2.5x10^4$particles/cell) and labelled with IFN-γ capture antibody, PE labelled anti IFN-γ and anti PE beads as per protocol. The figure shows the relative frequency of Ad-specific (a) CD4 and (b) CD8 T-cells prior to enrichment (pre-sort) and post enrichment. Pos sort and neg sort indicates the frequency of Ad-specific T-cells in the positively and negatively eluted cells respectively.

3.3.4 Discussion

Adenovirus infection and disease in HSCT recipients is a cause of significant morbidity and mortality. The role of cellular immunity in virus clearance is highlighted by the absence of Ad-specific T-cells in patients who succumbed to Ad disease in comparison to those who successfully cleared virus (Feuchtinger et al, 2005). The low frequency of Ad-specific CD8 T-cells makes detection by Elispot and intracellular cytokine assays difficult and their role in Ad infection is less well described. In a recent study, Ad-specific CD8 T-cells following hexon or minimal peptide stimulation could be identified in 6/16 or 3/16 healthy donors by intracellular IFN-γ staining (Zandvliet et al, 2010). Interestingly this group could also detect Ad-specific CD8 T-cells after culturing PBMCs for 1week in T-cell medium supplemented with IL-2 after hexon stimulation by the same method on 13/16 donors. Ad class I HLA epitopes identified to date are broadly cross-reactive across species (Zandvliet et al, 2010). It is possible that Ad-specific CD8 T-cells play a major role in virus control and elimination along with the CD4 T-cells. It may also be that the CD4 T-cells are both cytotoxic and recruit cytotoxic cells to sites of infection. Hence the combination of CD4 and CD8 T-cell therapy may be effective in preventing or controlling Ad infection.

The frequency of Ad pMHC tetramer-staining T-cells in peripheral blood has not been enumerated to date. My work has shown that 13/13 HLA A*01 donors had a mean of 0.18% of TDL pMHC tetramer staining CD8 T-cells and in 10/10 cases the tetramer staining T-cells were enriched on magnetic selection. Only 2 of the 4 Ad HLA A*02 epitopes (YVL, LLY) showed tetramer staining in 5/5 donors but in both cases the tetramer stained cells could not be enriched (Figure 3-9). 4/4 of the HLA B*07 donors screened showed a response to the Ad HLA B*07 pMHC tetramers and tetramer staining T-cells could be enriched in 3/4 donors

(Figure 3-11). Despite successful enrichment by magnetic selection they failed to proliferate in culture following stimulation with the respective peptides.

Leen *et al*, showed in 6 HLA A*01 donors tetramer staining T-cells in the range of 0.1-0.6% of CD8 T-cells (Leen et al, 2004b). Zandvliet *et al* observed a frequency ranging from 0.06-0.46% of CD8 T-cells in 5/6 HLA A*01 donors who had hexon-specific CD8 T-cells by intracellular IFN-γ staining (Zandvliet et al, 2010). The lower frequency in my experiments is probably due to a stringent gating strategy.

Two tetramer staining patterns were observed as shown in Figure 3-5. The clustered pattern was found in a stem cell transplant recipient with Ad infection who successfully cleared virus (Figure 5-9). The same pattern was observed in tetramer staining of PBMCs (maintained in culture) following peptide stimulation (Figure 4-2). I have not been able to explain the differences in staining patterns of fresh PBMCS, peptide stimulated PBMCs in culture and fresh PBMCs from stem cell recipient with Ad infection.

PBMCs of LD2 failed to show a response to MPN peptide on tetramer staining but subsequently showed a response in Elispot assays. MPN has been identified to have HLAB*07 and B*35 restriction (Leen et al, 2008; Zandvliet et al, 2010) and LD2 is restricted by HLA B*07 and B*35. It is possible that LD2-specific PBMCs recognise the peptide by HLA B*35 restriction rather than HLA B*07, hence the inconsistencies between pMHC tetramer staining and Elispot assay. LD14 had both MPN and KPY pMHC tetramer-staining cells in the peripheral blood which could be enriched. In all 3 donors with a positive response to the HLA B*07pMHC tetramers the PI staining revealed a higher proportion of dead cells approximately 2% on tetramer staining alone and 25% on sorting, above the negative control. It is possible that the MPN and KPY tetramers, in the process of engaging their respective TCR, bring about cellular activation and death.

In the Elispot assay, only 3/7 HLA*01 donors showed a TDL peptide-specific response and HLA *02 and *07 donors failed to show peptide-specific responses (Figure 3-13). In an elispot assay only up to $4x10^5$ cells can be loaded into one well. Higher cell numbers can increase the background as well as blur the spots formed by IFN-γ release as cells pile on top of each other. Given the low frequency of Ad peptide-specific T-cells this assay may not have a high enough threshold for detecting them. This has also been the experience of C Rooney (Houston, Texas; personal communication). Negatively selected CD8 T-cells, obtained by CD4 T-cell depletion, may increase the sensitivity of this assay to determine CD8 T-cells specific for Ad-specific peptides. Peptide-specific T-cell responses measured by IFN-γ CCS assay to TDL, LLY and YVL peptides on 5 donors (LD2, 3, 4, and 5) were also negligible (data not shown).

A blood donation (500ml) would yield approximately 10^8T-cells while lymphopheresis yields 10^9 T-cells. Given the mean frequency of TDL-specific T-cells (0.18%) (Figure 3-7), on average $2x10^3$ TDL-specific T-cells/ kg (for a 70kg patient) could be collected by tetramer selection. The advantage of this selection method is that class I and II tetramers can be pooled and all tetramer staining Ad epitope-specific T-cells selected together. For example LD2 had TDL staining (Figure 3-7) as well as MPN responsive (Elispot) T-cells (Figure 3-13). Use of an HLA B*35 MPN and HLA A*01TDL tetramer would allow simultaneous selection of TDL and MPN epitope specific T-cells from this donor. The mean purity of TDL selected T-cells was 86%, suggesting that approximately 14% of the selected T-cells are not antigen-specific and could potentially be alloreactive. The risk of GvHD is low, despite this alloreactive population as they will amount to only $4x10^2$ T-cells/kg. The minimum dose of T-cells used for DLI is $1x10^5$ T-cells /kg. Based on the HLA frequency in the Caucasian population approximately 50% of the HSCT donors will be eligible for antigen specific T-cell selection using tetramers (Table 3-5). Though donors may not have Ad pMHC tetramer-

staining T-cells for all known epitopes, the epitopes that they respond to could yield adequate number of cells for adoptive transfer purposes.

HLA type	Caucasians	Africans	Hispanics	Orientals
A*01	27.4	10	11.4	<5
A*02	45.6	22.3	37.1	47.4
A*24	12.1	<5%	24.9	33.7
B*07	18.1	15.8	13.2	<5

Table 3-5 Frequency of HLA class 1 alleles

Data from HLA Matchmaker, http://tpis.upmc.edu/tpis/HLAMatchmaker/

The frequency of Ad-specific T-cells responding to virus as determined by CCS assay was a mean of 0.15% and 0.1% of the CD4 and CD8 T-cells (Figure 3-17), in accord with data obtained by other groups. The mean frequency observed by Feuchtinger *et al* in a larger cohort of healthy donors (n=53) was 0.38% of CD3 T-cells by CCS assay (Feuchtinger et al, 2005). Intracellular IFN-γ staining on PBMCs from 73 donors showed a mean of 0.30% CD4 and 0.10% CD8 T-cells (Feuchtinger et al, 2008). IFN-γ Elispot analysis of PBMCs from healthy volunteers by Onion *et al* showed a mean frequency of 0.26% CD4 T-cells. Low CD8 T-cell frequency was detected in 5 out of 10 donors (Onion et al, 2007). My data are comparable to previously published data.

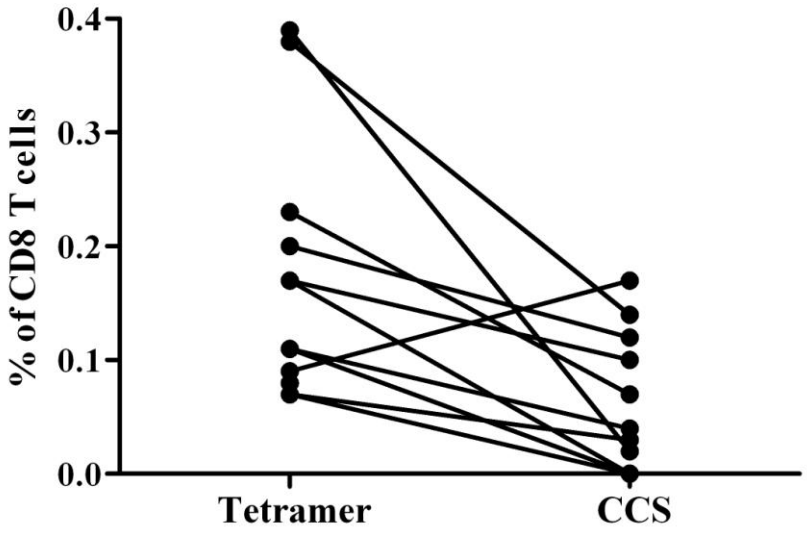

Figure 3-23 Comparison of CD8 T-cell frequency by tetramer stain and CCS.

The frequency of Ad-specific CD8 T-cells was determined by TDLtetramer staining and CCS on 9 healthy volunteers. No significant correlation was found between the 2 assays. Data obtained from Figure 3-7 and Figure 3-17

The frequency of CD8 T-cells determined by TDL tetramer and CCS were compared as shown in Figure 3-23. The tetramer frequency is higher than the Ad-specific CD8 T-cell frequency derived by CSS for most donors though no significant correlation was found.

Simultaneous determination of virus and hexon specific T-cells has shown Ad-specific T-cells in all donors (9/9) with a mean frequency of 0.07 and 0.04% of CD4 T-cells and 0.12 and 0.06% of CD8 T-cells to virus and hexon respectively (Figure 3-18). The differences in the CD4 T-cell frequencies were found to be statistically significant. Hexon-specific T-cell responses have been described by Feuchtinger *et al* (Feuchtinger et al, 2008) in a cohort of 76 healthy donors. Their analysis showed no statistically significant difference between the frequency of hexon and species C Ad lysate specific T-cells. 72.4% of donors responded to both Ad lysate and hexon protein. 10.5% of the donors responding to Ad lysate failed to respond to hexon. In a recent study (Zandvliet et al, 2010) the hexon-specific CD4 T-cell

frequency (range 0.03-0.39%), observed in 13/16 (81%) donors by intracellular IFN-γ staining, was higher than that I observed. Culturing PBMCs for 1 week in T-cell medium supplemented with IL-2 after hexon stimulation followed by intracellular IFN-γ staining showed hexon-specific CD4 and CD8 T-cell responses in all donors (Zandvliet et al, 2010). This indicates that these cells are at frequency below the threshold of detection directly *ex-vivo*.

Ad-specific T-cells could be enriched by CSS and the enriched cells have a mean purity of 39.58% for CD4 T-cells and 26% for CD8 T-cells (Figure 3-21). Given the mean frequency of Ad-specific T-cells, for a 70 kg adult, a clinical grade selection by CCS would yield $1x10^4$ T-cells /kg. 95% of these cells were viable post enrichment. This data is in accord with published data (Feuchtinger et al, 2004). Approximately 25% ($2.5x10^3$) of the cells may be alloreactive, is unlikely to cause significant GvHD and would be permitted for use.

My data has also shown that hexon or combined hexon, penton base and fibre can be used as alternative antigens to identify and enrich Ad-specific T-cells by CSS. The difference in frequencies of CD4 T-cells following hexon and virus stimulation in CCS assay was statistically significant. Generation of GMP grade virus is expensive and time consuming. GMP grade hexon is available as a pool of 15mer sequences with 11 overlapping amino acids covering the whole hexon sequence of Ad5 (PepTivator, Miltenyi Biotech, Bergish Gladbach, Germany). There is the possibility of competition for HLA molecules resulting in even lower responses if overlapping peptides are used (Kedl et al, 2003). Recently this Ad5 derived hexon peptide pool was successfully used as an antigen to enrich Ad-specific T-cells to clinical grade by CSS with a mean purity for CD4 T-cells of 56±20.8% and CD8 T-cells of 42±27%. Another observation was a 1.27 log reduction in the alloreactivity of the enriched cells (Aissi-Rothe et al, 2010).

In conclusion, multimers containing Ad epitopes and the CSS system can be used to select Ad-specific T-cells. Both methods have the potential to generate adequate number of Ad-specific T-cells on a clinical scale and according to GMP standards for purposes of adoptive transfer to HSCT patients with Ad infection refractory to antiviral therapy. A randomised controlled trial offers a suitable platform to compare the safety and efficacy of these methods.

4 Phenotype and functionality of Ad-specific T-cells

4.1 Introduction

Reconstitution of the adenovirus-specific adaptive immune system plays a key role in virus clearance and elimination in HSCT recipients. The inconsistent reports of success with antivirals have been attributed to variations in adaptive Ad-specific immunity (Tebruegge & Curtis, 2010). Adoptive transfer of Ad-specific T-cells would enable Ad-specific immune reconstitution in immunocompromised HSCT recipients. As discussed earlier Ad-specific T-cells can be selected using CSS or pMHC multimers (section 3).

CD45 or leukocyte common antigen is a leukocyte-specific transmembrane glycoprotein and a receptor-like protein tyrosine phosphatase. It lowers the threshold for receptor signaling, resulting in T and B cell activation as well as proliferation (Saunders & Johnson, 2010). CD45RO expressing cells are of low frequency in childhood and increase to account for about 50% of T-cells by age 20 (Michie et al, 1992). T-cells are broadly classified into naïve and memory cells based on the reciprocal expression of CD45RA (highest molecular mass) and RO (least molecular mass) isoforms (Akbar et al, 1988; Merkenschlager & Beverley, 1989). Naive cells migrate through secondary lymphoid organs recognise antigens presented by dendritic cells (Banchereau & Steinman, 1998; Butcher & Picker, 1996). Once they encounter antigen and become activated through the T-cell receptor, they proliferate and generate effector T-cells that are CD45RO+ with the ability to migrate into tissues (Mackay, 1993).

CCR7 is a chemokine receptor that controls homing of T-cells and dendritic cells to secondary lymphoid organs (Forster et al, 2008). A small proportion of these effector cells persist as memory cells which give an accelerated response upon a future encounter with the specific antigen (Dutton et al, 1998). Memory T-cells are divided into central memory (T_{CM}), effector memory (T_{EM}) (Sallusto et al, 1999) and revertant memory cells (T_{EMRA}) based on

CD45RA and CCR7 expression. Naïve cells are antigen inexperienced and hence CD45RA+ and CCR7-. Central memory cells (CD45RA-, CCR7+) are lymph node homing cells lacking inflammatory and cytotoxic function but have high proliferative capacity and high IL-2 production. Effector memory cells (CD45RA-, CCR7-) are tissue homing cells endowed with cytotoxic abilities but reduced proliferative potential and IL-2 production (Carrasco et al, 2006). Revertant memory T-cells (T_{EMRA}) were identified mainly in the CD8 T-cell subset and found to be terminally differentiated, with proliferative capacity in the presence of homeostatic cytokines IL-7 and IL-15, sensitive to apoptosis, secrete IFN-γ but not IL-2 and express high perforin levels (Lanzavecchia & Sallusto, 2000)(Figure 4-1).

CD27 and CD28 are costimulatory receptor molecules that belong to the tumour necrosis factor (TNF) and immunoglobulin super-family respectively. They are essential for the effective stimulation of lymphocytes. 90% of CD4+ T-cells and 50% of CD8+ T-cells express CD28 (Peggs & Allison, 2005). Both these costimulatory receptor molecules are expressed on most naïve T-cells and loss has been associated with repeated antigen experience. Van Lier and colleagues characterised memory T-cells as T_{CM} and T_{EM} based on CD27 expression. They demonstrated that CD45RA- CD27+ cells lack immediate cytolytic function whereas CD45RA+ CD27- cells have low proliferative capacity, lack CD62L but have high levels of perforin and cytotoxicity (Hamann et al, 1997).

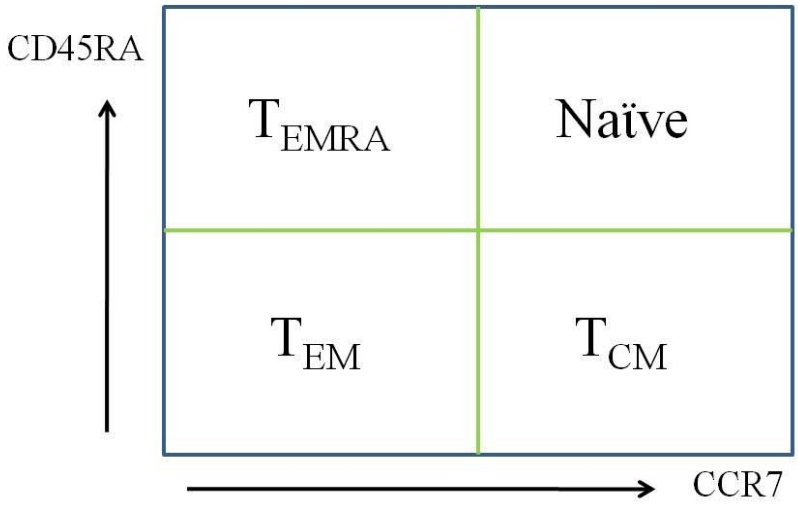

Figure 4-1 Phenotypic classification of T-cells

T-cells are classified into Naive (antigen inexperienced), T_{CM} (central memory T-cells), T_{EM} (effector memory T-cells) and T_{EMRA} cells (CD45RA expressing T_{EM} cells) based on the expression of CD45RA and CCR7

In chapter 3 the feasibility of selecting Ad-specific T-cells by CSS or pMHC tetramers was demonstrated. Ad epitope-specific T-cells have not been fully characterised with regards to their phenotype and functionality. This information hitherto would allow the determination of the homing capacities of these cells as well as indicate their *in vivo* proliferative potential. pMHC tetramer selected T-cells could have a significant population of functionally inert cells (Zajac et al, 1998). pMHC tetramers may also induce T-cell death by engaging the TCR (Cebecauer et al, 2005). Ad-specific T-cells enriched following antigenic stimuli and cytokine release using CSS may undergo differentiation resulting in altered function, proliferating capacity and possibly reduced lifespan (Obar & Lefrancois, 2010). Thus knowledge of the phenotype and functional properties of T-cells enriched by either method (tetramer and CSS) will help to understand whether selected T-cells are likely to be capable of effector function *in vivo*.

4.2 Aims of the chapter

The aim of this chapter is to characterise Ad-specific T-cells in terms of their phenotype and functionality following enrichment by cytokine capture or pMHC tetramers. This will give valuable information on the effector and proliferative potential of the enriched cells and thereby their suitability for adoptive transfer.

4.3 Results

4.3.1 Characterisation of Ad pMHC tetramer selected T-cells

TDL epitope containing tetramer gave consistent staining and enrichment results in 13/13 donors (Figure 3-7, Figure 3-8). This peptide and TDL pMHC tetramer were then used to characterise the Ad pMHC tetramer selected T-cells.

4.3.1.1 *TDL epitope-specific T-cells have high proliferative capacity*

The previous chapter showed that the frequency of the TDL epitope-specific T-cells in the peripheral blood of healthy volunteers was 0.18% (section 1.1.1.1). As these cells occur at a low frequency, they would need to significantly expand *in vivo* to achieve therapeutic efficacy. Hence the proliferative capacity of these T-cells in response to antigen is of interest. To assess antigen-specific T-cell proliferation, PBMCs from HLA *01 donors (LD3,4,5,12) were stimulated with TDL peptide (10µg/ml) and maintained in T-cell medium (10^6cells/ml) supplemented with IL-2 (100U/ml) for 29 days. Cells were restimulated with TDL peptide (10µg/ml) on day 14. As a negative control was maintained under the same conditions, after initial stimulation with irrelevant Ad HLA A*02 (YVL) or A*24 (TFY) mismatched peptide (10µg/ml). Cells were stained with anti CD3 and anti CD8 antibody, PI and TDL tetramer on days 0, 12, 20, 29 and analysed by flow cytometry. The number of PI-, CD3+, CD8+, TDL tetramer stained T-cells was determined as a percentage of the total PI-, CD3+, CD8+ve, T-cell population at each time point. No tetramer-specific response was seen in the unstimulated sample. The results for LD5 are shown (Figure 4-2). In this donor TDL-specific T-cells proliferated from a starting frequency of 0.08% of CD8T-cells to account for 82% of the culture at 4 weeks.

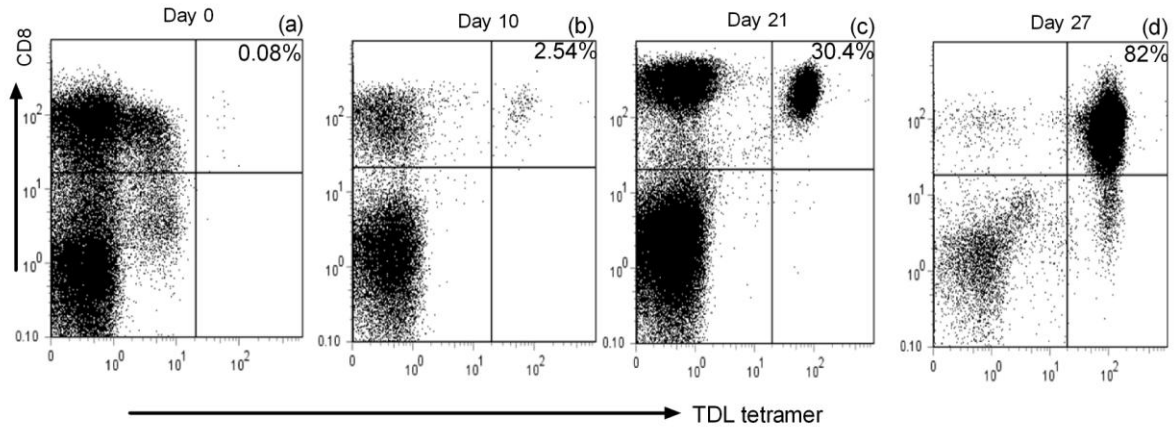

Figure 4-2 Proliferation of TDL-specific CD8 T-cells following TDL peptide stimulation

TDL- specific lymphocytes (LD5) maintained in T-cell medium supplemented with IL-2 (100U/l) were stimulated with TDL peptide (10µg/ml) on day 0 and 14. Stimulated T-cells were analysed after TDL tetramer, anti-CD3, anti-CD8 and PI staining. FACS plots are shown at days 0, 10, 21, 27 after gating on PI-, CD3+ cells. The number of PI-, CD3+, CD8+, TDL tetramer stained T-cells as a percentage of total PI-, CD3+, CD8+, T-cell population is shown in the top right hand corner of each FACS plot. 10^5 cells analysed per sample.

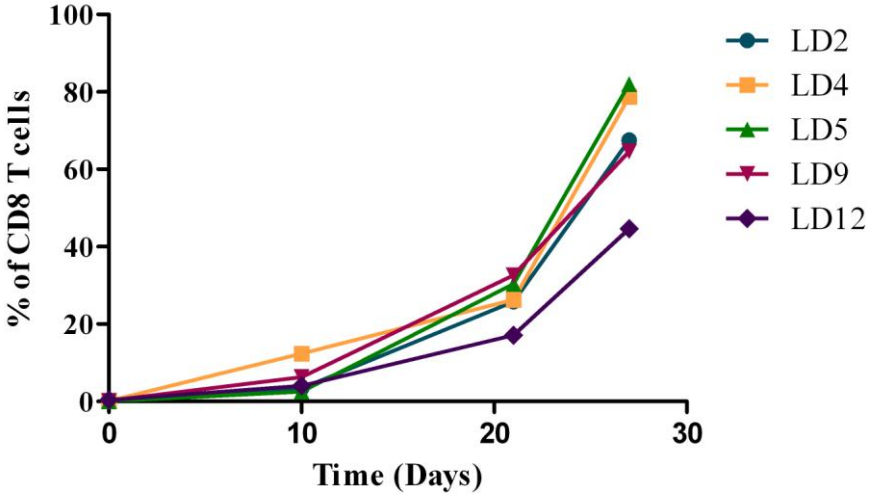

Figure 4-3 Collated data on proliferation of TDL-specific T-cells following TDL peptide stimulation

PBMCs of HLA A*01 lab donors (n=5; LD2, 4, 5, 9 and 12) were stimulated with TDL peptide (10µg/ml) on day 0 and 14 and maintained in T-cell medium supplemented with IL-2 (100U/l). $1x10^4$cells/sample were analysed after TDL tetramer staining for TDL-specific T-cells. The graph shows the number of PI-, CD3+, CD8+, TDL-tetramer stained T-cells as a percentage of total PI-, CD3+, CD8+, T-cell population determined on days 0, 10, 21 and 27.

The results for five HLA A*01 donors (LD2, 4, 5, 9 and 12) are summarised in Figure 4-3. The mean frequency of TDL-specific T-cells on days 0, 10, 21 and 27 was 0.17% ± 0.13% (SD), 5.73% ±3.96%, 26.46% ±5.94% and 67.49% ±14.72% respectively.

IL-2 is a T-cell growth factor that promotes T-cell-dependent immune responses [recently reviewed by(Dooms & Abbas, 2010)]. The experiment was repeated without supplementing the T-cell medium with IL-2 to remove the concomitant proliferative stimulation. PBMCs (donors LD2, 4, 5 and 13) were stimulated with TDL peptide (10μg/ml) and maintained in T-cell medium (10^6/ml) for 14 days. A negative control was maintained under the same conditions after initial stimulation with irrelevant Ad HLA A*02 (YVL) or A*24 (TFY) mismatched peptide (10μg/ml). Cells were analysed on days 0, 3, 7, 10 and 14 (Figure 4-4). No tetramer staining T-cells were seen in the negative control. The mean values ± SD on days 0, 3, 7, 10 and 14 were 0.16%±0.07%, 0.52%±0.18%, 2.28%±1.24%, 5.03%±2.66%, 3.96%±2.09% respectively. Despite the low starting frequency this percentage still represents a large fold increase. In the absence of IL-2, reduced proliferation as well as extensive T-cell death (< 80% of the cells viable, data not shown) was observed at day 14. This indicates that a conducive microenvironment is potentially required for the optimal proliferation of the T-cells *in vivo*.

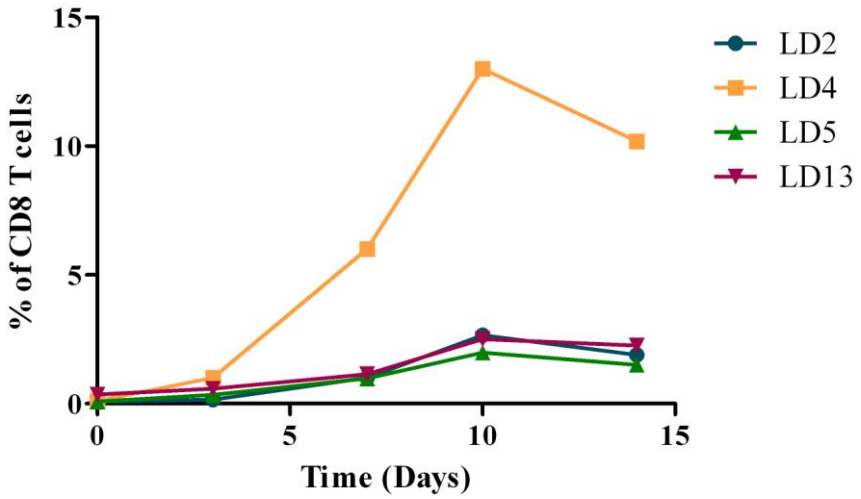

Figure 4-4 Percentage of TDL tetramer-specific T-cells following TDL stimulation

PBMCs (n=4; LD2, 4, 5 and 13) were stimulated with TDL peptide (10µg/ml) and maintained in T-cell medium. $1x10^4$cells/sample were analysed after TDL tetramer, antiCD3, anti CD8 antibody and PI staining, for TDL-specific T-cells. The above graph shows the shows the number of PI-, CD3+, CD8+, TDL tetramer stained T-cells as a percentage of total PI-, CD3+, CD8+, T-cell population on days 3, 7, 10 and 14 (n=4). No tetramer staining cells were seen at each time point in the irrelevant peptide stimulated negative control.

In order to confirm the proliferative potential of the TDL epitope-specific T-cells, PBMCs were labelled with CFSE according to protocol (section 2.5.1.4) prior to stimulation. This method allows visualisation of the proliferating cells for up to 7-10 days. The CFSE experiments were performed in the absence of IL-2. Fresh PBMCs from HLA*01 donors (n=4; LD2, 4, 5 13) were CFSE labelled and stimulated with TDL peptide (10µg/ml) or irrelevant peptide and maintained in T-cell medium. An unlabelled sample was also maintained under the same conditions. Cells were counted and labelled with TDL tetramer, anti-CD8 antibodies and PI on days 3, 5, 7 and 10. The number of PI-, CD8+, CFSE-, TDL tetramer stained T-cells as a percentage of total PI-, CD8+, CFSE- T-cell population was determined by flow cytometry. Figure 4-5 shows the FACS plots of the TDL peptide stimulated CFSE labelled cells (donor LD4). No tetramer positive cells were seen in the irrelevant peptide stimulated sample at any time point (data not shown).

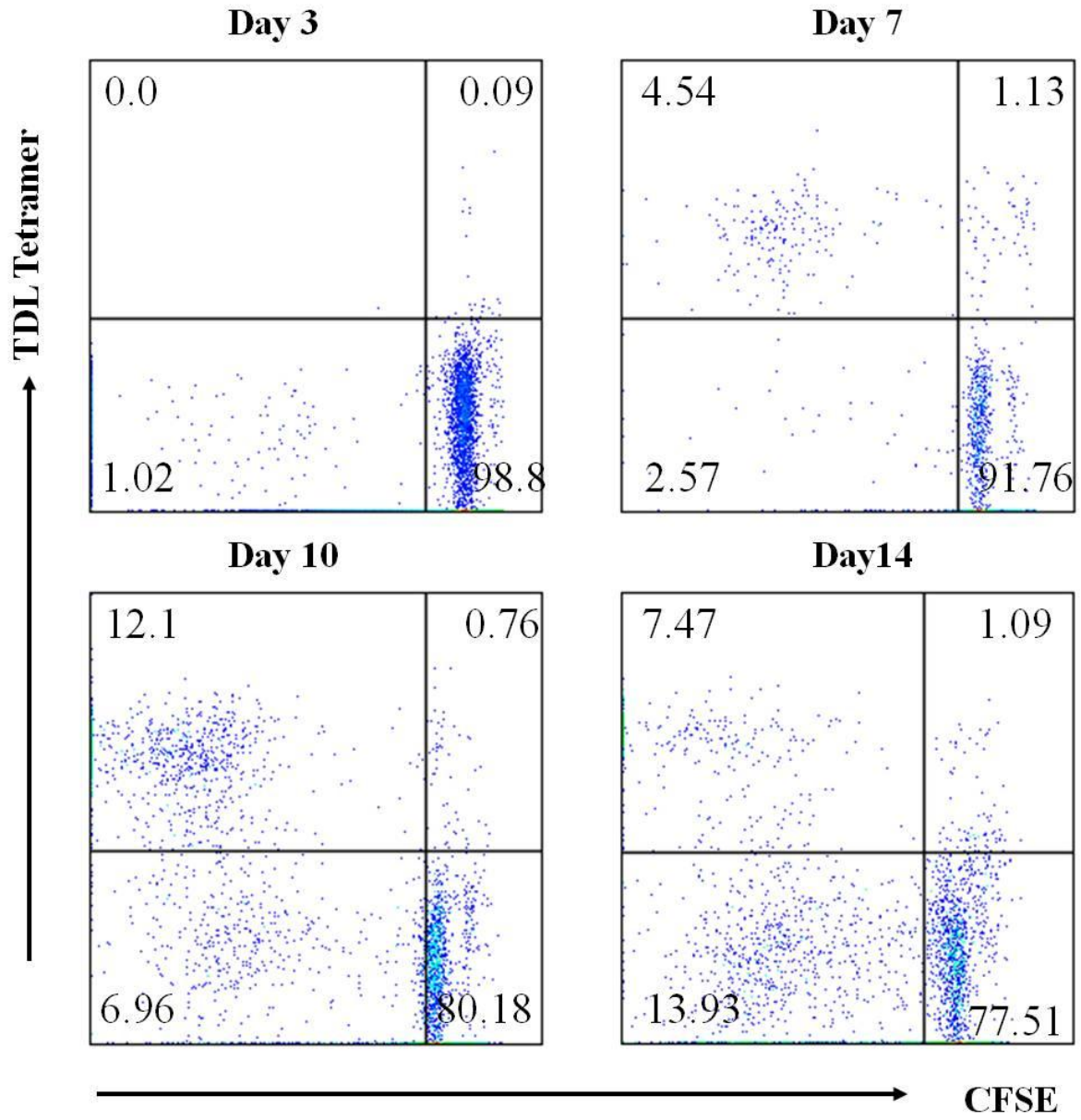

Figure 4-5 TDL peptide stimulated T-cell proliferation by CFSE labelling

PBMCs (LD4) were CFSE labelled and stimulated with TDL peptide (10µg/ml) and maintained in a T-cell medium. FACS plots of LD4 on days 3, 7, 10 and 14 respectively, after gating on PI- and CD8+ cells. The top left figures in each FACS plot represent the number of PI-, CD8+, CFSE-, TDL tetramer stained T-cells as a percentage of the total PI-, CD8+, CFSE- T-cell population. On day 0 the donor had 0.11% CD8+ Tetramer+ cells. No TDL tetramer-specific T-cells were seen in the unstimulated culture at all time points.

The percentage (PI-, CD8+, CFSE-, TDL tetramer+) obtained by flow cytometry was multiplied by the total cell count at each time point to calculate the actual number of proliferating cells. The number of proliferating TDL T-cells on days 3, 7 , 10 and 14 was then

divided by the number of TDL tetramer staining CD8 T-cells on day 0 to determine the fold proliferation of the TDL epitope-specific T-cells.

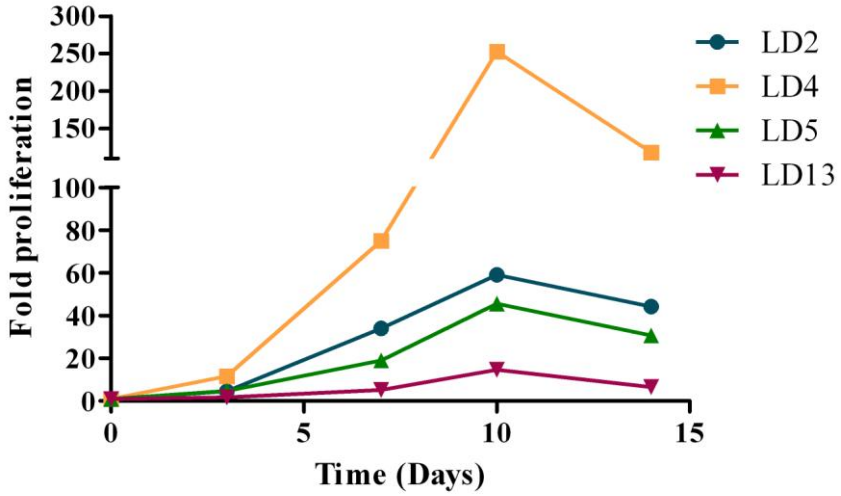

Figure 4-6 Fold proliferation of TDL stimulated TDL tetramer staining CD8 T-cells

PBMCs of healthy volunteers (n=4) were CFSE labelled, stimulated with TDL peptide (10µg/ml) and maintained in T-cell medium. The percentages (PI-, CD8+, CFSE-, TDL tetramer+) obtained by flow cytometry was multiplied by the total cell count at each time point to calculate the actual number of proliferating cells. The number of proliferating TDL T-cells on days 3, 7 , 10 and 14 was then divided by the number of TDL tetramer staining CD8T-cells on day 0 to determine the fold proliferation of the TDL epitope-specific T-cells.

Figure 4-6 shows the fold proliferation of TDL epitope-specific T-cells in healthy volunteers (n=4). Mean fold proliferation ±SD on days 3, 7, 10 and 14 are 5.69%±2.12%, 33.31%±15.1%, 93.04%±44.09%, 49.95%±24.05% respectively.

These experiments demonstrated the proliferation of TDL-specific T-cells to TDL peptide. The ultimate goal of my project is to adoptively transfer TDL or other adenovirus epitope-specific T-cells to HSCT recipients with active virus infection or disease. As these patients have circulating virus, the adoptively transferred T-cells will experience antigen presented by APCs as soon as they are within the recipient. These experiments demonstrate the proliferative response of TDL epitope-specific T-cells to TDL peptide. For the prospective

trial their potential to respond to antigen presenting cells infected with virus needs to be explored.

PBMCs of four healthy volunteers (LD2, 4, 5, 13) were CFSE labelled and maintained (10^6 cells/ml) in T-cell medium following no infection (mock) or exposed to heat inactivated CTL102 ($2.5x10^4$ particles/cell) or SEB (10μg/ml). Antigen-specific T-cells were detected by flow cytometry after labelling with PI, anti CD8 antibodies and TDL tetramer. Figure 4-7 shows FACS plots of LD4 on days 3, 7, 10 and 14 respectively demonstrating the percentage of TDL-specific T-cells proliferating in response to virus. No TDL tetramer-specific T-cells were seen in the controls. Fold proliferation of the virus stimulated TDL-specific T-cells was calculated. Mean fold proliferation ± SD on days 3, 7, 10 and 14 of the virus stimulated TDL-specific T-cells was 3.99%±0.49%, 11.98%±1.99%, 42.86%±7.41%, 27.30%±8.02% respectively (Figure 4-8). This enabled comparison of the proliferative response of TDL-specific T-cells in response to virus or TDL peptide (Figure 4-9). These experiments demonstrate that TDL-specific T-cells have high proliferative potential in response to antigen; virus or peptide.

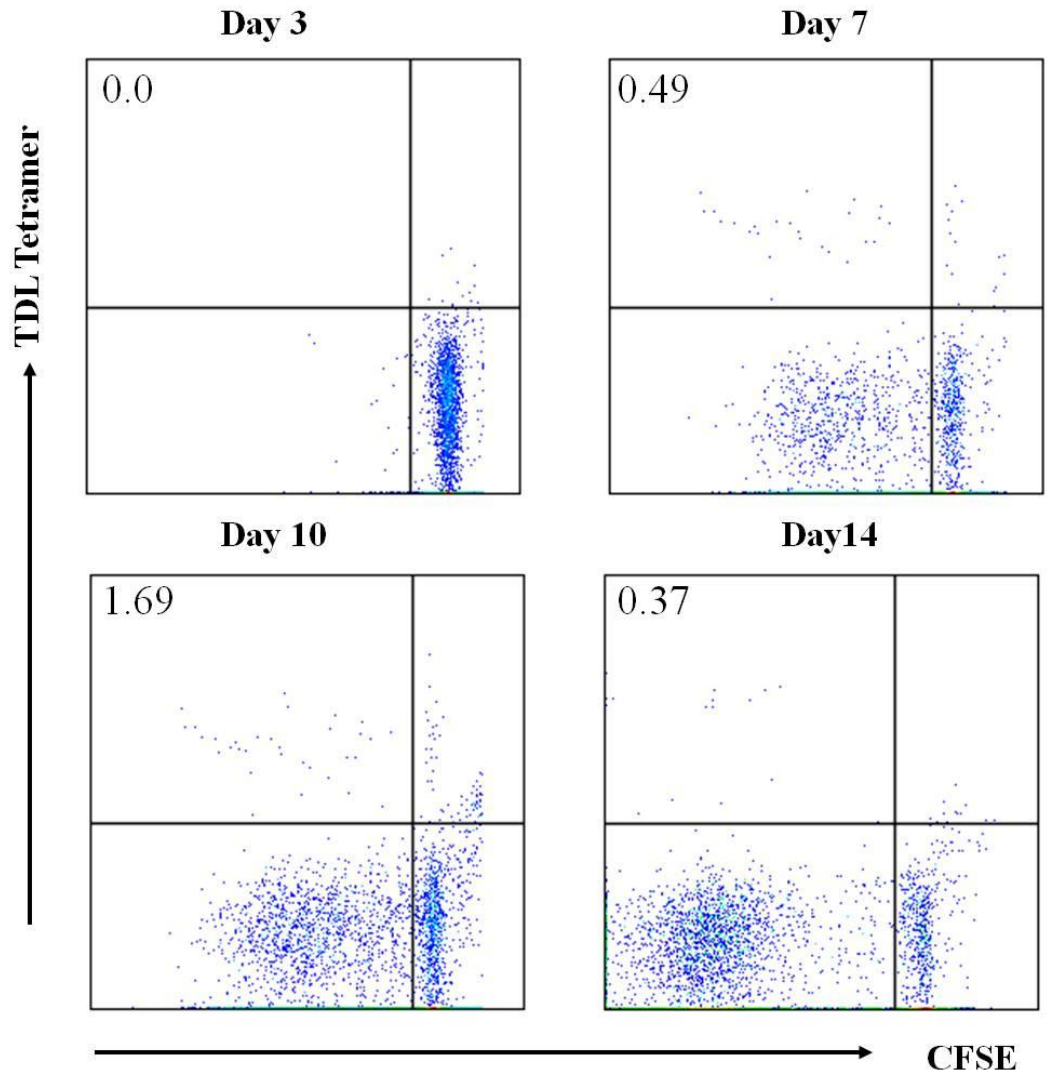

Figure 4-7 Proliferation of TDL-specific T-cells in response to adenovirus

PBMCs (LD4) were CFSE labelled and maintained (10^6 cells/ml) in T-cell medium following no infection (mock) or infection with CTL102 ($2.5x10^4$ particles/cell) or SEB (10µg/ml). FACS plots on days 3, 7, 10 and 14 respectively, after gating on PI- and CD8+ cells demonstrating the percentage of TDL-specific T-cells proliferating in response to virus. The top left figure in each FACS plot represents the number of PI-, CD8+ Tetramer +CFSE- cells as a % of total PI-, CD8+, CFSE- cells. On day 0 the donor had 0.11% CD8+ Tetramer+ cells.

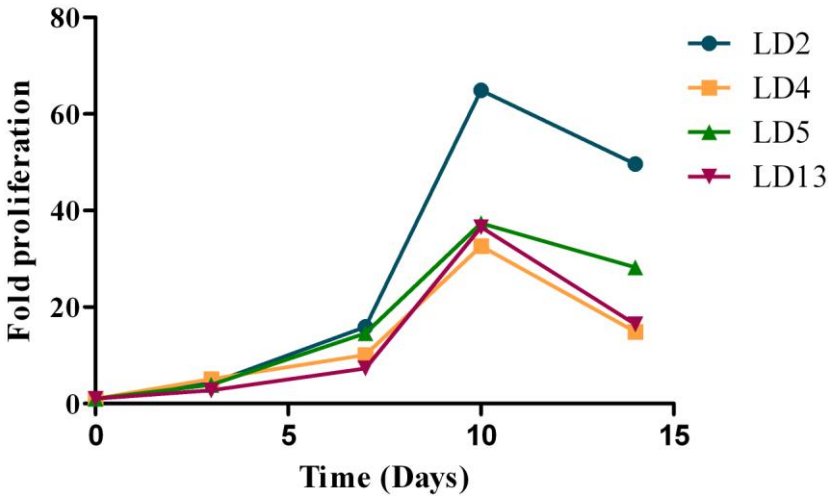

Figure 4-8 Fold proliferation of TDL-specific T-cells in response to adenovirus

PBMCs (n=4) were CFSE labelled and maintained (10^6 cells/ml) in T-cell medium following exposure to mock or CTL102 ($2.5x10^4$ particles/cell, 1.5hr) or SEB (10µg/ml, 1.5hr). From the percentages (PI-, CD8+, CFSE-, TDL tetramer+) the actual number of proliferating cells was calculated by multiplying with the total cell count at each time point. The number of proliferating TDL T-cells on days 3, 7, 10 and 14 was then divided by the number of TDL tetramer staining CD8T-cells on day 0 to determine the fold proliferation of the TDL-specific T-cells.

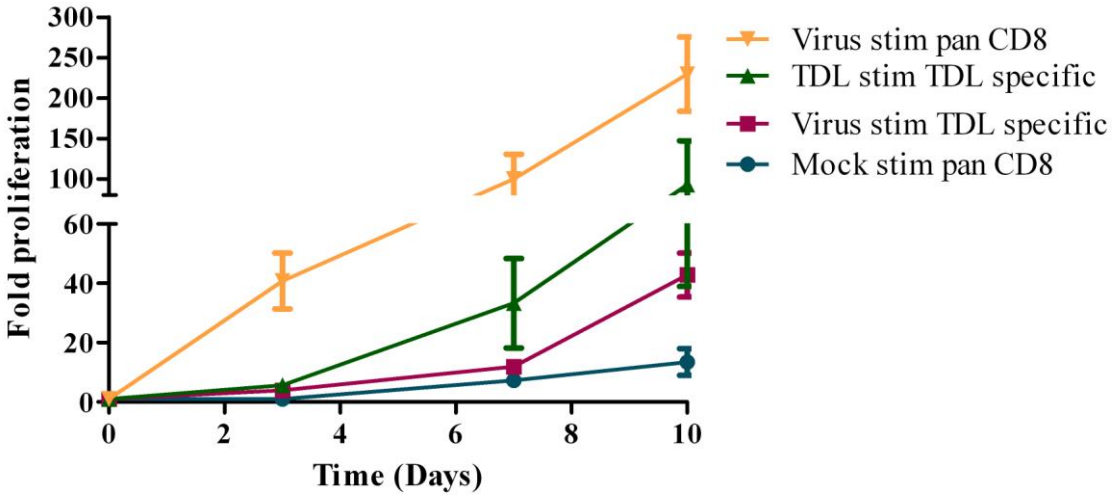

Figure 4-9 Comparison of fold proliferation of Adenovirus and TDL stimulated CD8 T-cells

PBMCs (LD2, 4, 5, 13) were CFSE labelled and maintained (10^6 cells/ml) in T-cell medium following exposure to mock or CTL102 ($2.5 x10^4$ particles/cell) or SEB (10µg/ml) or stimulation with TDL or irrelevant peptide (10µg/ml). The figure compares fold proliferation of CD8 T-cells to mock, TDL tetramer staining CD8 T-cells to Ad and TDL peptide and CD8 T-cells to Ad. Each symbol shows the mean values on days 0, 3, 7 and 10 respectively and the error bars the SD.

4.3.1.2 *TDL epitope-specific T-cells recognise antigen*

As pMHC tetramer selected cells may have a significant population of functionally inert cells, it was important to see if the Ad epitope-specific T-cells recognise antigen. PBMCs from HLA *01 donors were stimulated with TDL peptide (10μg/ml) and maintained in T-cell medium supplemented with IL-2 100U/ml at 10^6cells/ml for one month, to assess antigen-specific T-cell proliferation. Cells were restimulated with TDL peptide (10μg/ml) on day 14. As a negative control, an unstimulated sample was maintained under the same conditions. On day 29 cells were stained with TDL tetramer and enriched using magnetic beads to a purity of 99% (Figure 4-10a). To determine antigen recognition ability, these sorted TDL tetramer staining T-cells were incubated with autologous dendritic cells which had been pre-exposed to Ad (CTL102) or loaded with TDL peptide or left untreated as a negative control, and IFN-γ release measured by ELISA. Figure 4-10b shows that T-cells (donor LD5) selected with TDL tetramer recognise Ad5 infected and peptide loaded antigen presenting cells and secrete IFN-γ in response. This was repeated for LD2 with similar results (data not shown). This experiment demonstrates that the T-cells proliferating in response to antigen have not lost their functional ability to respond to cognate antigen following tetramer selection.

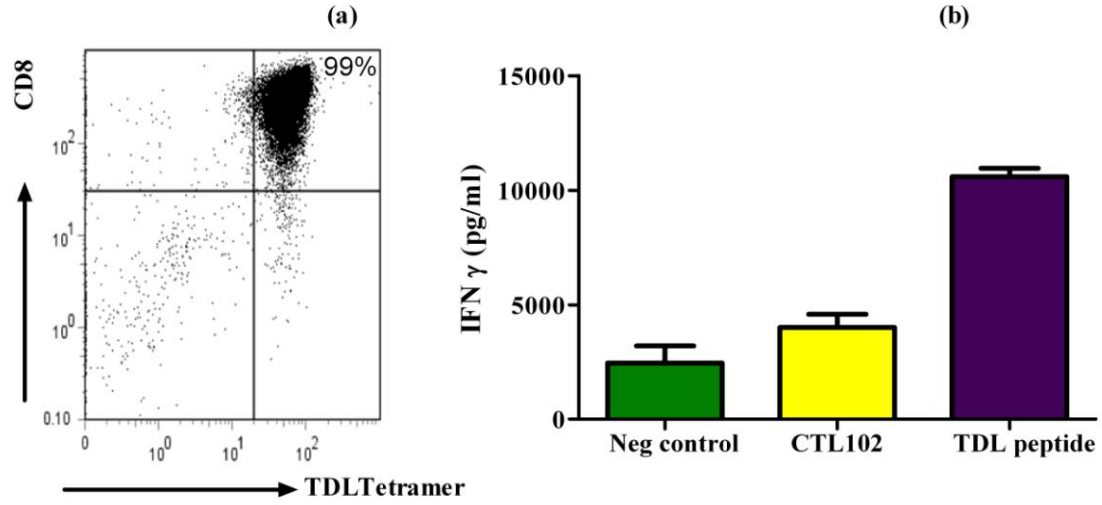

Figure 4-10 TDL tetramer-specific T-cells can recognise antigen

PBMCs of LD5 (HLA A*01) were stimulated with TDL peptide (10µg/ml) on day 0 and day 14 and maintained in T-cell medium supplemented with IL-2 (100U/l). After 29 days, TDL stained T-cells were magnetically enriched (section 2.5.2.3) (a). FACS plot following magnetic enrichment of TDL tetramer stained T-cells and anti CD3, anti CD8 and PI labelling. (b) IFN-γ release measured by ELISA on magnetically enriched TDL tetramer selected T-cells after overnight incubation with autologous dendritic cells left untreated as a negative control or exposed to CTL102 or loaded with peptide. All samples were run in triplicate. The mean IFN-γ release with standard deviations of each is shown.

4.3.1.3 *TDL epitope-specific T-cells have a minimally differentiated memory phenotype*

In addition to proliferation, the phenotype of T-cells has been shown be important to understand the homing potential and the long term ability to form a functional memory compartment. Determination of CD45RA, CCR7, CD27 and CD28 expression enable the categorisation of Ad-specific T-cells, i.e., T_{CM}, T_{EM} or T_{EMRA}. PBMCs (10^7) were stained with TDL tetramer (50µg, 37˚C, 15 min) followed by anti-PE magnetic beads labelling (4˚C for 20 minutes) and enriched according to protocol (section 2.5.2.3). Enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CCR7, CD27 and CD28 or PI and analysed by flow cytometry (DakoCyAN, Beckman Coulter). A non tetramer stained sample was used as the negative control and the total CD8 T-cells in this sample was analysed for expression of CD45RA, CCR7, CD27 and CD28. Figure 4-11 A shows the flow cytometry plots following enrichment (purity of 88.31%) and Figure 4-11 B shows the results in comparison with the total CD8 T-cells in the non-tetramer stained sample for LD5. The TDL-specific T-cells are $CD45RA^{high}$, $CCR7^{high}$, $CD27^{high}$ and $CD28^{high}$.

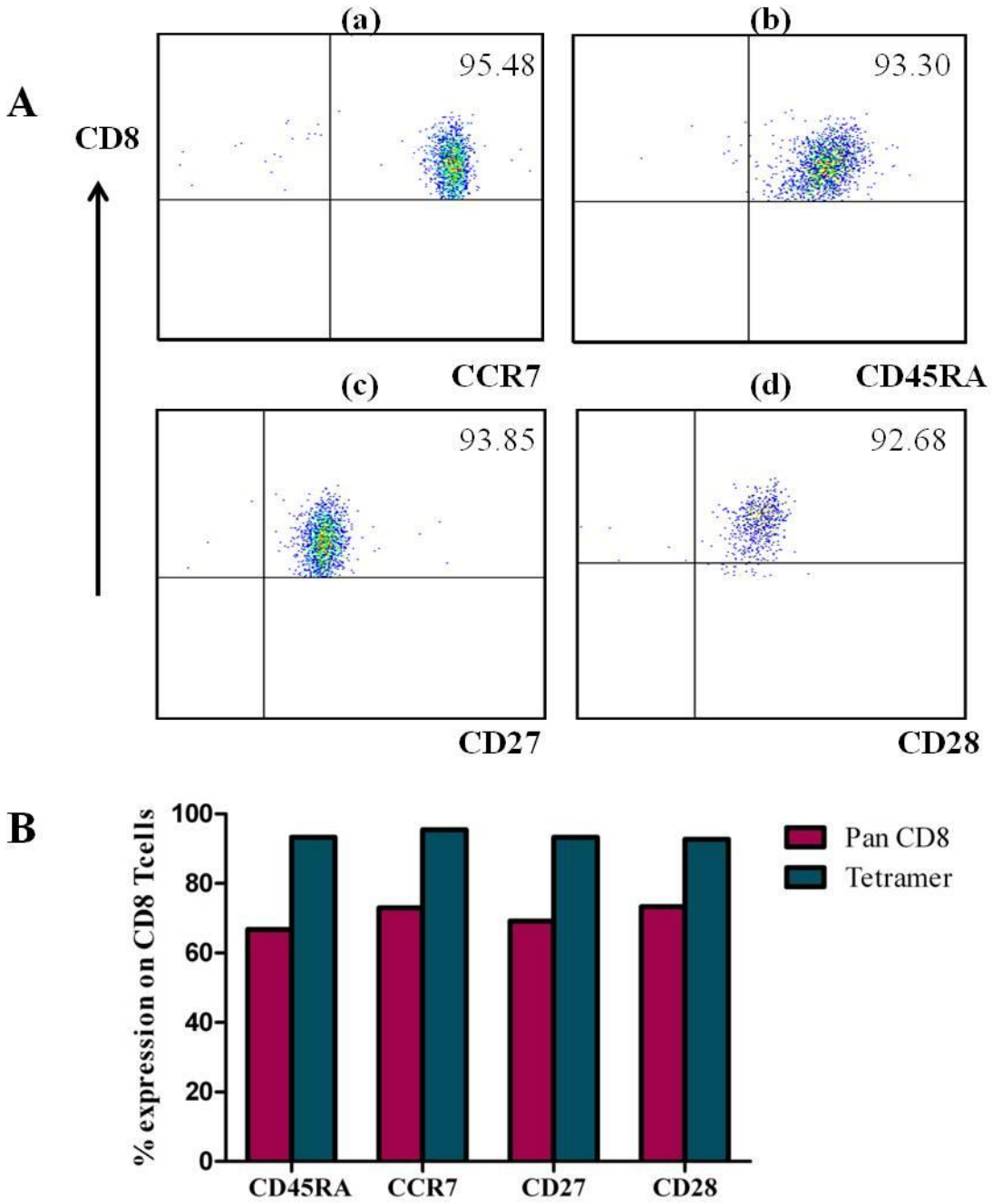

Figure 4-11 Phenotype of enriched TDL-specific T-cells

PBMCs LD5 ($1x10^7$) were stained with TDL tetramer (50µg, 37˚C, 15 min) followed by anti-PE magnetic bead labelling (4˚C, 20 min) and enrichment. Enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CCR7, CD27, CD28 or PI and analysed by flow cytometry (DakoCyAN). The PI-, CD8+, TDL tetramer stained T-cells were identified and the expression of CD45RA, CCR7, CD27 and CD28 on these cells determined. A: FACS plots showing the number of TDL tetramer staining T-cells expressing the corresponding receptor expressed as a percentage of the total. The purity of the tetramer enriched T-cells was 88.31%. B: Comparison of TDL specific T –cells expressing CD45RA, CCR7, CD27 and CD28 to total CD8 T-cells.

PBMCs of (LD2, 3, 4, 5, 6) donors were analysed as above (Figure 4-12). In all donors the enriched population of cells had > 80% purity. The difference in expression of the receptors was found to be statistically significant by 2 way Anova analysis for the collated data set and Bonferroni post test for the difference in CD45RA expression p=0.009. Initial analysis showed that the TDL-specific T-cells have a $CD45RA^{high}$, $CCR7^{high}$, $CD27^{high}$ and $CD28^{high}$ phenotype which would fit the description of a naive cell (section 4.1. A naive cell at this frequency and with this T-cell receptor expression is unlikely. As TDL epitope-specific T-cells respond to antigen by secreting IFN-γ (Figure 4-10b) they are not naive, but do not fit into established memory T-cell phenotypes.

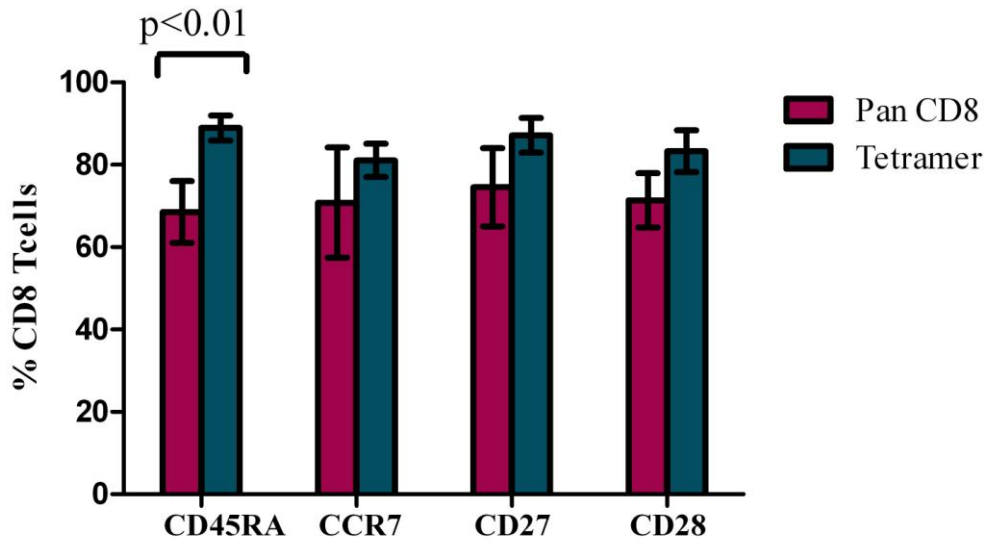

Figure 4-12 TDL-specific T-cells have an early memory phenotype

PBMCs (LD 2, 3, 4, 5, 6) were stained with TDL tetramer (50µg, 37˚C, 15 min) followed by anti-PE magnetic bead labelling (4˚C, 20 min) and enrichment. Enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CCR7, CD27, CD28 or PI and analysed by flow cytometry (DakoCyAN). The PI-, CD8+, TDL tetramer stained T-cells were identified and the expression of CD45RA, CCR7, CD27 and CD28 on these cells was determined. The frequency of receptor expression on total CD8 T-cells (pan CD8 T) and TDL epitope-specific T-cells are shown. The error bars show the standard deviation.

To investigate this further the panel was extended to include CD45RO, CD62L, CD57 and LFA-1. Determination of CD45 isoforms as well as the lymph node homing markers, CCR7 and CD62L expressions will allow the detailed phenotypic characterisation of these cells. CD11a (LFA-1) is a leukocyte function associated antigen (LFA) involved in leucocyte adhesion. LFA-1 expression is high on primed or non-naive T-cells and low on cord blood cells (Hviid et al, 1993). Expression of this integrin on tetramer-specific T-cells will aid in investigating whether they are naive or antigen experienced. CD57 expression indicates terminal differentiation or replicative senescence (Brenchley et al, 2003).

PBMCs (10^7) of healthy donors (LD2, 3, 4, 5, 10, 11, 12, and 13) were stained with TDL tetramer (50μg, 37˚C, 15 min). The cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, CD57 and LFA-1 and PI. $2x10^6$ cells were analysed by flow cytometry (LSR II, BD Biosciences). A non tetramer stained sample was used as the negative control and the total CD8 T-cells in this sample was analysed simultaneously. Figure 4-13 shows the flow cytometry results on LD5 for CD45RA, CD45RO, CCR7 and CD62L and Figure 4-14 the collated data for all 8 donors. This shows that the TDL-specific CD8 T-cells have a $CD45RA^{high}$, $CD45RO^{high}$, $CCR7^{high}$, $CD62L^{low}$, $CD27^{high}$, $CD28^{high}$, LFA-1^{high} and $CD57^{low}$ phenotype suggesting that they have a minimally differentiated central memory phenotype with effector capabilities. Paired t-test analysis showed that the differences between TDL-specific CD8T-cells and Total CD8 T-cells in CD45RA and CD57 expression were statistically significant with a p value <0.001. As the cells express both RA and RO isoforms of CD45 they are not naïve. High expression of LFA-1 confirms that they are antigen experienced and low CD57 expression indicates their proliferative potential in response to antigen.

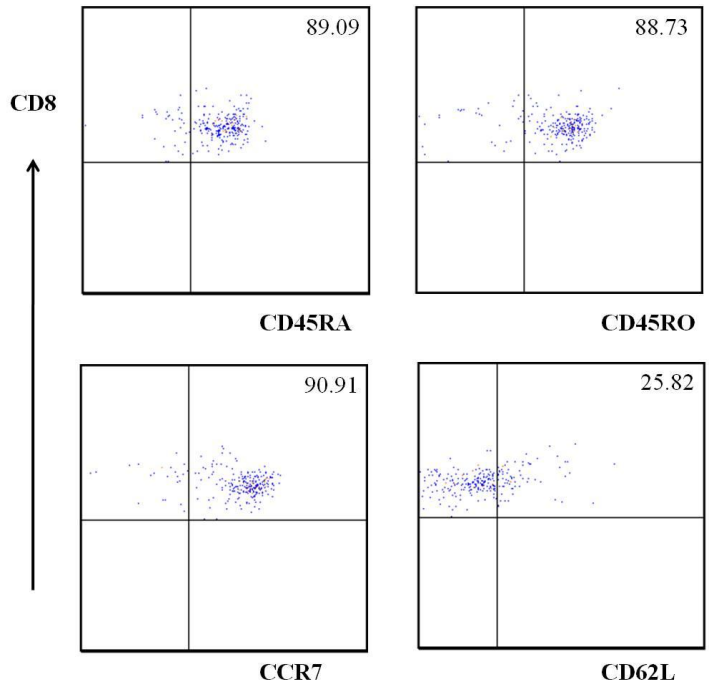

Figure 4-13 TDL-specific CD8 T-cells have a central memory phenotype

PBMCs LD5 (10^7) of healthy donors were stained with TDL tetramer (50µg, 37˚C, 15 min). The cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, CD57, LFA-1 or PI. $2x10^6$ cells were analysed by flow cytometry (LSR II). A non tetramer stained sample was used as the negative control. Flow cytometry plots with the frequencies of expression of CD45RA, CD45RO, CCR7 and CD62L receptor expression on TDL epitope-specific T-cells are shown.

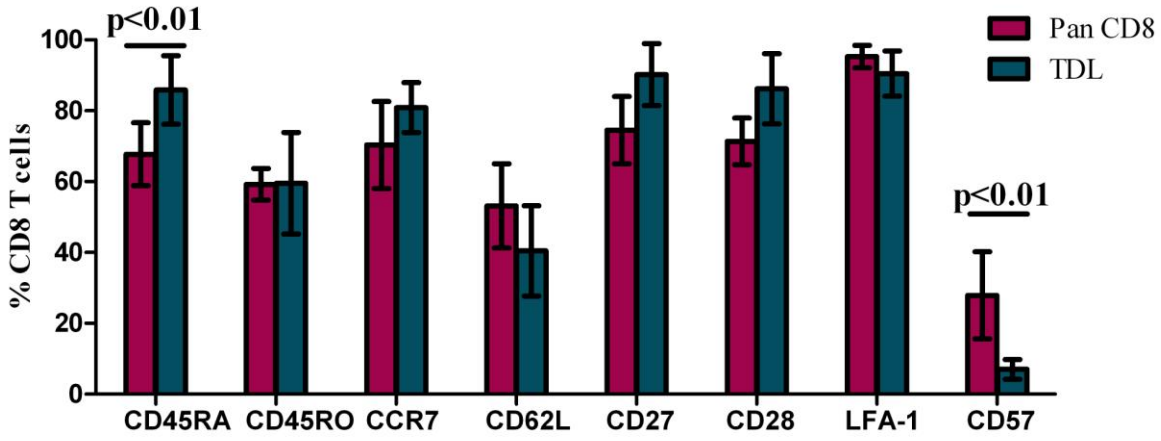

Figure 4-14 Phenotype of TDL epitope-specific T-cells

PBMCs (10^7) of healthy donors (n=8; LD2, 3, 4, 5, 10, 11, 12, 13) were stained with TDL tetramer (50µg, 37˚C, 15 min). The cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, CD57, LFA-1 or PI. $2x10^6$ cells were analysed by flow cytometry (LSR II). A non tetramer stained sample was used as the negative control. The figure shows mean frequency of expression of the respective receptors on total CD8 T-cells and TDL epitope-specific CD8 T-cells. The error bars indicate the standard deviation.

4.3.1.1 *TDL epitope-specific T-cells retain effector phenotype on proliferation*

In order to quantify the TDL specific T-cells proliferating in response to TDL, PBMCs from four donors (LD 2, 4, 5, 13) were CFSE labelled, stimulated with TDL peptide or irrelevant peptide (10μg/ml) and maintained in T-cell medium for 10 days. Cells were labelled with anti-CD3, anti-CD8, TDL tetramer, PI, anti-CD45RA, anti CCR7, anti CD62L and anti CD28 and analysed on day 0 and day 10 by flow cytometry. Figure 4-15 shows the day 10 flow cytometry plots on LD4 and Figure 4-16 the collated results of 4 donors (LD 2, 4, 5, 13). By day 10 the proliferating TDL tetramer staining T-cells switch their day 0 phenotype. Expression levels of CD45RA and CCR7 decreased, CD6L increased to intermediate levels and CD28 remained high. These experiments confirm the early minimally differentiated phenotype of the TDL-specific T-cells. Persistent high expression of costimulatory receptor molecules (CD28) highlights that they retain their effector capabilities following antigen stimulation.

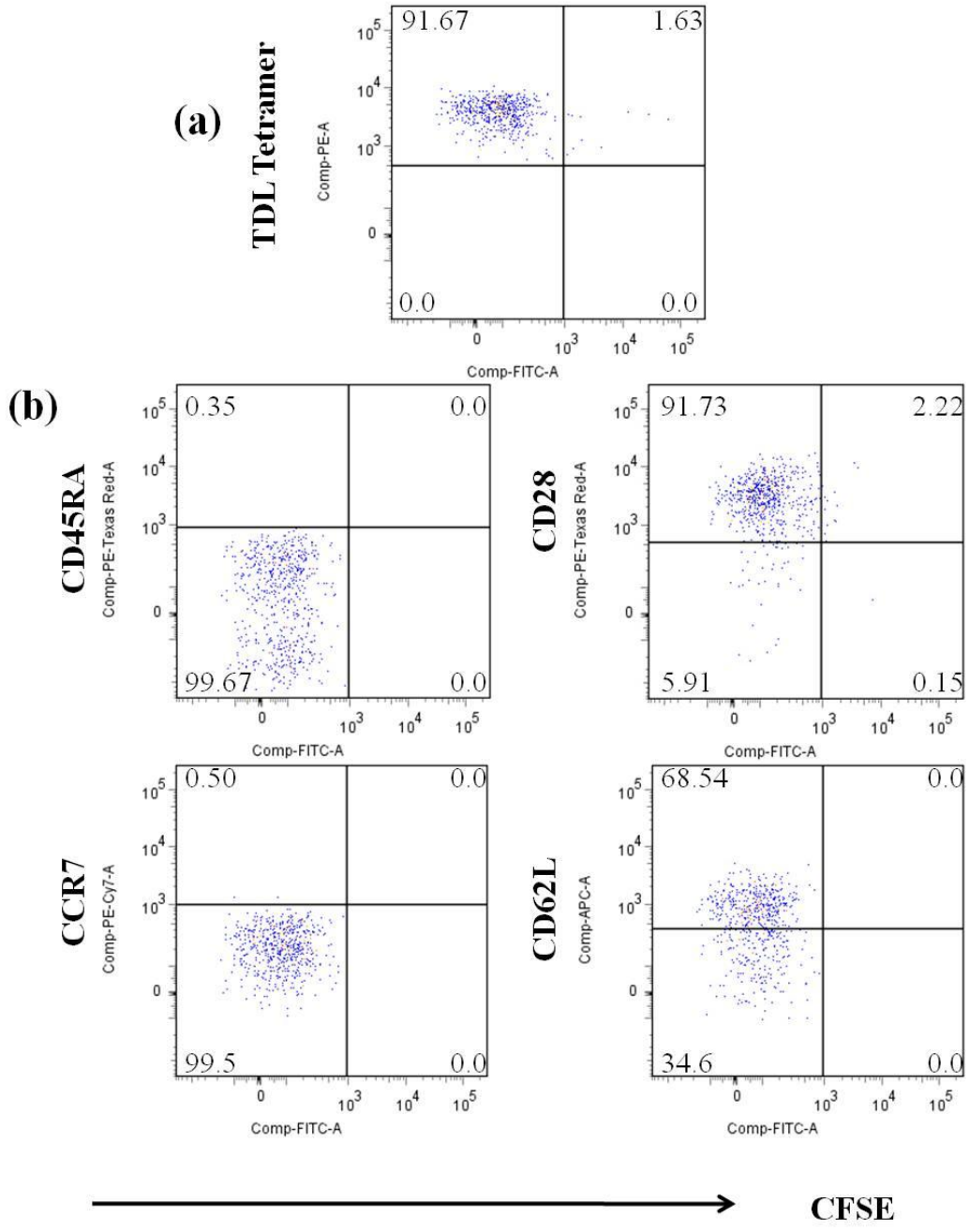

Figure 4-15 TDL-specific T-cells change their phenotype on proliferation

PBMCs were CFSE labelled, stimulated with TDL peptide or irrelevant peptide (10μg/ml) and maintained in T-cell medium for 10 days. Cells were labelled with anti-CD3, anti-CD8, TDL tetramer, PI, anti-CD45RA, anti CCR7 and anti CD62L. Flow cytometry plots on LD4 showing the (a) day 10 CFSE labelled tetramer stained T-cells gated on PI, CD3 and CD8. (b) Expression of CD45RA, CCR7, CD62L and CD28 on the CFSE-ve tetramer stained T-cells (91.67% as shown in (a)).

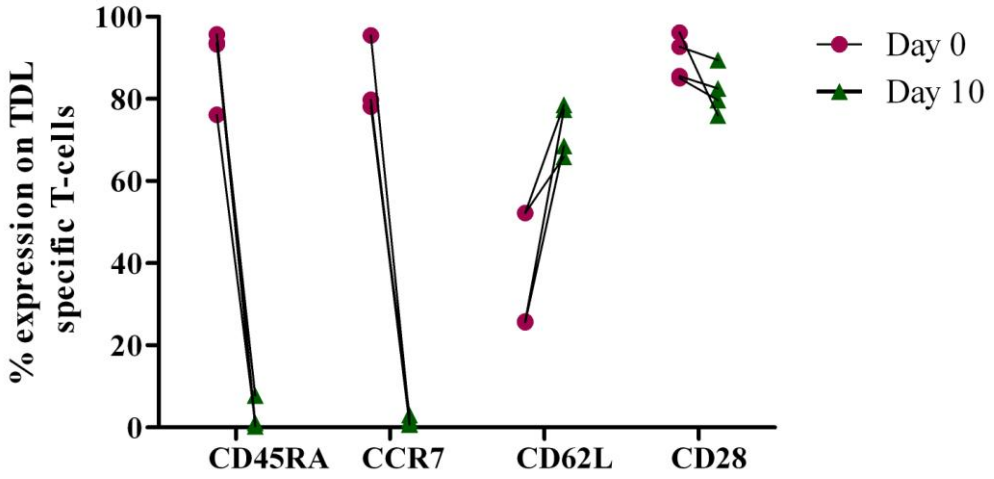

Figure 4-16 TDL-specific T-cells switch phenotype on proliferation

PBMCs (n=4, LD2, LD4, LD5 and LD13) were CFSE labelled, stimulated with TDL peptide or irrelevant peptide (10µg/ml) and maintained in T-cell medium. Cells were labelled with antibodies to CD3, CD8, CD45RA, CCR7, CD62L and CD28, TDL tetramer and PI. The figure compares the frequencies of expression of CD45RA, CCR7, CD62L and CD28, on proliferating TDL tetramer staining T-cells (PI-, CD8+, TDL tetramer +and CFSE-ve), on days 0 and 10.

4.3.1.2 *TDL epitope-specific T-cells recognise multiple adenovirus serotypes*

Ad infection and disease in HSCT recipients are usually not due to the more commonly occurring serotypes (section 1.4.7.1). Thus a therapeutic strategy that is effective against a broad range of serotypes is desirable. Sequence comparisons of the TDL epitope across different serotypes have shown minor variations suggesting the possibility of cross reactivity across species. To study this matter further Ad 3, 5, 11, 12, 19a and Ad40 viruses were obtained. These viruses belong to B1, C, B2, A, D and F species respectively and are responsible for the majority of infections in HSCT recipients.

TDL peptide stimulated CTLs were maintained in T-cell medium and 100U/l IL-2 until TDL tetramer staining T-cells accounted for >50% of the culture. Autologous and mismatched LCLs were infected with Ad 3, 5, 11, 12, 19a or 40 (100pfu (plaque forming units)/cell) or mock at 37°C overnight. The following day these infected LCLs were loaded with Cr^{51} and

used as targets in chromium release assays (section 2.5.5). Targets were incubated for 16hr at 37°C with TDL peptide stimulated T-cells (effectors) at two effector: target ratios (10:1 and 5:1). The supernatants were sampled and γ-emission quantified using the Packard Cobra gamma counter. Figure 4-17 shows the results for LD2 after 4 weeks culture; the percentage of TDL tetramer staining T-cells prior to the assay was 62%. TDL-specific T-cells demonstrated cytotoxicity to LCLs infected by serotypes from all species. The experiment was repeated on the same donor (data not shown) with similar results. There is only a single aminoacid variation between the peptide sequences of the different serotypes and based on the SYFPEITHI scoring system (Rammensee et al, 1999) this results in minimal changes in the predictive binding of the epitope (Table 4-1). This experiment also highlights the potential of these T-cells to clear infections of a different serotype (other than Ad5) in a HSCT recipient following adoptive transfer.

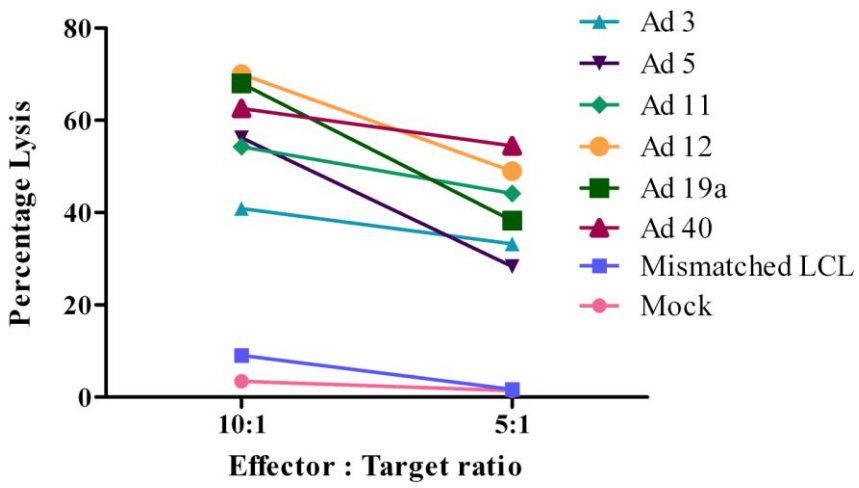

Figure 4-17 TDL peptide CTLs recognise multiple adenovirus serotypes

TDL peptide stimulated CTLs (LD2) maintained in T-cell medium supplemented with IL-2 (100U/l) and had 62% TDL tetramer staining T-cells prior to this assay. Autologous or mismatched LCLs were infected with Ad 3, 5, 11, 12, 19a or 40 (100pfu/cell) and mock at 37°C overnight (targets). The following day infected LCLs are loaded with Cr51 and used as targets in a chromium release assay. Targets are incubated for 16hr at 37°C with TDL peptide or mock stimulated CTLs (effectors) at effector: target ratios (10:1 and 5:1). The supernatants were sampled and γ- emission quantified using the Packard Cobra gamma counter. Results are expressed as percentage lysis of targets lysed with SDS (total release) compared to spontaneous release from targets incubated without effectors.

Serotype	Species	Position	Sequence	SYFPEITHI Score
5	C	886-894	TDLGQNLLY	20
3	B1	878-886	TDLGQN**M**LY	19
11	B2	881-890	TDLGQN**M**LY	19
12	A	853-861	TDLGQN**M**LY	19
19a	D	885-893	TDLGQN**M**LY	19
40	F	857-865	TDLGQN**M**LY	19

Table 4-1 Conservation of TDL epitope within human Ad hexon sequences

The hexon sequences of the respective serotypes available from NCBI were aligned. Variation in the peptide sequence is highlighted in red. The SYFPEITHI scores were obtained from http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm

In summary, TDL-specific T-cells are able to recognise antigen presenting cells that have either been loaded with peptide or that have been infected with heat inactivated virus. They have a high proliferative potential; 43 and 98 fold in response to virus and peptide respectively over 7 days in the absence of IL-2 (Figure 4-9). The tetramer staining cells have a minimally differentiated central memory phenotype, $CD45RA^{high}$, $CD45RO^{high}$, $CCR7^{high}$, $CD62L^{low}$, $CD27^{high}$, $CD28^{high}$, LFA-1 ^{high} and $CD57^{low}$ (Figure 4-14). The cells switch their phenotype on proliferation in response to antigen stimulation ($CD45RA^{low}$, CCR7 ^{low}, $CD62L^{intermediate}$, $CD28^{high}$) (Figure 4-16). The cells retain their costimulatory receptor molecules, on proliferation thereby maintaining their potential to clear virus. TDL-specific T-cells demonstrate significant cytotoxicity to the six serotypes tested, which predominantly affect the HSCT recipients.

4.3.2 Characterisation of Ad-specific T-cells enriched by CSS

Ad-specific T-cells enriched by CSS are capable of antigen recognition as they are selected based on cytokine release secondary to antigen recognition. For immunotherapeutic purposes knowledge of the phenotypic and proliferative properties of these cells is valuable. In this section experiments characterising the cells selected by CSS will be described.

4.3.2.1 *Ad-specific T-cells have high proliferative capacity*

PBMCs were CFSE labelled and left uninfected (mock) or stimulated with heat inactivated CTL102 (2.5 x10^3particles/ cell) or SEB (10μg/ml) and maintained in T-cell medium. Cells were counted on days 3, 7 and 10 using a haemocytometer. Percentages of Ad-specific CD4/8T-cells on all 6 donors were determined by flow cytometry after labelling with PI, anti CD4 and anti-CD8 antibodies. Figure 4-18:A shows FACS plots on CFSE–ve Ad-specific T-cells of LD4 on day 7 and Figure 4-18: B shows histograms of LD4 on days 3, 7 and 10 respectively comparing PI-, CD4 /CD8+ mock, SEB and CTL102 exposed cell lines. Ad-specific CD4-T-cells proliferated predominantly in comparison to CD8T-cells. The proliferation though above background, is lower than that seen in the SEB stimulated positive control sample.

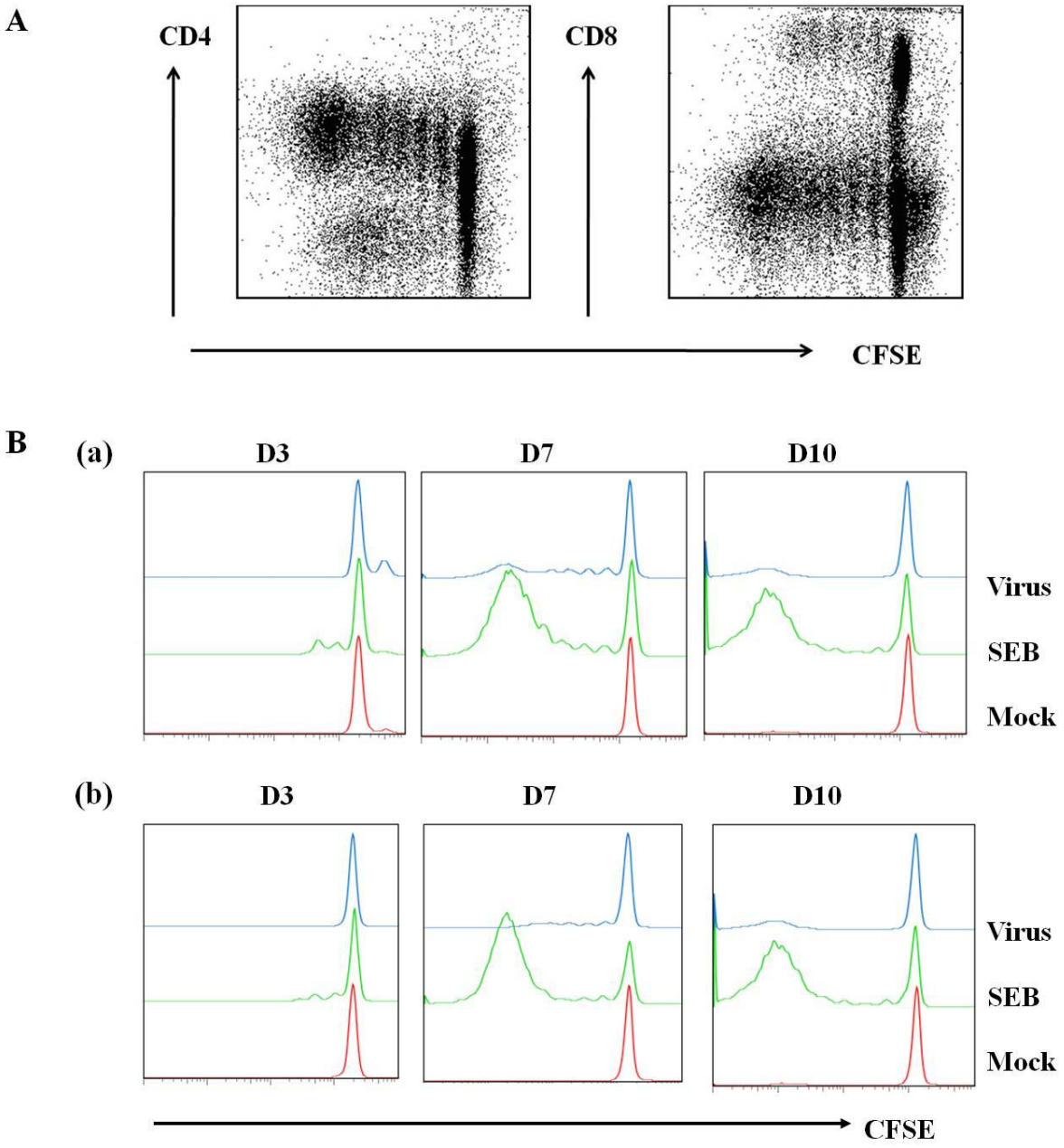

Figure 4-18 CFSE proliferation of virus stimulated T-cells

PBMCs (LD4) were CFSE labelled and not stimulated (mock) or stimulated with heat inactivated CTL102 ($2.5x10^4$particles/ cell) or SEB (10µg/ml) and maintained in T-cell medium. Percentages of virus-specific T-cells were determined by flow cytometry after labelling with PI, anti-CD4 and anti-CD8 antibodies. A: FACS plots showing CFSE-ve CD4 and CD8 Ad-specific T-cells on day 7. B: histograms days 3, 7 and 10 respectively comparing Mock, SEB and CTL102 stimulated cell lines. (a) shows the PI-ve, CD4 and (b) the PI-ve, CD8 Ad-specific T-cells.

The number of proliferating cells at each time point was calculated by multiplying the percentage of PI-, CD3+, CD4/8+, CFSE-ve cells determined by flow cytometry with the total cell count. The number of virus-specific CD4/8 T-cells on day 0 was calculated from the percentage of IFN-γ secreting T-cells determined by CCS assay. Fold proliferation was obtained by dividing the number of proliferating T-cells by the Ad-specific T-cells on day 0. Figure 4-19 shows the fold proliferation of CD4 and CD8 T-cells in six donors. The mean fold proliferation ± SD on days 3 and 7 are 52.67±17.65 and 325.67±52.97 respectively for CD4 T-cells and 57.5±23.65 and 114.5±30.72 fold respectively for CD8 T-cells. Figure 4-20 compares the mean values for mock, SEB and virus stimulated CD4 and CD8 proliferating T-cells of six donors. In all 6 donors CD4 and CD8 T-cells proliferated, though CD4T-cells were predominant. The mean fold proliferation on day 7 of the CD4 T-cells was 325 in comparison to 114 fold proliferation of the CD8 T-cells. Virus stimulated T-cell proliferation was above back ground proliferation of the unstimulated cells though lower than SEB stimulated T-cells. On 2 way Anova analysis the differences were statistically significant: results shown in Table 4-2.

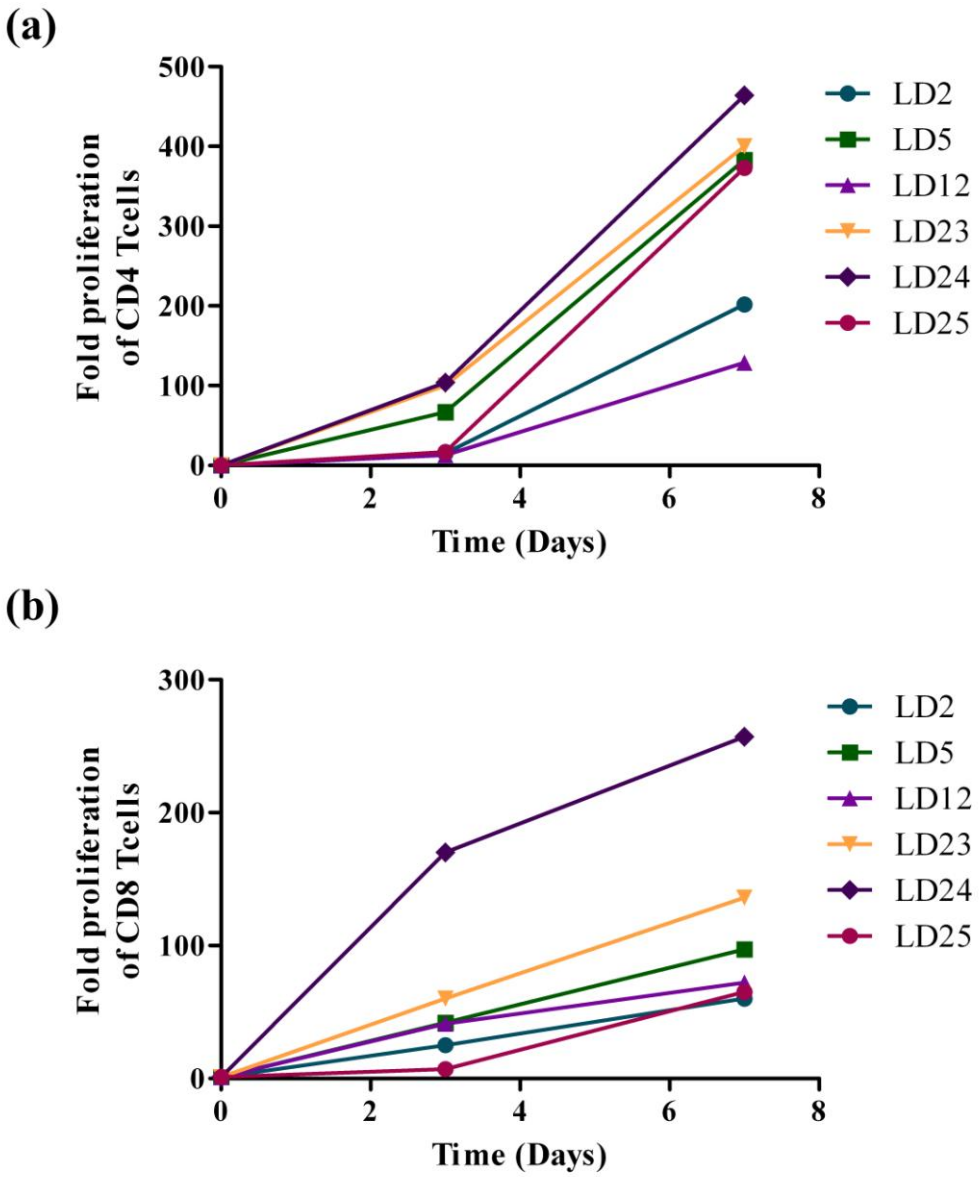

Figure 4-19 Fold proliferation of virus stimulated T-cells

PBMCs from 6 lab donors were labelled with CFSE and not stimulated (mock) or stimulated with heat inactivated CTL102 ($2.5x10^3$ particles/cell) or SEB (10µg/ml) and maintained in T-cell medium. Percentage of PI-, CD3+, CD4/8+, CFSE-ve cells was determined for days 3 and 7 by flow cytometry. The number of proliferating cells was then calculated from the total cell count at each time point. The number of virus-specific T-cells on day 0 was calculated from the percentage of IFN-γ secreting T-cells determined by CCS. Fold proliferation was obtained by dividing the number of proliferating T-cells by the Ad-specific T-cells on day 0.

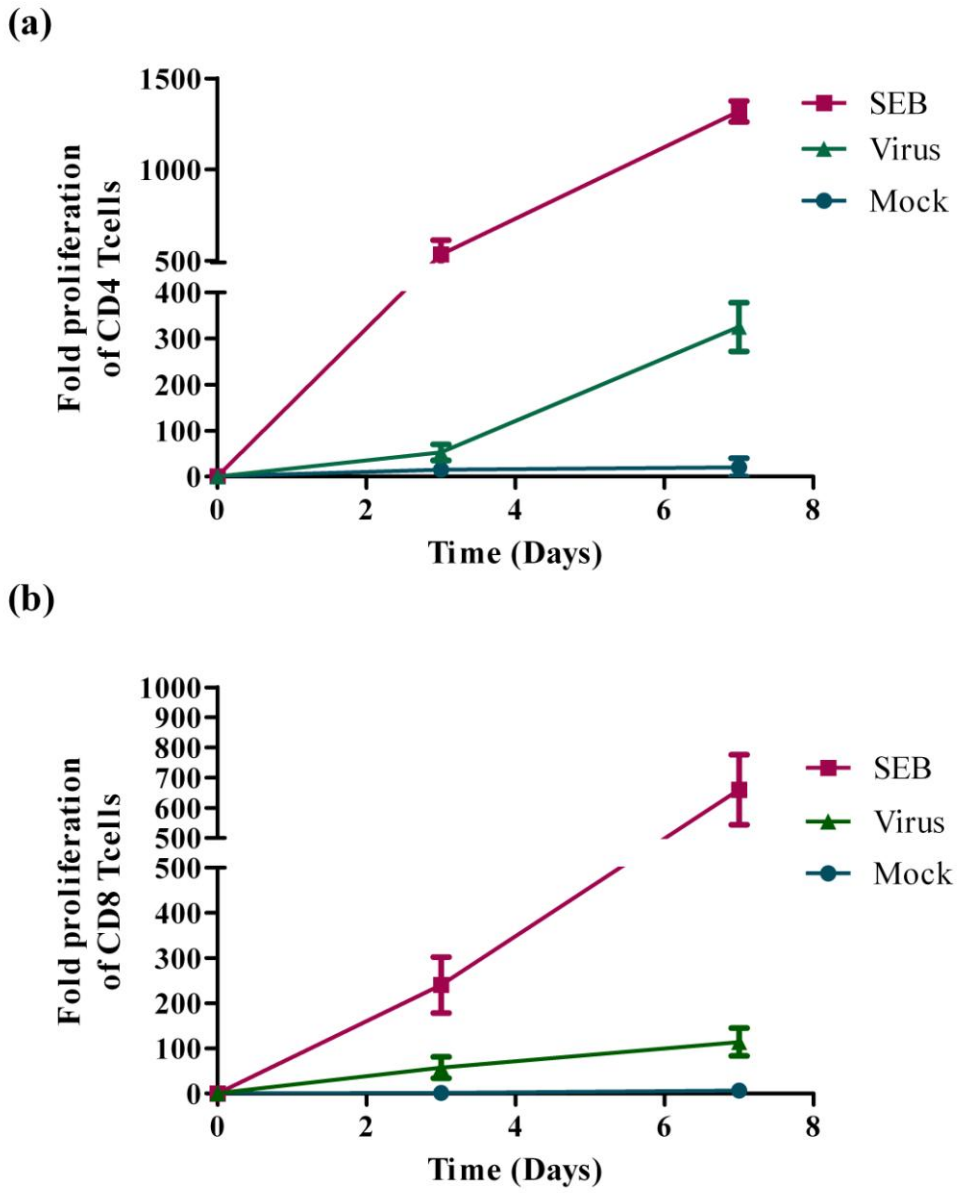

Figure 4-20 Fold proliferation of virus stimulated T-cells

PBMCs of 6 lab donors were labelled with CFSE and not stimulated (mock) or stimulated with heat inactivated CTL102 ($2.5x10^3$ particles/cell) or SEB (10µg/ml) and maintained in T-cell medium. Percentage of PI-ve, CD3+ve, CD4/8+ve, CFSE-ve cells was determined for days 3 and 7 by flow cytometry. The number of virus-specific T-cells on day 0 was calculated from the percentage of IFN-γ secreting T-cells determined by CCS. Fold proliferation was obtained by dividing the number of proliferating T-cells by the Ad-specific T-cells on day 0. Fold proliferation of (a) CD4 T-cells and (b) CD8 T-cells following infection with CTL102, mock or SEB. Error bars represent one SD.

		Virus v Mock	Virus v SEB
CD4 T-cells	Day 0	p>0.05	p>0.05
	Day 3	p>0.05	p<0.001
	Day 7	p<0.001	p<0.001
CD8 T-cells	Day 0	p>0.05	p>0.05
	Day 3	p>0.05	p<0.05
	Day 7	p>0.05	p<0.001

Table 4-2 Statistical significance of fold proliferation

Fold proliferation of mock, SEB and virus stimulated samples on six donors showed statistical significance by 2-way Anova analysis. The results of Bonferroni post-test shown and significant results highlighted.

4.3.2.2 *Ad-specific T-cells have a minimally differentiated memory phenotype*

Characterisation of the phenotype provides valuable information with regards to the homing potential as well as their long term ability to form a functional memory compartment. Ad-specific T-cells enriched by CSS were characterised for the expression of CD45RA, CCR7, CD27 and CD28. PBMCs (10^7) were exposed to heat inactivated CTL102, SEB or mock infected and Ad-specific T-cells enriched by the CSS system and AutoMACS technology (section 2.5.2.3.). Enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CCR7, CD27, CD28 antibodies or PI and analysed by flow cytometry (DakoCyAN). Figure 4-21 shows the expression of CD45RA, CCR7, CD27 and CD28 on PI-ve, CD4 (a) or CD8 (b) +ve T-cells for LD2 and Figure 4-22 shows the collated data on 6 donors.

Analysis showed that the Ad-specific CD4 and CD8 T-cells have a $CD45RA^{high}$, $CCR7^{high}$, $CD27^{high}$ and $CD28^{high}$ phenotype similar to that of TDL-specific CD8 T-cells. As these cells were enriched based on their antigen recognition ability it was unlikely that circulating naive cells were analysed, implying that they are non-naive. 2 way Anova analysis on the collated data showed statistically significance and a Bonferroni post test was performed. The differences between CD45RA ($p<0.001$) and CCR7 ($P<0.01$) expression on virus versus mock stimulated CD4 and CD8T-cells achieved statistical significance. CD45RA ($p<0.001$), CCR7 ($p<0.01$) and CD28 ($P<0.001$) expression on virus versus SEB stimulated CD4 T-cells was statistically significant. With respect to CD8 T-cells CCR7 ($p<0.01$), CD27 ($p<0.01$) and CD28 ($p<0.01$) expressions on virus versus mock stimulated CD8 T-cells achieved statistical significance. For virus versus SEB stimulated T-cells expressions of CD45RA, CCR7, CD27 and CD28 on CD4 and CD8 T-cells were statistically significant ($p<0.001$) by Bonferroni post test.

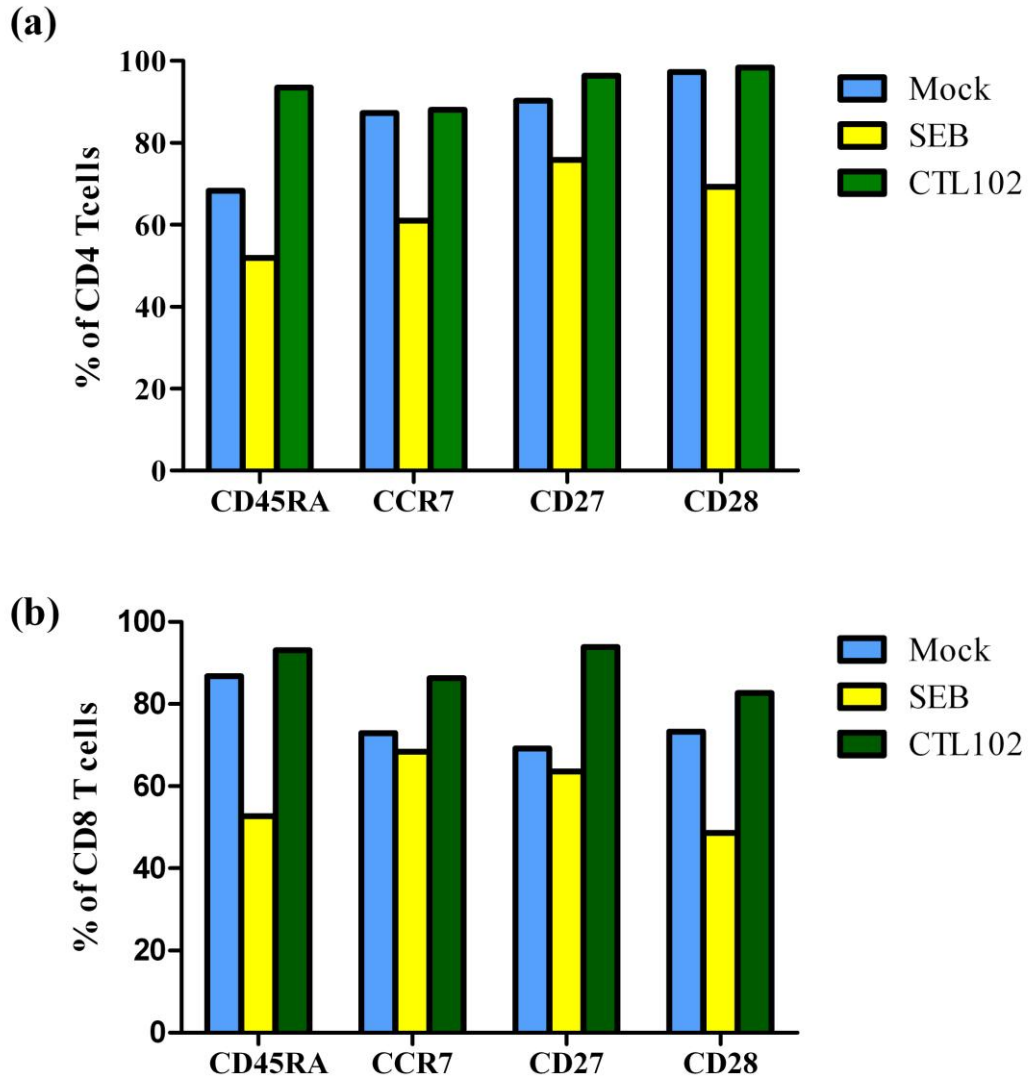

Figure 4-21 Ad-specific T-cell initial phenotype

PBMCs (10^7) LD2 were treated with heat inactivated CTL102, SEB or mock infected and Ad-specific T-cells enriched by the Miltenyi CSS system and AutoMACS technology (section 2.5.2.3.). Enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CCR7, CD27, CD28 antibodies or PI and analysed by flow cytometry (DakoCyAN, Beckman Coulter). The expression of CD45RA, CCR7, CD27 and CD28 on PI-, CD4 (a) or CD8 (b) +T-cells was determined.

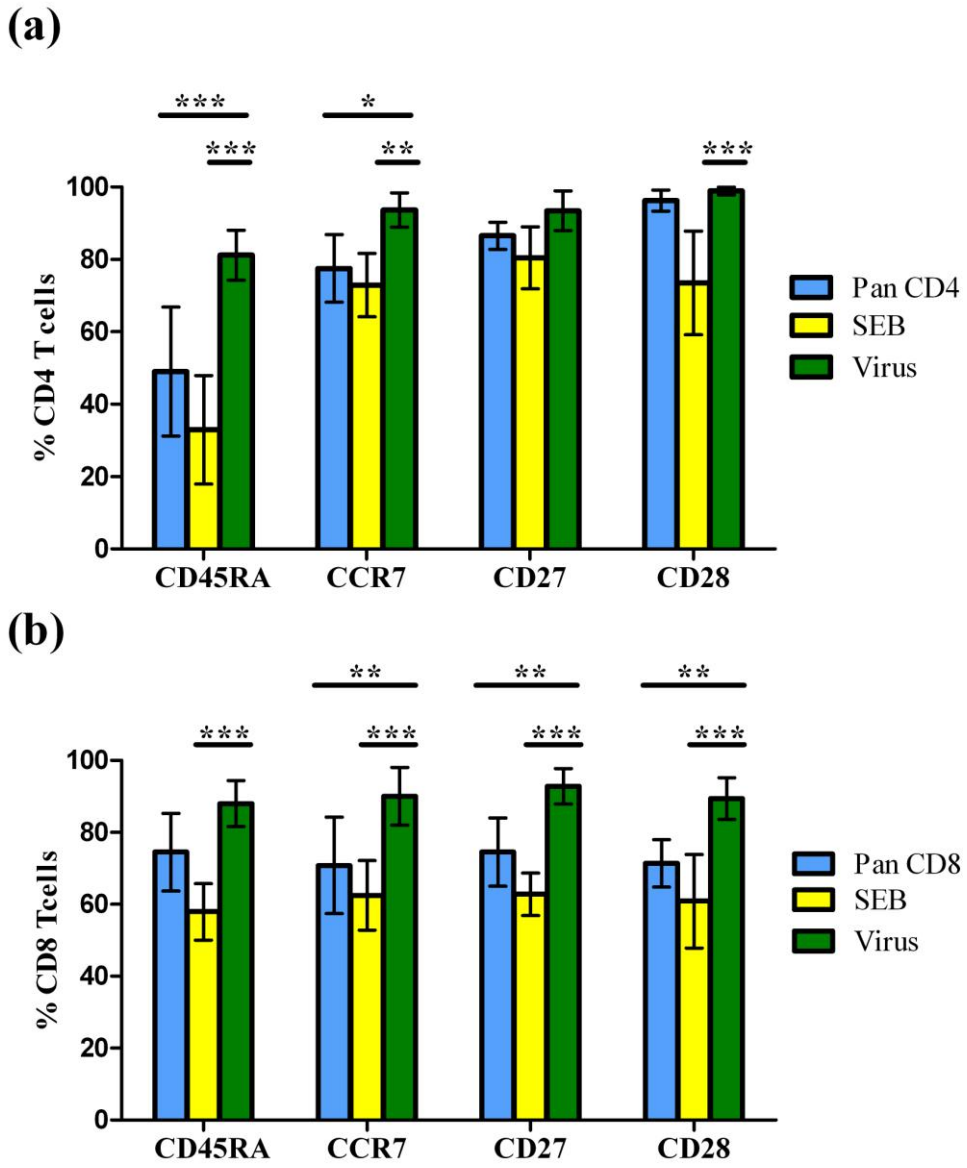

Figure 4-22 Ad-specific T-cells have minimally differentiated phenotype

PBMCs (10^7) on 5 lab donors were treated with heat inactivated CTL102, SEB or mock infected and Ad specific T-cells enriched by the Miltenyi CSS system and AutoMACS technology (section 2.5.2.3.). Enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CCR7, CD27, CD28 antibodies or PI and analysed by flow cytometry (DakoCyAN). The expression of CD45RA, CCR7, CD27 and CD28 on PI-, CD4 (a) or CD8 (b) + T-cells is shown. Bonferroni post test results that showed statistical significance are shown- *** $p<0.001$, **$P<0.01$

The panel was extended to include CD45RO, CD62L, CD57 and LFA-1. These parameters will help ascertain the degree of differentiation of these T-cells (section 4.1). Ad-specific T-cells were enriched following stimulation with CTL102 by CSS from a starting population of $2x10^7$ PBMCs of (n=10) healthy donors. All enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, CD57, LFA-1 or PI and analysed by flow cytometry on LSR II. The PI-, CD3+, CD4/CD8+ve T-cells were determined and the expression of each molecule on these cells ascertained. The total PI-, CD4/ CD8 T-cells in the uninfected sample (negative control) and the PI-, CD3+, CD4/ CD8+ SEB-specific T-cells releasing IFN-γ in the SEB stimulated sample (positive control) were analysed for the same parameters. Figure 4-23 and Figure 4-24 show the mean percentage expression of CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, LFA-1 and CD57 expression in mock, virus and SEB stimulated CD4 and CD8 T-cells respectively. The CD4 and CD8 T-cells showed $CD45RA^{high}$, $CD45RO^{high}$, $CCR7^{high}$, $CD62L^{low}$, $CD27^{high}$, $CD28^{high}$, $LFA\text{-}1^{high}$ and $CD57^{low}$; a minimally differentiated central memory phenotype similar to that observed with TDL-specific T-cells.

2 way Anova analysis showed statistically significant ($p=<0.001$) difference on the collated data which on Bonferroni post test analysis was between Ad-specific and SEB-specific CD4 T-cells (CD45RA, CCR7 and CD62L and CD28 expression) and CD45RA, CD62L and CD28 on CD8 T-cells.

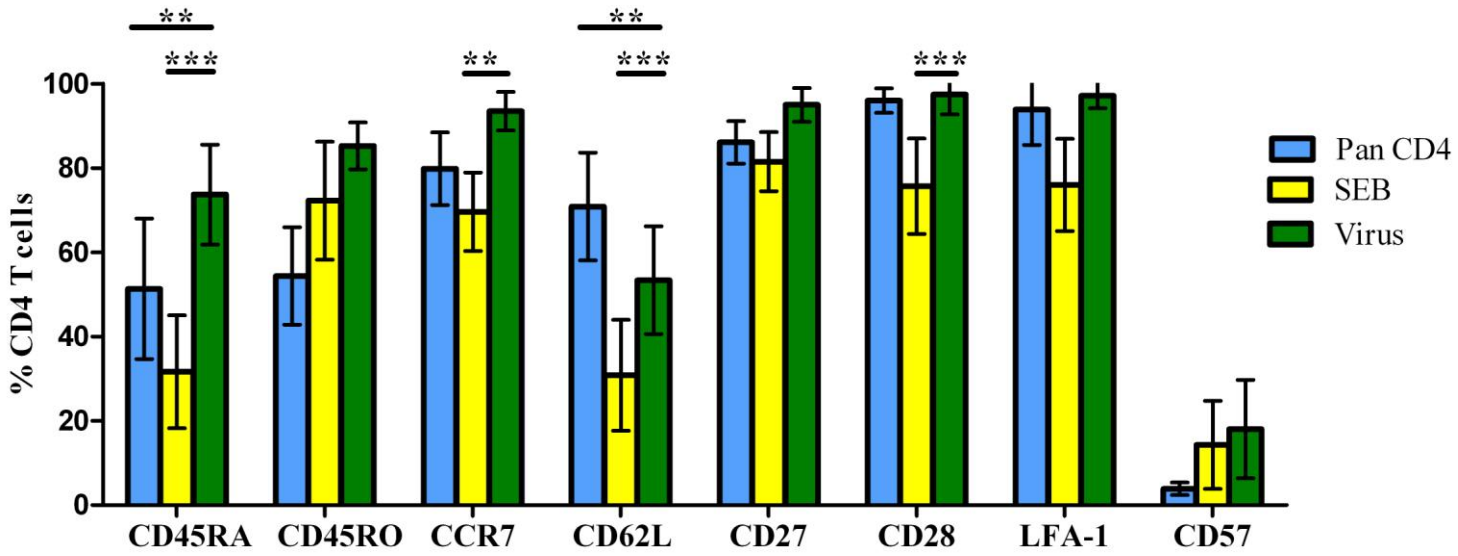

Figure 4-23 Ad-specific CD4 T-cells have a minimally differentiated phenotype

Ad-specific T-cells were enriched from a starting population of $2x10^7$ PBMCs of (n=10) healthy donors following stimulation with CTL102 by CSS. All enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, CD57 and LFA-1 or PI and analysed on LSR II. The PI-, CD3+, CD4/CD8+ T-cells were first determined and the expression of each receptor on these cells was then ascertained. The total PI-ve CD4/ CD8 T-cells in the uninfected sample, negative control, and the PI-, CD3+, CD4/ CD8+ SEB-specific T-cells releasing IFN-γ in the SEB infected sample, positive control, were analysed similarly. Mean percentage expression of CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, LFA-1 and CD57 receptors on the mock, virus or SEB stimulated total, Ad-specific and SEB-specific CD4 T-cells shown. The error bars represent the standard deviation.

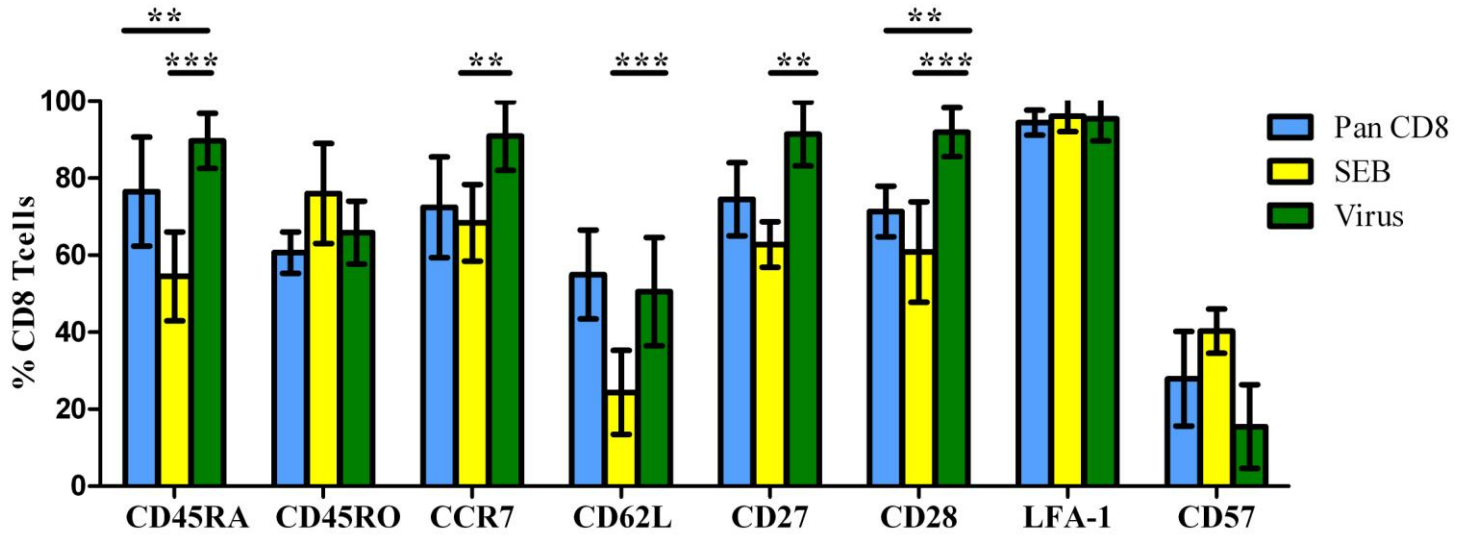

Figure 4-24 Ad-specific CD8 T-cells have a minimally differentiated phenotype

Ad-specific T-cells were enriched from a starting population of $2x10^7$ PBMCs of (n=10) healthy donors following stimulation with CTL102 by CSS. All enriched cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, CD57 and LFA-1 or PI and analysed on LSR II. The PI-, CD3+, CD4/CD8+ T-cells were first determined and the expression of each receptor on these cells was then ascertained. The total PI-ve CD4/ CD8 T-cells in the uninfected sample, negative control, and the PI-, CD3+, CD4/ CD8+ SEB-specific T-cells releasing IFN-γ in the SEB infected sample, positive control, were analysed similarly. Mean percentage expression of CD45RA, CD45RO, CCR7, CD62L, CD27, CD28, LFA-1 and CD57 receptors on the mock, virus or SEB stimulated total, Ad-specific and SEB-specific CD8 T-cells shown. The error bars represent the standard deviation.

4.3.2.1 *Ad-specific T-cells retain effector phenotype on proliferation*

In order to study the changes in the phenotypic characteristics following antigen stimulation, PBMCs from four donors (LD 2, 4, 5, and 13) were CFSE labelled, stimulated with virus or mock and maintained in T-cell medium. Cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CCR7, CD62L and CD28, TDL tetramer, PI, and analysed on day 0 and day 10 by flow cytometry. On day 10 the proliferating (CFSE-ve) CD4 and CD8 T-cells switch the day 0 phenotype (Figure 4-25 and Figure 4-26). Expression levels of CD45RA, CCR7 decrease, CD62L increased to intermediate and CD28 remained high. Figure 4-25 and Figure 4-26 show CD45RA, CCR7, CD62L and CD28 expressions on Ad-specific CD4 and CD8 T-cells on day 10 (LD4), Figure 4-27 the collated results of 4 donors (LD 2, 4, 5, 13) for CD4 T-cells (CD8 T-cells not shown). These experiments confirm the early minimally differentiated phenotype of the Ad-specific T-cells. Persistent high expression of costimulatory receptor molecule (CD28) highlights that they retain their effector capabilities following antigen stimulation.

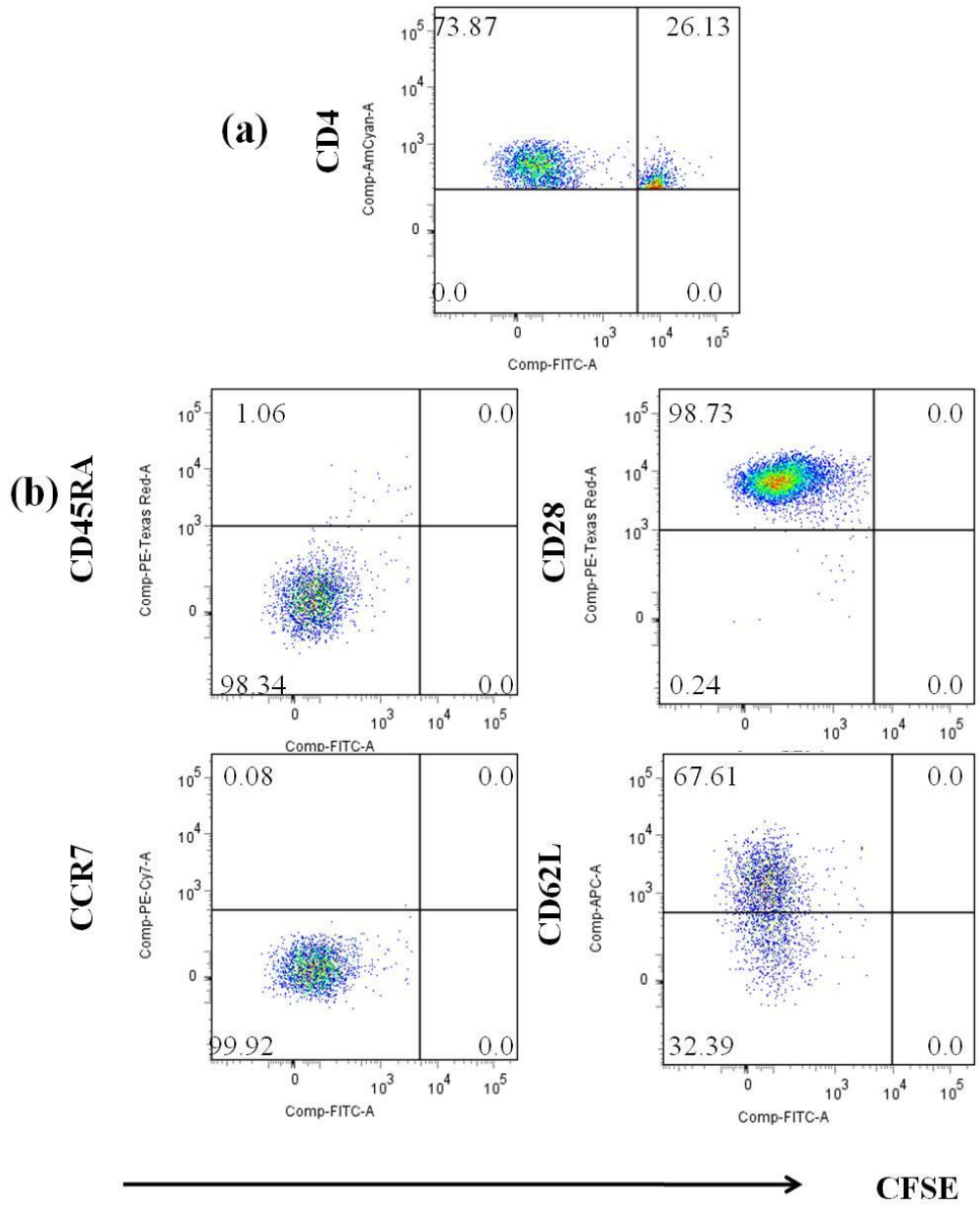

Figure 4-25 Phenotype switch of virus stimulated CD4 T-cells

PBMCs (LD2) were CFSE labelled, treated with CTL102 and maintained in T-cell medium. Cells were labelled with antibodies to CD3, CD4, CD45RA, CCR7, CD62L and CD28 or PI on day 10. (a) CFSE-ve CD4 Tcells on day 10 gated on PI, CD3 and CD4. (b) Expressions of CD45RA, CCR7, CD62L and CD28 on CFSE-ve CD4 T-cells (73.87% of total CD4 T-cells as shown in (a)).

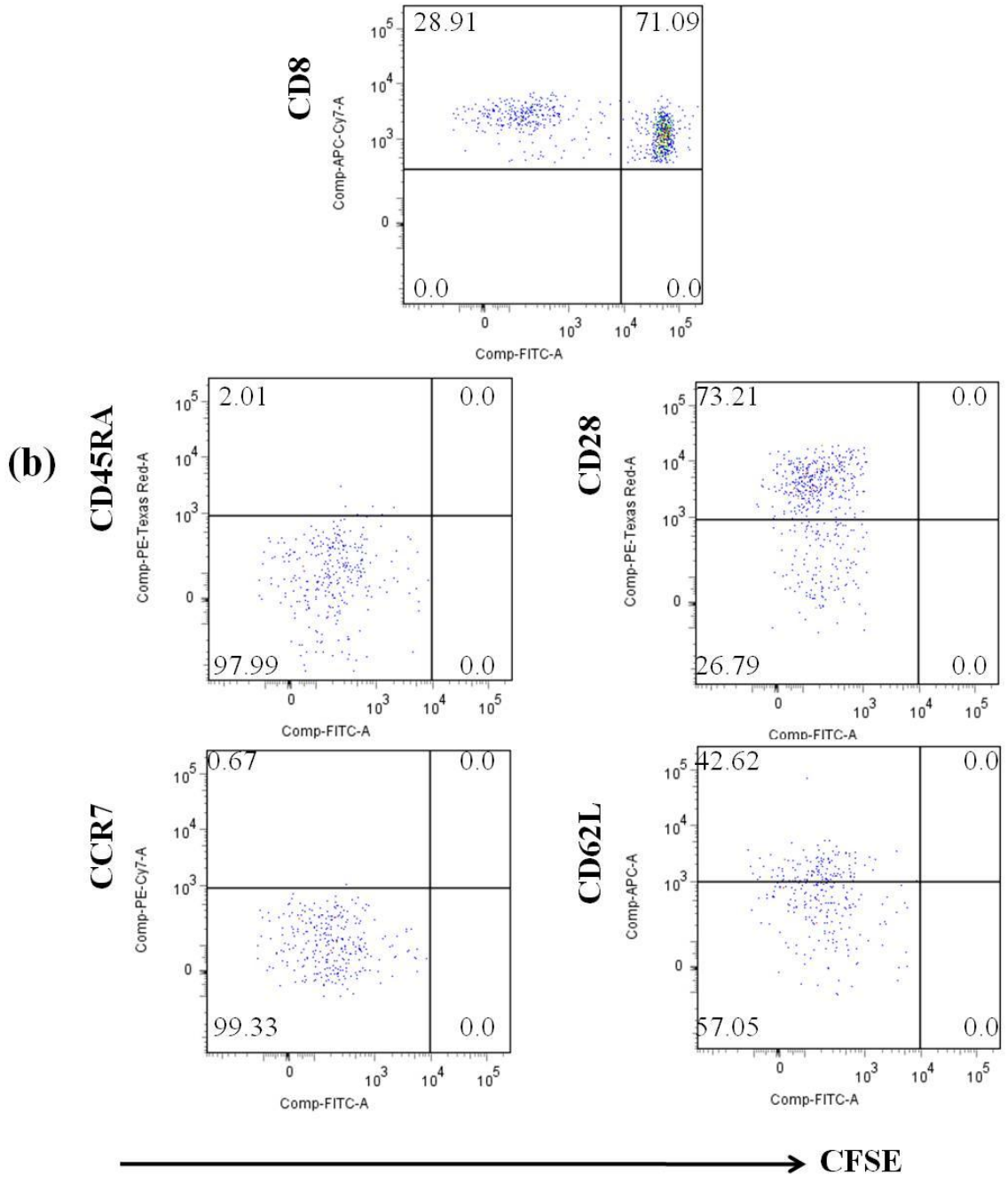

Figure 4-26 Phenotype switch of virus stimulated CD8 T-cells

PBMCs were CFSE labelled, treated with CTL102 and maintained in T-cell medium. Cells were labelled with antibodies to CD3, CD8, CD45RA, CCR7, CD62L and CD28 or PI on day 10. (a) CFSE-ve CD8 Tcells on day 10 after gating on PI, CD3 and CD8. (b) Expressions of CD45RA, CCR7, CD62L and CD28 on CFSE-ve CD8 T-cells (28.91% of total CD4 T-cells as shown in (a)).

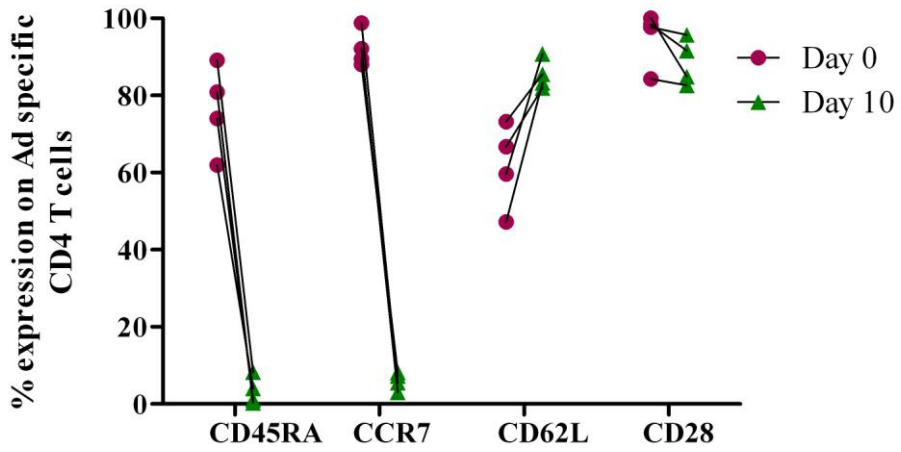

Figure 4-27 Change in phenotype of proliferating CD4 T-cells

PBMCs four donors (LD2, LD4, LD5 and LD13) were CFSE labelled, stimulated with CTL102 or mock and maintained in T-cell medium. Cells were labelled with antibodies to CD3, CD4, CD45RA, CCR7, CD62L and CD28 or PI. The figure compares the frequencies of expression of CD45RA, CCR7, CD62L and CD28, on proliferating CD4 Ad-specific T-cells (PI-, CD4+, TDL tetramer +and CFSE-ve), on days 0 and 10.

4.3.2.2 *Ad-specific T-cells can recognise multiple adenovirus serotypes*

T-cell clone isolation allows detailed analysis of antigen and epitope targets of the T-cell. These can then be linked with the HLA restriction, phenotype and functional characteristics of the T-cell. To increase the chance of cloning an Ad specific T-cell, polyclonal lines were first established. PBMCs from healthy volunteers were stimulated with CTL102 or TDL peptide and plated out at $1x10^6$ cells/ml. Medium was refreshed twice a week with a half medium change. After 7 days the medium was supplemented with IL-2 (50 IU/ml). Cultures were maintained for up to 28 days and samples were tested on day 14, 21 or 28.

During culture of a polyclonal line, only T-cells that are stimulated receive sufficient signals to survive and proliferate. Hence as the polyclonal T-cell line becomes older, it should contain higher frequencies of Ad-specific T-cell clones. To increase the frequency of Ad-specific T-cells, polyclonal T-cell lines were enriched using CSS or tetramer and used for cloning by limiting dilution (section 2.6.2).Unfortunately clones generated were shown not be Ad-specific on screening by IFN-γ Elisa. After 6 attempts polyclonal T-cell lines were used without enrichment by tetramer or CSS. Out of 55 clones generated two (clone 5 and 9) were shown to be Ad-specific by IFN-γ Elisa and CD4 T-cells by flow cytometry. I was unsuccessful in generating Ad-specific or peptide-specific CD8 T-cell clones despite 9 attempts.

Clones 5 and 9 were analysed by measuring IFN- γ release in response to autologous LCLs infected with different serotypes of Ad. Clone 5 recognised Ad4 and Ad5 (Figure 4-28a) Clone 9 recognised Ad5 (Figure 4-28b). Both the clones showed some recognition of Ad19 and Ad40. Surprisingly neither recognised Ad5 hexon nor Ad 5 hexon, penton base and fibre (Figure 4-29 a and b). Clone5 failed to recognise any known hexon derived epitope whereas clone 9 recognised QWS epitope. No IFN-γ was detected from APC's or the clones on their

own. The IFN-γ release in these experiments was too low to interpret accurately and caution should be used in interpreting these data. In chromium release assays both clones demonstrated cytotoxicity to Ad5 and Ad11 loaded autologous LCL's in 2 separate experiments (Figure 4-30).

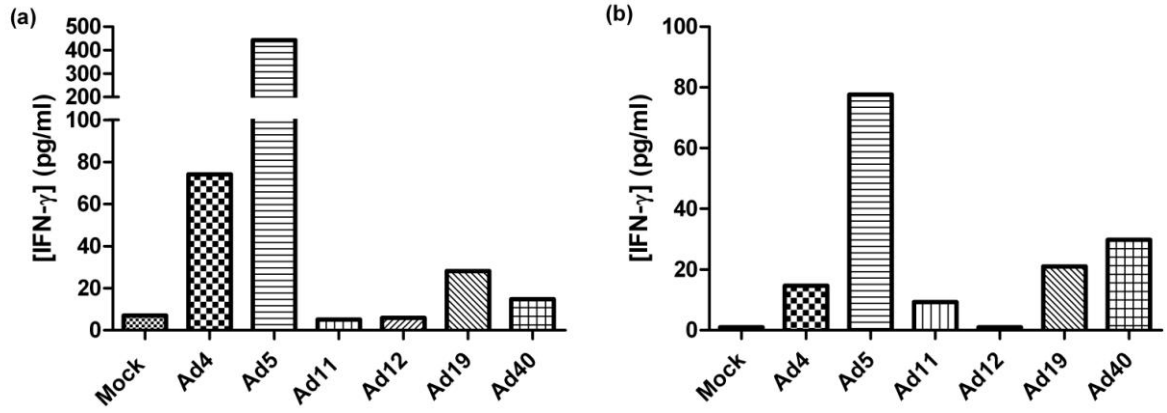

Figure 4-28 Clone 5 and Clone 9 recognition of different species

Autologous LCLs were infected with mock, Ad4, Ad5, Ad 11, Ad12, Ad19a and Ad40 (100pfu/cell) for 1½ hours, washed and incubated at 37°C overnight. These were then used as APCs ($5x10^4$/well) in an IFN-γ Elisa ($5x10^3$T-cell clones/well). (a) Clone 5 (b) Clone 9

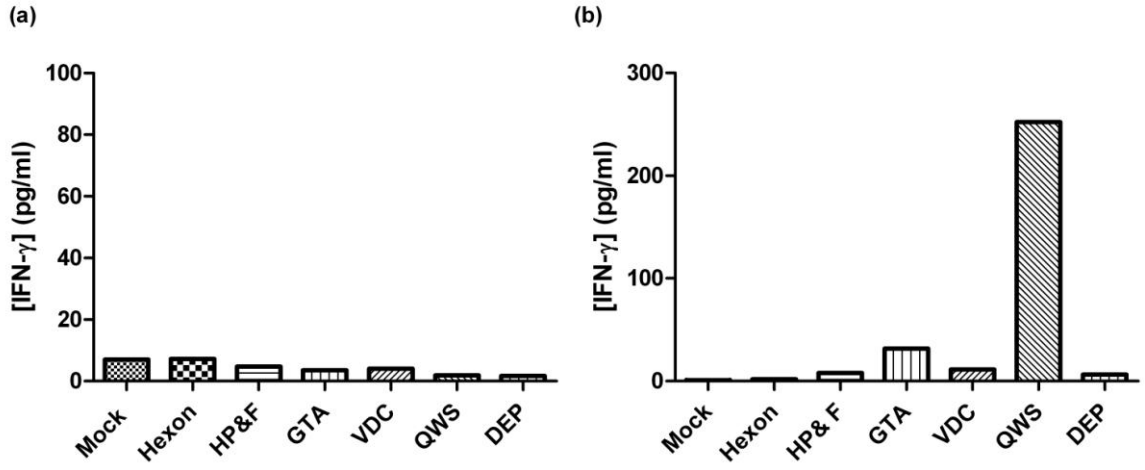

Figure 4-29 Clone 5 and 9 recognition of hexon and known hexon derived epitopes

Autologous LCLs were loaded with hexon (5μg/10^6cells), hexon, penton base and fibre (HP&F) (5μg/10^6cells), GTA, VDC, QWS or DEP peptide (5μg) and equivalent DMSO for 3 hours and washed. These were then used as APCs ($5x10^4$/well) in an IFN-γ Elisa ($5x10^3$T-cell clones/well). (a) Clone 5 (b) Clone 9

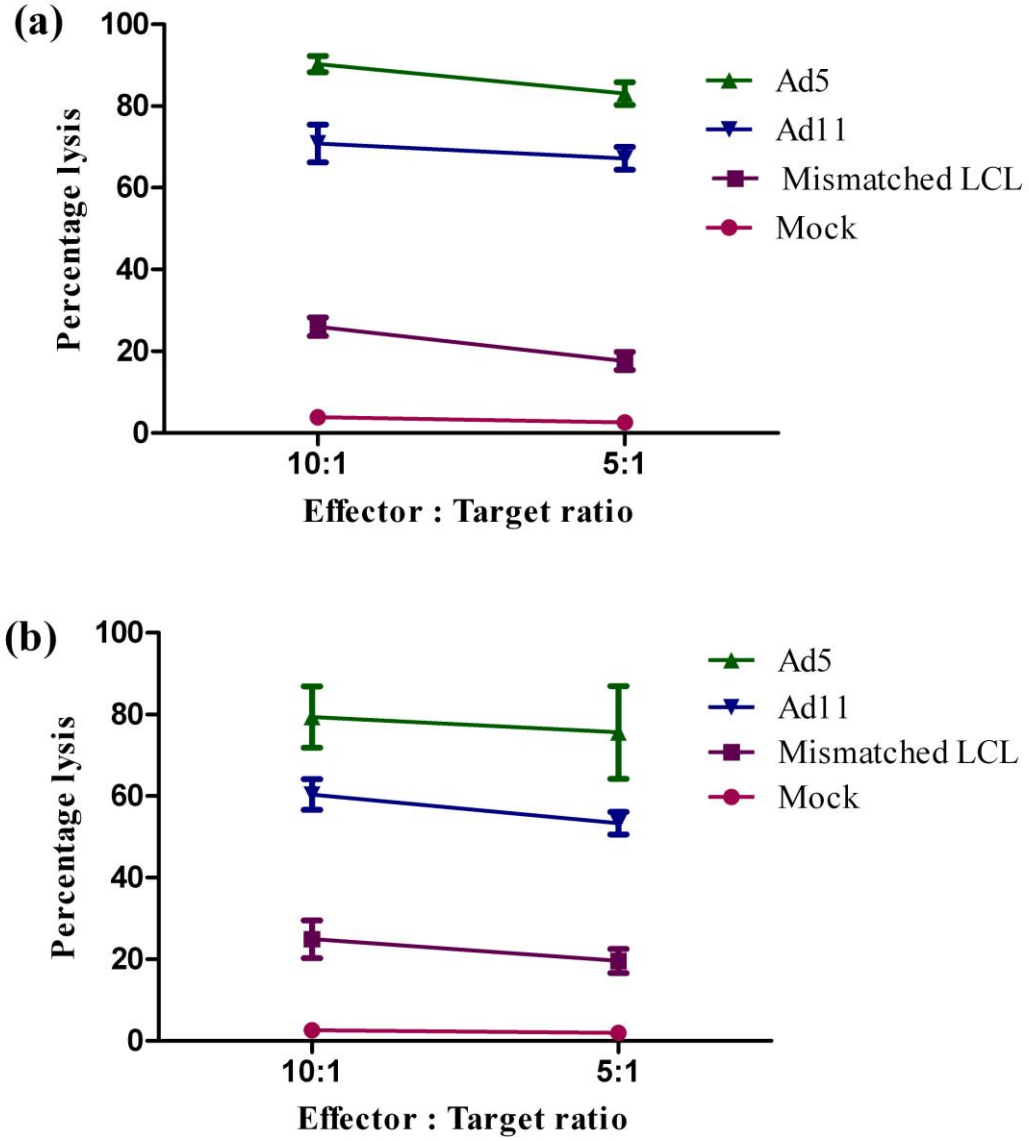

Figure 4-30 Ad-specific T-cell clones are cross reactive across species

Mock, Ad5 or Ad11 infected autologous LCLs (37°C, 90min) were incubated (37°C, overnight). LCLs were loaded with chromate and used as targets in a Cr^{51} release assay. Targets were incubated in triplicate (37°C, 16hr) with the T-cell clones (effector) cells at two different effector to target ratios. Supernatants were sampled and γ- emission quantified using T Packard Cobra gamma counter. Results were expressed as percentage lysis of targets as described in 2.5.5. (a) Clone 5 (b) Clone 9

4.3.3 Ad- specific T-cells can limit virus replication

Ad specific T-cells identified by tetramer or CCS have high proliferative potential, a minimal differentiated phenotype, recognise antigen and are cytotoxic to target antigen loaded APCs. For the adoptive transfer of enriched Ad-specific T-cells, it is of importance to see if they can control or limit virus replication. Not only are B-cells refractory to Ad virus entry but also Ad replication in the presence of EBV can affect the virus-specific T-cell response and alter the usual course of Ad infection (Lavery et al, 1987). Thus fibroblasts and not LCLs were considered as suitable antigen presenting cells for this experiment. They allow adenovirus replication following infection and IFN- γ treatment of fibroblasts results in up-regulation of class I and II MHC molecules, thereby increasing their antigen presenting ability (Umetsu et al, 1986). Enriched antigen-specific T-cells were added to infected fibroblasts and their ability to control virus replication assessed by quantifying the viral copy number using real time QPCR (RT-QPCR).

In order to determine a suitable multiplicity of infection (MOI), primary human fibroblasts were plated out into 96 well plates ($5x10^3$cells/well) in T-cell medium supplemented with IFN-γ (200 IU/ml) and after 72 hours infected with Ad–GFP (see section 2.3.1) at different MOI - 30, 300 and 3000 particles/cell. Virus was washed off after 1½ hours to establish synchronous infection of fibroblasts as well as to avoid infecting T-cells when added. 48 hours later virus transduction efficiency was determined by fluorescent microscopy (Figure 4-31). <30%, 30-60, >75% of cells were transduced at this time point in the wells infected with 30, 300 and 3000 MOI respectively.

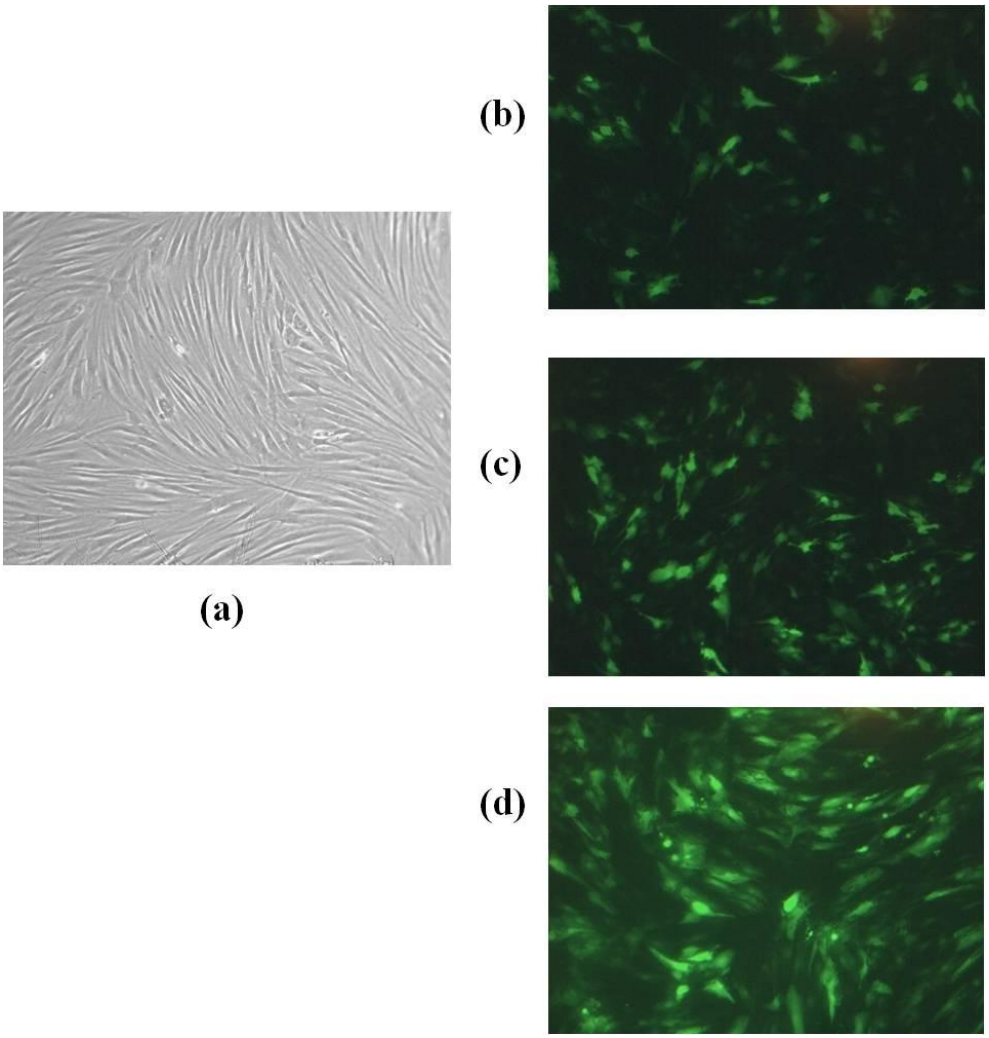

Figure 4-31 Transduction of fibroblasts at different multiplicity of infection

Confluent primary human fibroblast from LD12 were mock infected (a) or infected with AdGFP at different multiplicity of infections (b) 30 (c) 300 and (d) 3000 (particles/cell).with 42x, overall magnification.

Having established the transduction efficiency using Ad-GFP, the replicative cycle of Ad5WT in fibroblasts was monitored by RT-QPCR (see section 2.3.3) at 2, 4, 6, 8, 12, 24, 48 and 60 hours following infection (Figure 4-32). A 10 fold difference was observed between 30 and 300 MOI and virus DNA replication plateau after 12hrs. The difference between the virus genome copies at different MOI was thought to be adequate to recognise reduction in virus replication secondary to Ad-specific T-cells. This model system was then utilised to study the effect of Ad-specific T-cells on Ad replication.

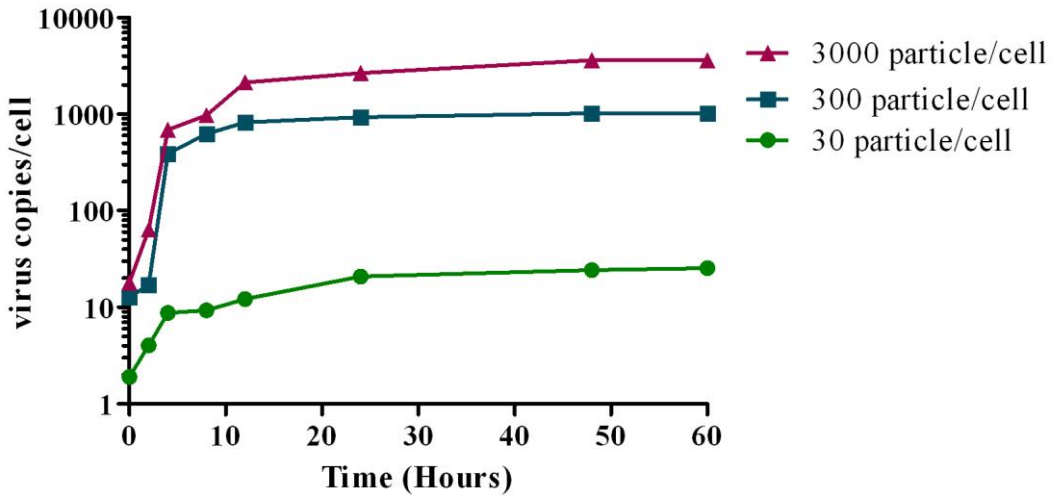

Figure 4-32 Time course of Ad5 WT in infected fibroblasts

Fibroblasts (LD12) were plated ($5x10^3$ cells/ well) in a 96 well plate in T-cell medium supplemented with IFN-γ (200IU/ml). Day 3 confluent fibroblasts were infected with Ad5 WT at MOI 30, 300 and 3000. The cells were harvested at different time points and adenovirus genomes and cellular DNA quantified by RT-QPCR (section 2.3.3). The virus genome copies were normalised to cellular DNA copies.

Primary human fibroblasts were plated ($5x10^3$cells/well) into 96 well plates in T-cell medium supplemented with IFN-γ (200 IU/ml, 3 days). Day 2 autologous PBMCs were stimulated with heat inactivated CTL102 (2.5 $x10^4$ particles/cell) and Ad-specific T-cells were enriched using MS columns as per protocol 2.5.2. The following day, fibroblasts in replicate wells were counted and test wells infected with Ad5WT at 30, 300 and 3000 MOI. Cells were washed after 1½ hours. 8 hours following infection, autologous PBMCs or Ad-specific T-cells were added to triplicate wells at a ratio of 1:10 (Fibroblast: T-cell). Samples were lysed on day 4 and day 6, i.e. day 1(36 hours) and 3 (56 hours) following infection and DNA extracted using Qiagen kit (Qiagen, Crawley, UK). A control sample was lysed (0 hours) following infection. Copies of Ad5WT genome, CTL102 virus genome as well as cellular DNA were quantified using RT-QPCR according to protocol (section 2.3.3). Previous work by Sarah Bonney (Gene and Immunotherapy group, School for Cancer Sciences, UK

(unpublished data)), showed that enriched Ad-specific T-cells have high copy numbers of CTL102 virus genome detected by Ad5WT virus PCR. Ad5WT virus copies were determined by subtracting CTL102 copies from total virus genome copies normalised to cellular DNA copies (Figure 4-33). The figure shows the results of this experiment on primary human fibroblasts of LD12. Enriched Ad-specific T-cells had 83% CD3+ve Ad-specific T-cells on flow cytometry analysis. Wells with Ad5WT infected fibroblasts to which Ad-specific T-cells were added showed lower Ad copy numbers compared to wells to which the autologous PBMCs or no PBMCs were added. The differences observed on day 1 and 3 were not statistically significant by 2-way Anova analysis. This experiment was repeated on the same donor with similar results.

These experiments demonstrate that these cells are capable of limiting virus replication *ex vivo*. Data on CD4 T-cell clones show cross reactivity across species. These characteristics are favourable for the use of these cells for the purposes of adoptive transfer to HSCT recipients with active Ad infection to limit virus replication.

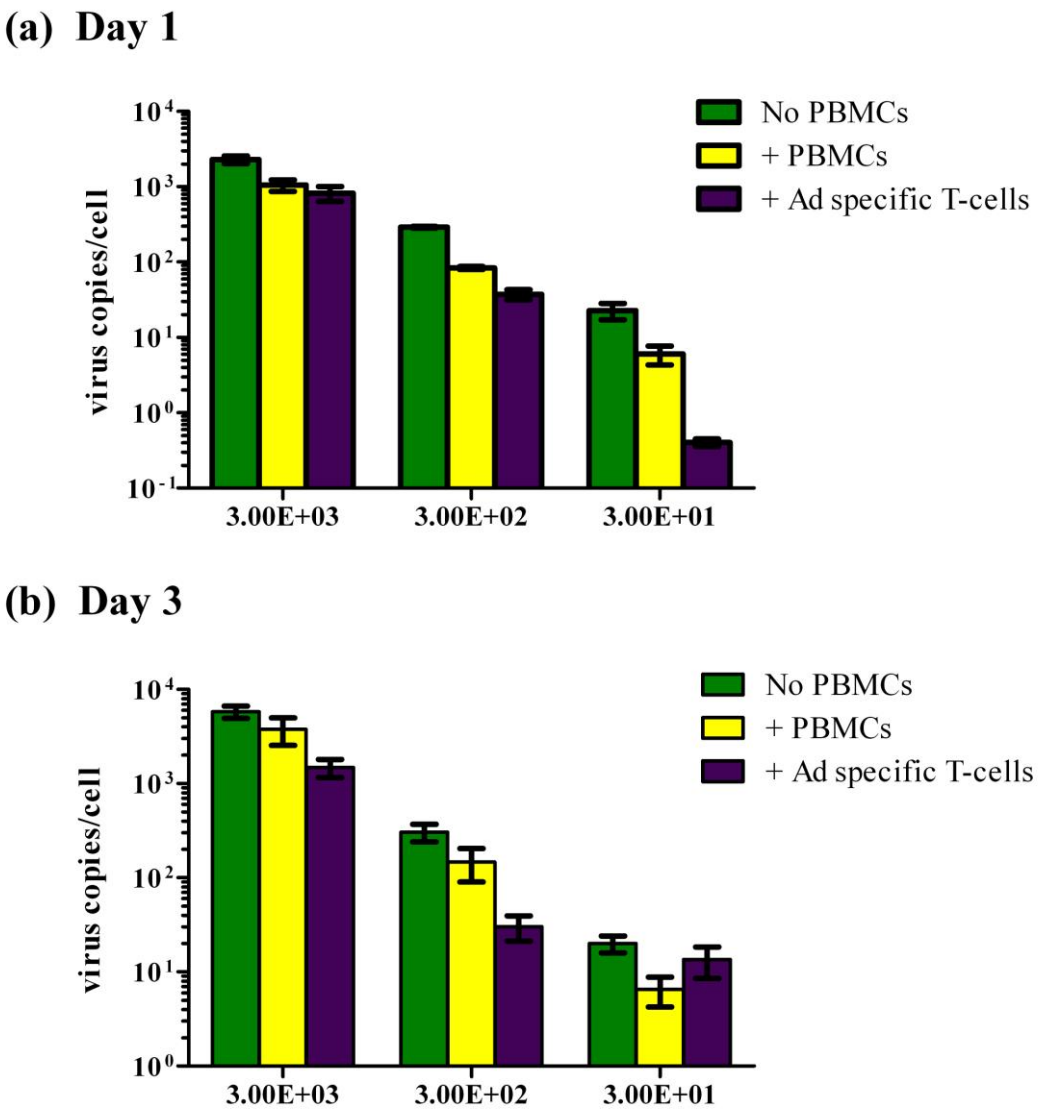

Figure 4-33 Ad-specific T-cells limit adenovirus replication in fibroblasts

Primary human fibroblasts (LD12) passage 15 were plated ($5x10^3$cells/well) in a 96 well plate and treated with IFN-γ (200IU/ml, 3 days). Day 3 fibroblasts were infected with Ad5WT at 30, 300 and 3000 particles/cell. Virus was washed off after 1½ hours and no PBMCs, autologous PBMCs or autologous Ad-specific T-cells (enriched by MS columns) were added to triplicate wells of infected fibroblasts 8hrs post infection. DNA was extracted from cells harvested on days 1 (a) and 3 (b). Adenovirus and CTL102 genomes and cellular DNA were quantified by RT-QPCR. The virus genome copies were normalised to cellular DNA copies. Each bar represents the mean value of triplicate wells. Enriched Ad-specific T-cells had 83% CD3+ve Ad-specific (IFN-γ +ve) T-cells on day 0.

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4.3.4 Discussion

The functional characteristics of Ad-specific tetramer staining T-cells have not been studied. As all HLA A*01 donors (13/13) showed TDL staining T-cells (Figure 3-7), experiments to study the homing and effector characteristics were performed using the TDL tetramer staining T-cells. The experiments show that TDL-specific T-cells can recognise antigen loaded APCs (Figure 4-10) and have a high proliferative potential (Figure 4-6), accounting for 67% of the CD8 T-cells in a polyclonal T-cell line at 4 weeks following TDL peptide stimulation (Figure 4-3). Cognate antigen recognition was demonstrated after tetramer selection suggesting that tetramer selection does not result in loss of functional ability. Suitability of these cells for the purposes of adoptive transfer to a HSCT recipient with acute virus infection is supported by the ability of these cells to recognise APCs loaded with peptide or virus.

IL-2 is a T-cell growth factor that promotes T-cell-dependent immune responses (Smith, 1988). Removal of this cytokine from the culture medium abrogates proliferation or induces cell death (<80% viable cells by day 14 (Figure 4-4)). Even in the absence of IL-2, TDL-specific T-cells proliferated in 4/4 donors in response to virus and peptide by 43 and 98 fold respectively in 10 days (Figure 4-9). The higher proliferation in response to peptide is probably due to complete saturation of antigen attained with peptide, as well as because of the differences in antigen presentation between peptide and virus antigen. CD4 T-cells proliferated predominantly in response to virus; this may have resulted in a proliferative disadvantage for the CD8 T-cells as they have to compete with their CD4 T-cell counterpart for survival. This experiment highlights the importance of a conducive microenvironment (presence of exocrine growth factors) for the optimal growth of these cells *in vivo*.

CD4 and CD8 T-cells replicated by 325 and 114 fold in 6/6 donors over a period of 7 days following virus stimulation (Figure 4-20). A proliferative disadvantage was observed in the absence of IL-2 in concordance with the data on TDL tetramer staining T-cells. The high proliferative potential of Ad-specific T-cells has previously been demonstrated (Feuchtinger et al, 2004; Onion et al, 2009; Zandvliet et al, 2010). Feuchtinger *et al* (Feuchtinger et al, 2004) demonstrated that Ad-specific T-cells enriched by clinical grade selection, cultured in T-cell medium supplemented with IL-2 (100 IU/ml), proliferated to >100 fold (determined by intracellular IFN-γ staining) over a median of 18 days. They also observed an increase in the CD4 T-cell cohort with a CD4:CD8 ratio of 52:21. A rise in frequency of Ad vector-specific T-cells was observed in 11/11 patients following gene therapy by Onion *et al* (Onion et al, 2009). These observations confirm the *in vitro* proliferative potential of Ad-specific T-cells following antigen stimulation.

TDL-specific T-cells and Ad-specific T-cells enriched by CSS showed a minimally differentiated phenotype: $CD45RA^{high}$, $CD45RO^{high}$, $CCR7^{high}$, $CD62L^{low}$, $CD27^{high}$, $CD28^{high}$, LFA-1^{high} and $CD57^{low}$. The $CD45RA^{high}$, $CCR7^{high}$ phenotype did not fit into the classical definition of an antigen experienced cell (Lanzavecchia & Sallusto, 2002). The frequency of these cells as well as the fact these were enriched antigen experienced cells made the possibility of these being naive unlikely. A switch in the surface expression of CD45, from CD45RA to RO isotype is frequently found following transition of a naive cell to an antigen experienced cell (Mackay, 1993). Simultaneous expression of CD45RA and RO on the TDL and Ad-specific T-cells suggests that these are antigen experienced and that the switch to a memory cell is not yet complete. Hamann *et al* (Hamann et al, 1997) described $CD45RA^{+}CD27^{+}$ CD8 T-cells that in the peripheral blood with characteristics of naïve cells such as $CD45RO^{negative}$, $CD62L^{high}$, $CD28^{high}$ and LFA-l ^{low}. The discriminators for naïve T-cells in their study were CD45RO, CD62L and LFA-1. Expression of CD45RO and LFA-1 as

well as down regulation of CD62L confirm that the TDL-specific and Ad-specific T-cells have experienced antigen.

LFA-l is universally expressed by human T-cells, and the expression is bimodal, allowing the partition of these cells into a LFA-1^{low} and LFA-1^{high}subsets (Sanders et al, 1988). This bimodal pattern of expression is only observed on CD8 T-cells and its relevance is disputed (Okumura et al, 1993). Therefore LFA-1 expression was determined as high or absent. LFA-1 expression reflects the degree of cellular activation and supports that these cells are antigen experienced (Hviid et al, 1993). Low CD57 expression, a marker of terminal senescence, supports the high replicative potential of these cells (Brenchley et al, 2003). Low expression of CD62L on the enriched Ad-specific T-cells that are $CD45RA^{high}$ further confirms that these cells are not naive (Roederer et al, 1995). CD62L is usually down regulated on T-cell activation with chronic and persistent viral infections (Wherry et al, 2003b). High CCR7 expression may be due to low antigen load and brief antigen exposure.

My data is in accord with recently published data. Zandvliet *et al* observed a $CD45RO^{+}$, $CD62L^{intermediate}$, $CD27^{+}$, $CD28^{+}$ phenotype on CD4 and CD8 +ve Ad hexon-specific T-cells in healthy volunteers (Zandvliet et al, 2010). They demonstrated that on proliferation, the CD62L expression increased and then fell with no change in other markers, confirming the central memory phenotype, effector potential and ability to proliferate (lack of terminal differentiation) of these cells. Feuchtinger *et al* on Ad hexon-specific T-cells isolated by CCS on a clinical grade found a $CD45RA^{+/-}$, $CCR7^{-}$, $CD62L^{+}$, $CD27^{+}$ and $CD28^{-}$ (Feuchtinger et al, 2008). The CCR7 and CD28 expression was different in comparison to my data and Zandvliet *et al* (Zandvliet et al, 2010).

Classification of T-cells based on CCR7 (a lymph node homing marker) and CD62L (L-selectin, a cell adhesion molecule, involved in homing to high endothelial venules)

expression into T_{CM} (CCR7+ve, CD62L+ve) and T_{EM} (CCR7-ve, CD62L-ve) (Geginat et al, 2001) was based on observations in mice; further phenotypic heterogeneity has been observed in the human T-cell memory pool (Unsoeld & Pircher, 2005). Unsoeld *et al* observed lymphocytic choriomeningitis virus (LCMV)-specific memory T-cells which were $CD62L^{low}$, $CCR7^{high}$ and named them Intermediate Memory T-cells (T_{IM}). Similar cells ($CD62L^{low}$, $CCR7^{high}$) have been identified in influenza A virus infected mice (Debes et al, 2004). The role of these cells has not been elucidated though Unsoeld *et al* found them in blood, spleen and non- lymph node tissue. The level of viral persistence and localisation may be responsible for the different phenotypes. The latency of LCMV and CMV has been shown to influence the phenotypic characteristics of the respective virus-specific T-cells (Obar et al, 2006; Wherry et al, 2003a).

TDL-specific CD8 T-cells (Figure 4-13) and Ad-specific CD4 and CD8 T-cells(Figure 4-23 and Figure 4-24) had high CD27 and CD28 expression levels that remained high following stimulation when analysed on day 10 (Figure 4-15, Figure 4-25 and Figure 4-26). T-cells with low CD27 expression are functionally differentiated as a result of persistent antigen stimulation (Baars et al, 1995; De Jong et al, 1992). CD28 is a potent transducer of proliferation IL-2 secretion and acquisition of effector functions (Croft, 2003a). Loss of CD28 expression is a marker of immune senescence indicating reduced proliferative potential and is associated with chronic infections and inflammatory syndromes (Vallejo, 2005). High levels of expression of both the costimulatory receptor molecules may be due to low levels of antigenic persistence. The ability of the TDL and Ad-specific T-cells to proliferate in the presence of IL-2 suggest an early minimally differentiated phenotype with high proliferative and effector potential.

Further to the characterisation of Ad-specific CD4 T-cells for their phenotypic and homing potential they were analysed for the expression of the HIV co-receptor CCR5 and HIV gp120-binding integrin $\alpha_4\beta_7$ directly *ex vivo* by CCS and after proliferation following antigen stimulation (data not shown). The impact of preexisting Ad-specific cellular immunity on the efficacy of recombinant Ad vectors is still unanswered. Conflicting reports on the impact of Ad gene therapy trials exist (Gahery-Segard et al, 1997; Molnar-Kimber et al, 1998; Onion et al, 2009) but the problem was most acutely highlighted by the recent cessation of a Phase II trial (Step Trial) of recombinant Ad HIV vaccine vector (adenovirus type 5-based gag/pol/nef construct) where HIV seroconversion was observed amongst individuals with high titres of Ad antibodies (Buchbinder et al, 2008). This led to the suggestion that Ad 5 vaccination may increase the frequency of Ad-specific T-cells which could represent a pool of cells susceptible to HIV infection. Ad-specific CD4 T-cells showed high levels of expression of the HIV co-receptor CCR5 and HIV gp120-binding integrin $\alpha_4\beta_7$ when checked directly *ex vivo* by CCS and after proliferation following antigen stimulation. The frequency of Ad-specific T-cells did not correlate with the titre of neutralising Ad antibodies. This indicates that the phenotype and proliferative potential of Ad5-specific T-cells can support HIV infection and may explain the seroconversion amongst vaccinated subjects (Chakupurakal et al, 2009). Recent work by Benlahrech et al (Benlahrech et al, 2009) confirmed the mucosal homing phenotype of the CD4 T-cells with no correlation with serotype status, in line with our experience.

In acute virus (EBV, HIV and CMV) infections, virus-specific T-cells characterised by tetramer staining typically are $CD45RO^{high}$, $CCR7^{high}$, $CD27^{high}$ and $CD28^{high}$ (van Lier et al, 2003). While influenza virus-specific T-cells retain this phenotype in the latency phase, EBV and Hepatitis C virus-specific T-cells (HCV) lose their CCR7 expression.

CMV-specific T-cells lose CCR7, CD27, and CD28 expression and re-express CD45RA (Appay et al, 2002). Low CD27 and CD28 expression as well as high CD57 expression of the CMV-specific T-cells indicate a terminally differentiated state (confirmed by the possession of short telomeres) and is attributes to the refractoriness to apoptosis and poor proliferative capacity (Effros et al, 2005). EBV-specific-T-cells are $CD45RA^{low}$, $CD45RO^{high}$, $CCR7^{low}$, $CD62L^{low}$, $CD27^{high}$, $CD28^{pos/neg}$, and $CD57^{low}$ (Callan, 2003). These cells had higher proliferative potential in comparison to CMV-specific T-cells. T-cells specific to EBV lytic phase proteins are predominantly of the T_{EMRA} phenotype. Ad-specific T-cells have a phenotype closer to the influenza virus-specific T-cells, indicating that the antigen is persisting at very low levels and the T-cells are not chronically exposed to the virus as in the case of CMV and EBV.

Virus	CD45RA	CCR7	CCR7	CD27	CD28
Influenza	Neg	Pos	Pos	Pos	Pos
EBV	Neg	Neg	Neg	Pos	Pos
CMV	Pos	Neg	Neg	Neg	Neg
HCV	Neg	Neg	Neg	Pos	Pos
HBV	Neg	Neg	Neg		
HIV	Neg	Neg	Neg	Pos	Neg
HTLV1	Neg			Pos	

Table 4-3 Comparing virus-specific T-cell memory phenotypes

Influenza memory T-cells as well as memory T-cells specific for chronic and latent viruses are compared in this table. EBV-Epstein Barr virus, CMV-Cytomegalovirus, HCV-Hepatitis C virus, HBV-Hepatitis B virus, HIV- Human immunodeficiency virus, HTLV1-human T-cell lymphotropic virus-1. Adapted from (van Lier et al, 2003)

Recently Turtle *et al* (Turtle et al, 2009) described a distinct subset of CD8 T-cells which are both T_{CM} ($CD62L^{high}$) and T_{EM} ($CD62L^{low}$) as well as $CD45RO^{high}$, $CD45RA^{low}$, $CD27^{high}$, $CD28^{high}$ and $CD57^{low}$accounting for <0.05% of the total PBMCs in healthy volunteers. Based on CD161 expression these CD8 T-cells were further classified. [CD161binds two ligands; lectin-like transcript-1 (LLT1) or proliferation-induced lymphocyte associated receptor (PILAR) and inhibits or augments cytokine secretion and proliferation mediated by TCR signalling (Huarte et al, 2008; Rosen et al, 2008)]. They found these cells in acute myeloid leukaemia patients treated with anthracyclines and identified that the $CD161^{high}$ subset of cells were virus (influenza, CMV, EBV) specific and resistant to chemotherapy. Further characterisation of the TDL and Ad-specific T-cells with markers like CD161, IL-18Rα and Ki-67 (proliferation markers) may assist in studying the functionality of these cells.

Ad-specific CD4 T-cell clones recognising conserved hexon epitopes have been shown to limit replication of multiple serotypes of Ad *in vitro* (Heemskerk et al, 2006). Ad-specific T-cell clones were added to IFN-γ treated autologous B-LCLs infected with Ad5 (100 MOI, 1hour, ratio of 10:1) and cells harvested on day 3. The virus titres of cell lysates determined by $TCID_{50}$ assay were reduced by 1000 fold when Ad-specific T-cell clones were co-cultured with virus loaded B-LCLs in comparison to those without-cells. Although Ad-specific T-cells (CD4 and CD8 T-cells), enriched from fresh PBMCs, limited virus replication, the 10 fold reduction measured by RT-QPCR (Figure 4-33) was not as profound as reported by the Heemskerk group. This may be due to the differences in the methods used. The potential of Ad-specific T-cells to limit virus replication and allow virus clearance *in vivo* is demonstrated in the phase I/II clinical trial by Feuchtinger *et al* (Feuchtinger et al, 2006). Both TDL-specific polyclonal T-cell lines and the Ad-specific CD4-Tcells demonstrated cytotoxicity to serotypes across species. TDL-specific polyclonal T-cells showed cytotoxicity to all species responsible for infections in HSCT (Figure 4-17).

In summary, the proliferative potential of Ad-specific T-cells enriched by CSS or tetramer selection suggest that these cells may rapidly expand to therapeutic frequencies in a HSCT recipient following adoptive transfer and thus enable Ad-specific immune reconstitution. The presence of IL-2, other Th_1 cytokines and growth factors in the HSCT recipient at the time of adoptive transfer support a favourable conducive microenvironment (Abu-Ghosh et al, 1999). The ability of these cells to limit virus replication as well as their cytotoxic potential across Ad species allow successful therapy for most HSCT recipients irrespective of the species responsible for infection enabling virus clearance.

5 Clinical grade selection of Ad-specific T-cells

5.1 Introduction

Cellular immunity has been shown to enable virus clearance and prevent progression of Ad infection to disease (Feuchtinger et al, 2005). Alternative therapeutic strategies are required for Ad infections in HSCT recipients especially when anti-adenovirus agents fail. Immunotherapy is a valuable tool in this setting as it allows Ad-specific immune reconstitution and thereby facilitates virus clearance (Tebruegge & Curtis, 2010). The work in my previous chapters demonstrates that the frequency of Ad-specific T-cells, as determined by CSS or tetramer selection, is low but adequate for clinical grade selection and effective adoptive transfer. These cells have a high proliferative potential, a minimally differentiated phenotype, are cytotoxic across Ad species and capable of limiting Ad replication. As a result it is anticipated that following adoptive transfer they would efficiently proliferate and aid in virus clearance in the HSCT recipient.

5.2 Aims of the chapter

In this chapter, standard operating procedures developed for the clinical grade selection of adenovirus-specific T-cells by CSS as well as difficulties in developing cell selection of adenovirus-specific T-cells by means of pMHC multimers according to Good Manufacturing Practice will be discussed. Methods to monitor adoptively transferred T-cells in HSCT recipients are also detailed. A trial design and protocol- Adoptive immunotherapy for adenovirus infections in HSCT recipients (AdIT) is also proposed.

5.3 Clinical grade multimer selection of Ad-specific T-cells

5.3.1 Streptamer

pMHC multimers have enabled the isolation as well as characterisation of antigen-specific T-cells as discussed (Casalegno-Garduno et al, 2010). For therapeutic purposes it is a requirement that cell selection complies with GMP standards and that the materials used have CE certification, [CE **(***conformité européenne*) marking certifies that a product has met European Union consumer safety, health or environmental requirements]. GMP grade isolation of antigen-specific T-cells using pMHC tetramers requires anti-PE/APC fluorophore labelled magnetic beads. Alternative options for clinical grade multimer selection of antigen-specific T-cells were investigated due to the unavailability of GMP grade magnetic beads.

Strep-tag II, is a short amino acid peptide (H_2N–WSHPQFEK–COOH) with an affinity (Kd ~$1x10^{-6}$ M) for the biotin binding site of streptactin (Voss & Skerra, 1997), a modified streptavidin molecule (Schmidt & Skerra, 1993). In 2002, a new pMHC multimer, Streptamer, facilitating clinical grade enrichment of antigen-specific T-cells was developed (Knabel et al, 2002). Streptag III (referred to as streptag from now), a fusion protein generated between one HLA protein and two Streptag II sequences arranged sequentially, has a higher affinity for streptactin than Streptag II (Neudorfer et al, 2007). pMHC Streptag molecules multimerised on a fluorophores labelled streptactin backbone, streptamers, demonstrated antigen-specific T-cell staining comparable to tetramer stained T-cells (Knabel et al, 2002) (Figure 5-1).

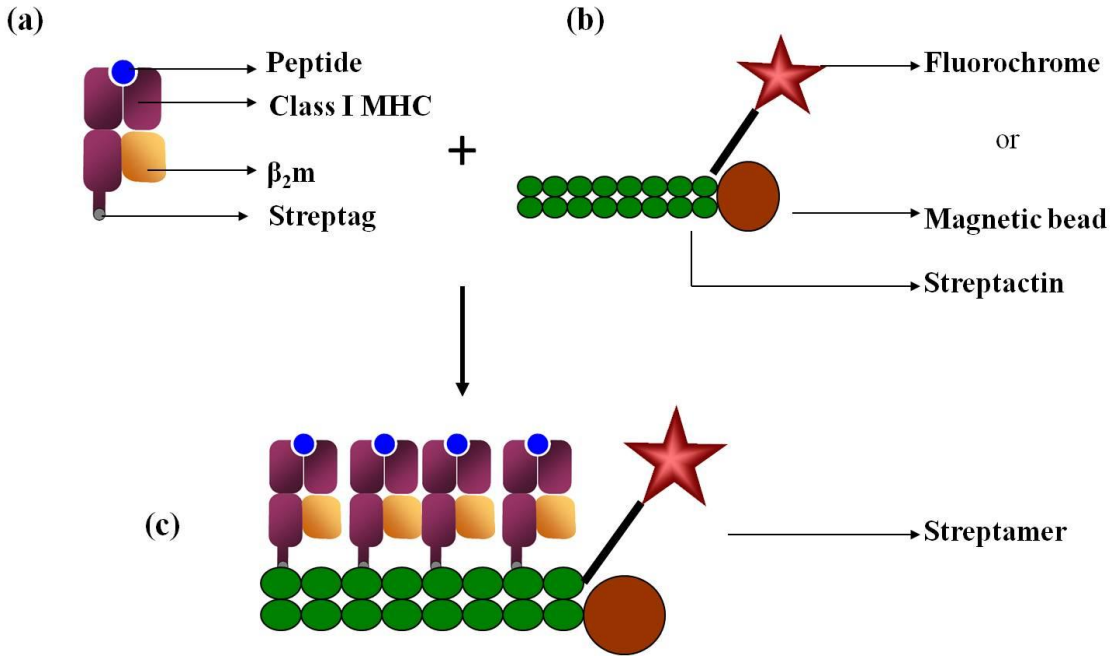

Figure 5-1 Schematic representation of Streptamer technology

(a) Streptag labelled class I MHC protein, β_2m and relevant peptide are refolded to generate pMHC monomers. These are incubated with streptactin (b) which is either flourochrome or magnetic bead labelled to generate pMHC multimers called Streptamers(c).

D-biotin has a higher affinity for Streptactin (Kd ~$1x10^{-13}$ M) than Streptag and so addition of D-biotin results in rapid disassembly of the multimers and removal of the streptactin backbone. Subsequently, due to weak interaction between the MHC and TCR, pMHC monomers dissociate from the T-cell surface (Knabel et al, 2002). This results in isolation of functional antigen-specific T-cell without impairing their functionality (Figure 5-2). Availability of GMP grade reagents (IBA GmbH, Gottingen, Germany) means that this method could be used for the clinical grade enrichment of adenovirus-specific T-cells.

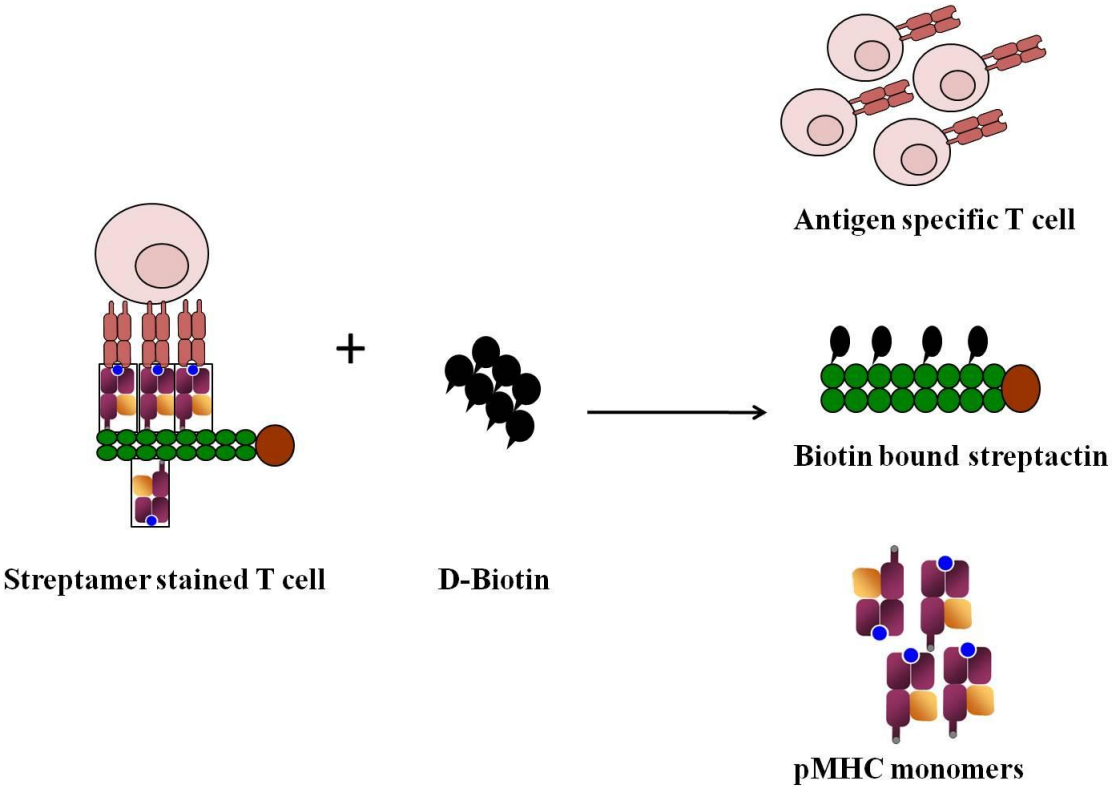

Figure 5-2 Isolation of antigen-specific T-cells by Streptamer technology

T-cells are stained with streptamers labelled with a fluorochrome or magnetic bead. Antigen-specific T-cells labelled by magnetic beads can be enriched using the Miltenyi MACS technology. The enriched cells are then incubated with D-biotin. Dissociation of the Streptag-Streptactin complex due to competitive binding of D-biotin results in disassembly of the pMHC monomer and T-cell TCR allowing isolation of antigen-specific T-cells without impairing their functionality.

MHC class I HLA A*01 containing the Ad epitope LTDLGQNLLY peptide, Streptactin-PE, Streptactin magnetic beads and D-biotin were purchased (IBA GmbH, Gottingen, Germany). Manufacturers' instructions were followed for streptamer staining. To assess the suitability of streptamers as an alternative source to isolate and enrich Ad-specific T-cells, streptamer and tetramer staining was compared. PBMCs of HLA A*01 donors ($5x10^6$ cells) (LD2, 3, 5, 12) were stained using streptactin PE (0.75µg) and class I MHC (1µg) in a final volume of 50µl Buffer IS (0.5% BSA (w/v) in PBS pH 7.4) and incubated (45 minutes, dark, 4°C). After two washes in Buffer IS, an aliquot was incubated with D-biotin (1mM, 20 min, 4°C) followed by a wash. This step was repeated and the eluted cells were washed 4 times prior to

labelling with TDL tetramer (37°C, 5% CO_2, 15min). All cells were then labelled with antibodies to CD3, CD8 or PI and analysed by flow cytometry (LSR II). The percentage of PI-, CD3+, CD8+, multimer-specific T-cells was evaluated. Failure to detect Ad-specific T-cells may be due to the low frequency of these cells.

Peptide stimulated polyclonal T-cell lines will have a high percentage of TDL-specific T-cells in comparison to fresh PBMCs (Figure 4-3). Hence after 5 unsuccessful experiments on fresh PBMCs, polyclonal T-cell lines following stimulation with TDL peptide maintained in T-cell medium supplemented with IL-2 (100IU/ml) were used. TDL-specific polyclonal T-cell lines (LD3) maintained in T-cell medium supplemented with IL-2 were stained on day 17 with (a) TDL tetramer, (b) TDL streptactin PE and (c) TDL streptactin PE followed by D-biotin elution and TDL tetramer staining (Figure 5-3). The frequency of TDL streptactin PE stained T-cells was lower than the TDL tetramer stained T-cells. Tetramer staining following elution improved results despite the extra washes involved in elution. This experiment was repeated on LD3 and LD5 TDL-specific polyclonal T-cell lines with similar results (data not shown). MACS Buffer, regularly used in tetramer staining and enrichment was used as an alternative buffer to see if this would change staining pattern but no improvement in streptamer staining pattern was observed.

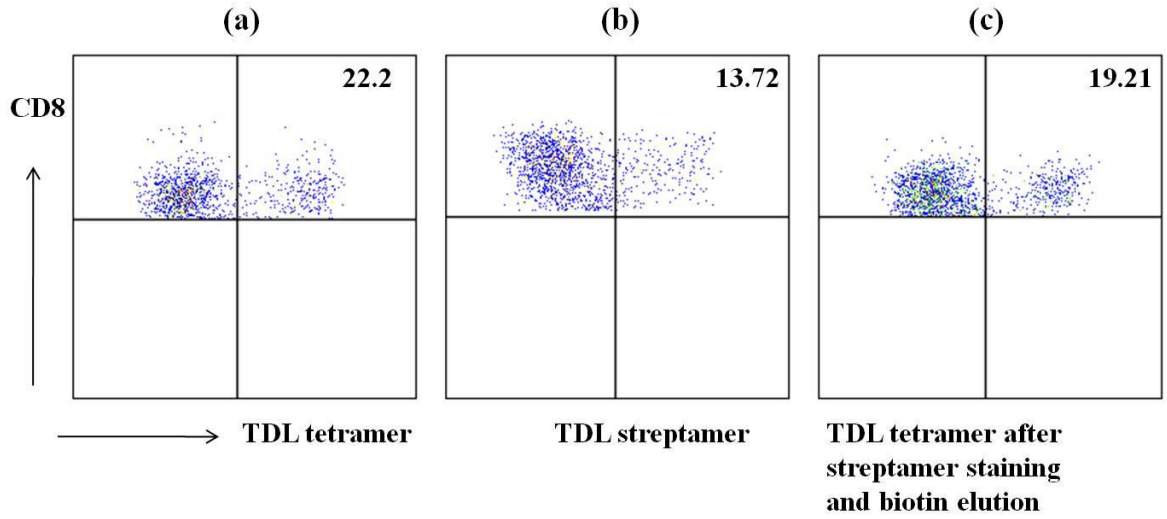

Figure 5-3 Streptamer staining on TDL-specific polyclonal T-cell line

PBMCs ($5x10^6$) of LD3 maintained in T-cell medium supplemented with IL-2 (100IU/ml) following stimulation with TDL peptide (10µg/ml) were stained on day 17 with (a) TDL tetramer (37°C, 5% CO_2, 15min) (b) (0.75µg) streptactin PE and (1µg) MHC in a final volume of 50µl Buffer IS (45minutes, dark, 4°C) or (c) (0.75µg) streptactin PE and (1µg) MHC in a final volume of 50µl Buffer IS (45minutes, dark, 4°C). After two washes, cells were incubated with d-biotin (1mM, 20 min, 4°C) followed by a wash. This step was repeated and the eluted cells were washed 4 times prior to labelling with tetramer (37°C, 5% CO_2, 15min). All cells were labelled with antibodies to CD3, CD8 or PI and analysed on LSR II. The numbers in the top right hand corner represent the % of PI-, CD3+, CD8+, multimer-specific cells.

After establishing that streptamers can stain TDL-specific T-cells, TDL streptamer labeled magnetic beads were used to enrich T-cells, according to manufacturers' instructions. Streptamer magnetic beads and (2µg) MHC were incubated in Buffer IS (overnight, dark, 4°C) and applied to an MS column placed in a magnetic field. Beads retained on the column were eluted and incubated with TDL-specific polyclonal T-cells (45 minutes, dark, 4°C) in Buffer IS. After a wash (300g, 5 min, 4°C) streptamer labelled T-cells were applied to the MS column placed in the magnetic field. Eluted cells were washed twice with Buffer IS (300g, 5 min, 4°C). The cells were then incubated with 1mM d-biotin (20 min, 4°C) followed by a wash (300g, 5 min, 4°C). This step was repeated and the cells were washed 4 times prior to labelling with streptactin PE or tetramer (37°C, 5% CO_2, 15min). All cells were labelled with anti CD3, antiCD8 antibodies and PI and analysed by flow cytometry (LSR II).

TDL-specific polyclonal T-cell lines of LD03, maintained in a T-cell medium supplemented with IL-2 (100IU/ml), were stained on day 22 with TDL tetramer (Figure 5-4 a) followed by enrichment (Figure 5-4 b). Cells were stained simultaneously with TDL streptamer magnetic beads and streptamer staining T-cells enriched by MS columns. After d-biotin elution the T-cells were stained with TDL streptactin PE or TDL tetramer (Figure 5-4 c &d).

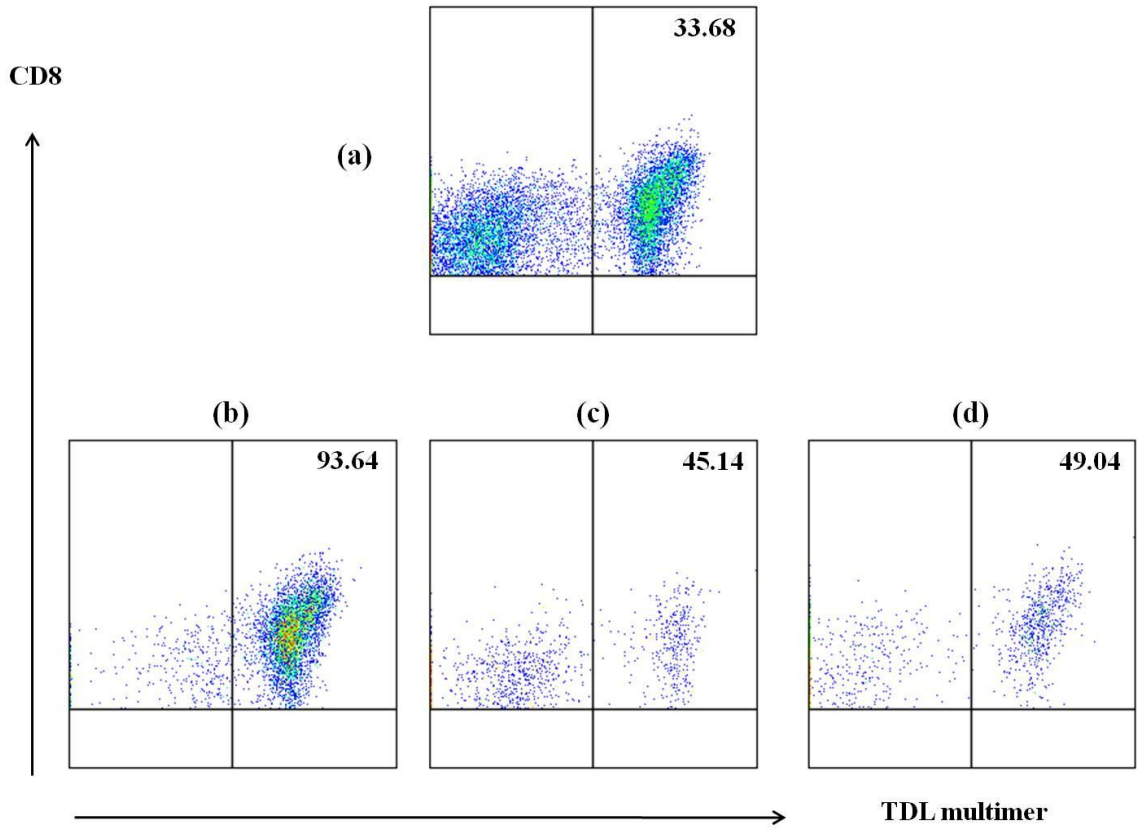

Figure 5-4 Enrichment of Ad-specific T-cells with streptamers

PBMCs ($5x10^6$) of (LD3) maintained in T-cell medium supplemented with IL-2 (100IU/ml) following stimulation with TDL peptide (10µg/ml) were stained on day 22 with (a) TDL tetramer (37°C, 5% CO_2, 15min) and (b) enriched using MS columns. (c) Streptamer magnetic beads and (2µg) MHC were incubated (overnight, dark, 4°C) and applied to an MS column placed in a magnetic field. Retained beads on the column were eluted and incubated with T-cells (45minutes, dark, 4°C) and applied to the MS column placed in the magnetic field after a wash. Eluted cells were washed twice with Buffer IS (300g, 5 min, 4°C). The cells were incubated with d-biotin (1mM, 20 min, 4°C) followed by a wash (300g, 5 min, 4°C). This step was repeated and the cells were washed 4 times prior to labelling with streptactin PE or tetramer (37°C, 5% CO_2, 15min). All cells were labelled with anti CD3, antiCD8 antibodies and PI and analysed by flow cytometry (LSR II). FACS plots of each sample with the % of PI-, CD3+, CD8+, multimer-specific cells shown. (a) tetramer stain (b) tetramer sort (c) streptamer sort streptactin-PE stain (d) streptamer sort tetramer stain

Though TDL streptamer staining was comparable to tetramer staining, enrichment of Ad-specific T-cells by streptamer was reduced by half in comparison to tetramer. Repeated experiments on TDL-specific polyclonal T-cell lines of LD3 and LD5 showed similar or reduced purity. Similar experiments on fresh PBMCs of 3 HLA *01 lab donors (LD3, 5 and 13) were unsuccessful (data not shown). The disparity in the results could be explained by the differences in the staining intensity (Figure 5-3). Due to the low purity of the streptamer selected T-cells in comparison to tetramer selected T-cells this method was felt not to be appropriate for the clinical grade selection of cells.

5.4 Clinical grade enrichment of adenovirus-specific T-cells by CCS

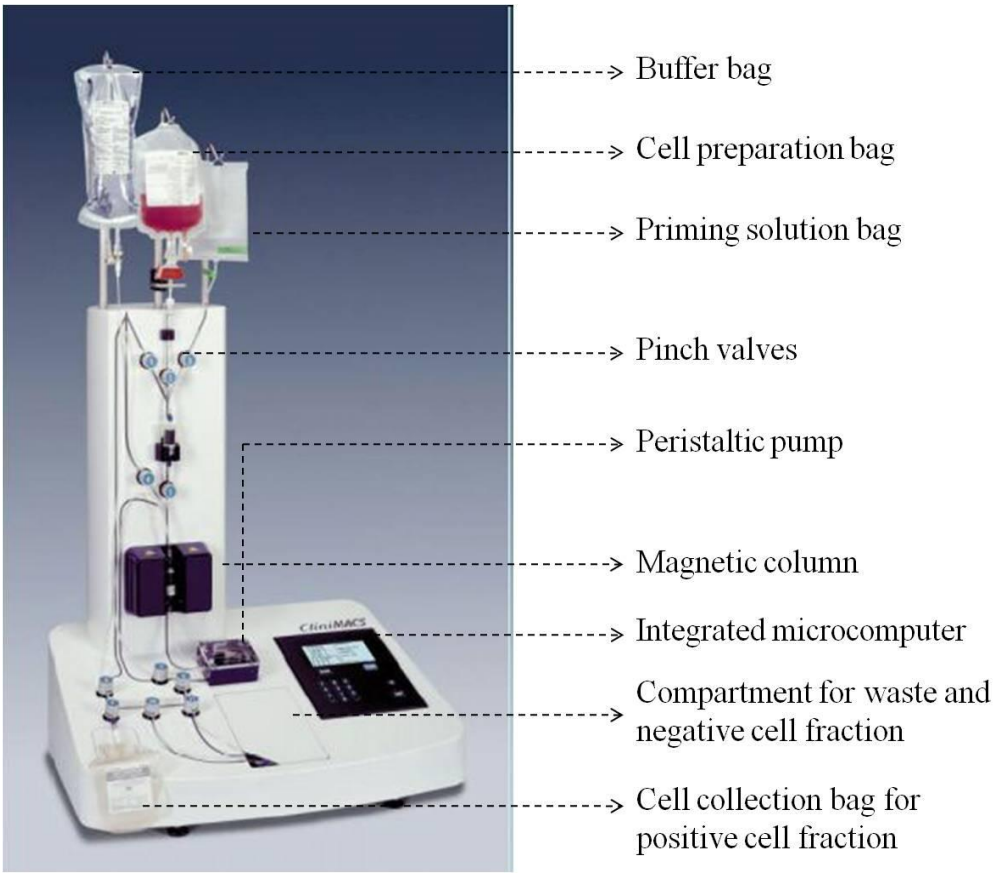

Figure 5-5 CliniMACS ® cell separation system

The CliniMACS ®Cell Selection System provides a closed, sterile system for the clinical scale enrichment or depletion of specific cell populations. This cell selection system comprises CliniMACS Tubing Set, CliniMACS Reagent and the CliniMACS PBS/EDTA Buffer.

The CliniMACS®Cell Selection System (GmbH, 2007) (Figure 5-5), is a closed sterile system for the clinical scale enrichment or depletion of specific cell populations and is based on MACS Technology (see Figure 2-1). The cell selection system comprises CliniMACS Tubing Set, a CliniMACS Reagent, and the CliniMACS PBS/EDTA Buffer. It is CE-marked for clinical use in Europe. In the EU it can be used for separation of human cell types including stem and progenitor cells, monocytes, NK cells, B-cells, and T-cell subsets. In the

US it can be used for research purposes only as the approval is granted only as an investigational new drug (IND) or investigational exemption device (IDE) (GmbH, 2007).

The key components of the CliniMACS ®cell separation instrument include a computer, the magnetic separation unit, the peristaltic pump and various pinch valves (Figure 5-5). All components of the instrument are controlled by the computer and procedures can be repeated in a standardised manner. A permanent magnet and a holder for the selection column comprise the magnetic separation unit. The peristaltic pump controls the flow rate throughout the tubing set and, together with the pinch valves, ensures the controlled flow of buffer through the system and continual suspension of cells.

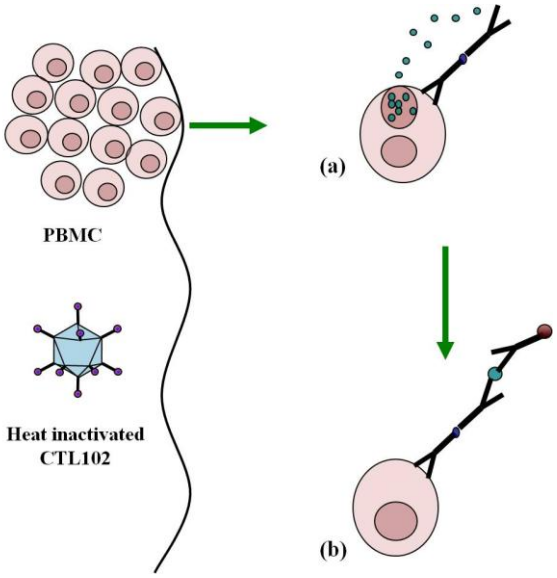

Figure 5-6 Cytokine secretion selection and enrichment on a clinical scale

PBMCs were incubated at a density of 10^7cells/m^2 at (37°C, 5% CO_2) with heat inactivated CTL102 antigen ($2.5x10^4$ particles/cell, 16 hours). (a) IFN-γ catch reagent was added to the cytokine releasing cells and after initial incubation in cliniMACS buffer (5min, 4°C) the cells were incubated in T-cell medium containing human albumin (45min, 37°C, 5% CO_2). Cells were washed and (b) cliniMACS IFN-γ enrichment reagent was added (20min, 4°C). A sample was taken as pre-sort for analysis following a wash. The resulting cells in the cell preparation bag were applied to the cliniMACS separation instrument.

Clinical grade selection of IFN-γ selected cells (Figure 5-6) is a modification of the protocol for CCS (section 2.5.2.2) using the cliniMACS cytokine capture system (CCS). Cells labelled with the cliniMACS reagents were enriched using CliniMACS ®Cell Selection System. PBMCs were incubated at a density of 10^7cells/m^2 at (37°C, 5% CO_2) with heat inactivated CTL102 antigen ($2.5x10^4$ particles/cell, 16 hours). IFN-γ catch reagent and cliniMACS IFN-γ enrichment reagents were used to label the cells. The resulting cells were applied to the cliniMACS separation instrument as per standard operating procedures (Appendix)

For the purposes of the trial, training was obtained to use the facilities in the stem cell laboratories at the National Blood Service, Birmingham to generate clinical grade products by cell selection to GMP standards. Training was provided by Dr John Campbell, Miltenyi Biotech GmbH for the use of CliniMACS cytokine (IFN-γ) capture system to GMP standards. In October 2009, the cost of all the necessary reagents for one clinical grade selection was £4000. For each selection, reagents should be ordered in duplicate in case of any unexpected adverse events during the procedure.

500ml blood was obtained from healthy volunteers (LD 20, 21, 22, 26) and PBMCs were extracted by lymphoprep (section 2.4.1.1). An accurate cell count was obtained from Horiba ABX Pentra 60+ cell analyser at the NBS, Birmingham and heat inactivated clinical grade CTL102 ($2.5 x10^4$ particles /cell) was added to cells maintained at a density of 10^7/ml in T-cell medium containing 10% human albumin. 5 selections were performed as per manufacturer's instructions as summarised in Table 5-1.

Cell selection no:	Date	LD	PBMCs	Pre-sort CD4%/CD8%	Sort CD4%/CD8%
1	22/04/08	2xBuffy	$1.27x10^8$	0.00/0.00	0.00/0.00
2	13/08/08	LD20	$1x\ 10^8$	0.03/0.07	21.54/14.7
3	19/08/08	LD22	$2.4x10^8$	0.27/0.21	0.42/0.03
4	02/09/08	LD21	$2.43x10^8$	0.03/0.12	16.67/3.77
5	14/10/08	LD26	$2.37x10^8$	0.14/0.14	3.05/1.15

Table 5-1 Collated data on clinical grade selection

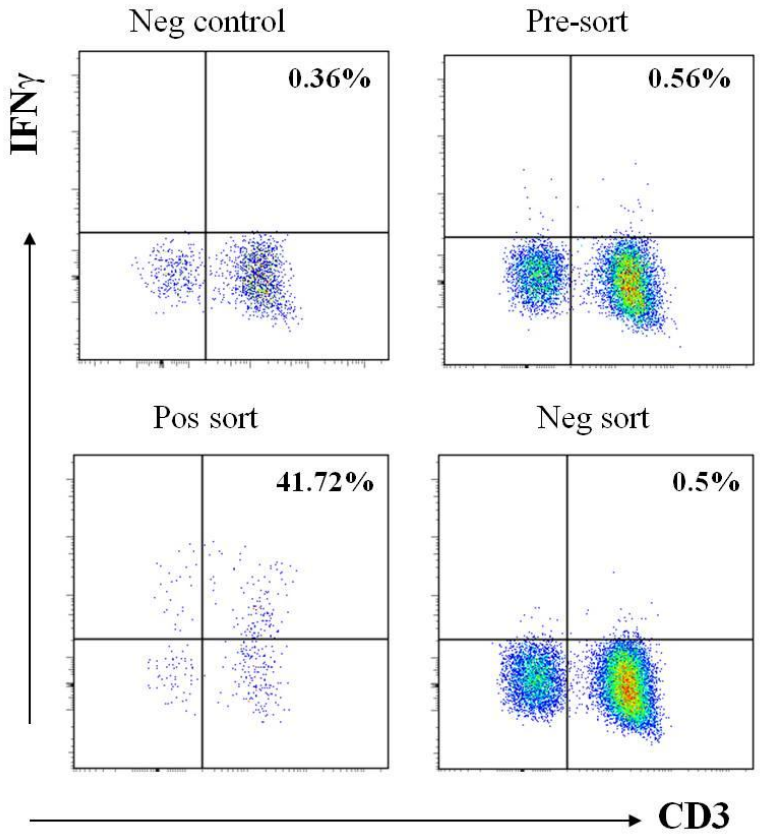

Figure 5-7 Clinical grade selection by CSS on LD20

PBMCs (10^8) (LD20) were incubated with CTL102 (2.5 $x10^4$ particles/cell) in T-cell medium with 10% human albumin. Cells were labelled with clinical grade cytokine capture system according to protocol and applied to the CliniMACS ® cell separation system. Pre-sort, positively (pos sort) and negatively (neg sort) enriched cells were labelled with anti-CD3, anti-IFN-γ and PI and analysed on Coulter Epics flow cytometer. An unstimulated sample was the negative control. The number of Ad-specific T-cells secreting IFN- γ as a percentage of total CD3 T-cells is shown in each FACS plot.

Total mononuclear cells	$3.9x\ 10^5$
Total live lymphocytes	$2.5x10^5$
Non CD3 T-cells	$1.4x10^5$
Total CD3 T-cells	$1.92x10^5$ (76.65%)
Total IFN-γ producing CD3 T-cells:	$1.04\ x10^5$ (41.72%)
CD3 T-cells not producing IFN-γ	$8.73x10^4$ (34.92%)

Table 5-2 Results on LD20 following clinical grade CSS

The non CD3 T-cells are the mononuclear cells which are not lymphocytes. The percentages are that of total live lymphocytes.

Although 5 selections were undertaken only one (LD20) was successful. The flow cytometry results on the pre-sort, pos-sort and neg-sort samples of donor LD20 are shown in Figure 5-7 and details of the enriched cells summarised in Table 5-2. The number of possible alloreactive T-cells was below the cut-off of $1x10^5$ set for the trial. Despite a suboptimal cell selection on LD21, the CD4 and CD8 T-cells enriched by 16% and 3% respectively. The three other selections failed due to technical glitches (Table 5-3). These experiences were taken into account to generate an SOP for the clinical grade selection of cells-SOP/BIR/SB/089 NBS, Birmingham (section 8.1.). These errors can be avoided by further trial runs and expertise gained following multiple selections.

Cell selection no:	Problems
1	2 buffy coats were obtained from 2 different donors. Following Ficoll separation the cells were mixed and all steps followed according to manufacturers' guidance. No Ad-specific T-cells were found in the enriched sample This was thought to be due to alloreaction between the 2 donor PBMCs
2	Successful cell selection
3	Tubing was defective and possibly contaminated as culture of the enriched sample showed bacteria the next day. A high (10%) proportion of PI-ve cells were seen on flow cytometry in comparison to previous experiments in the laboratory.
4	Cell bag was not clamped prior to starting selection hence the priming solution washed some cells into the cell collection bag, waste bag and negative selection bag. Despite attempts to salvage cells, the procedure was not successful.
5	The tubing set was not attached properly to the valves, introducing air into the system. As the cell bag was not affected the procedure was repeated with the same kit due to the unavailability of a spare kit.

Table 5-3 Technical problems encountered during clinical grade CSS

5.5 Adenovirus-specific T-cells in HSCT recipients with adenovirus infection

Samples were obtained from three patients with acute Ad infections, under 'Study of the immune responses to haematological disorders' approved by the Birmingham, East, North and Solihull Research Ethics Committee in June 2003 (Rec Number 1148). Demographics of these patients is summarised in Table 5-4.

Patient ID	Age	Sex	Diagnosis	Donor type	HLA matching	Conditioning	T-cell depletion
PID 01	57	M	Follicular lymphoma	UD	HLA matched	Fludarabine Melphalan Campath	Yes
PID 02	56	F	Relapsed AML	UD	One antigen mismatch	Fludarabine Melphalan Campath	Yes
PID 03	36	M	Multiple myeloma	UD	HLA matched	Melphalan TBI	No

Table 5-4 Demographics of patients with adenovirus infection following HSCT

AML –Acute myeloid Leukaemia, UD- Unrelated, TBI- Total body irradiation

Ad infection was detected in PID 01 on day 12 (day 0= day of stem cell infusion) and was commenced on cidofovir 5mg/kg once weekly. Despite virus clearance the patient died on day 71 post HSCT from multi-organ failure. Virus load obtained from the Virology Laboratory, Heart of England, UK are shown in Figure 5-8. Although blood samples were obtained, the lymphocyte numbers were too low to study an Ad-specific T-cell response. This patient was not HLA A*01 and hence not a candidate to check for TDL specific T-cells.

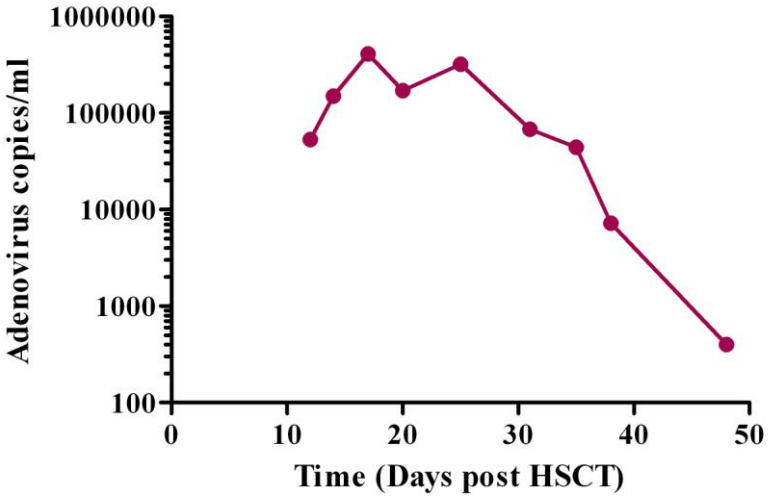

Figure 5-8 Ad copies/ml on patient PID 01

Virus load in the serum was quantified by PCR by the virology laboratory, Heart of England Hospital, and plotted against the days post HSCT.

Ad infection was detected in PID 02 on day 24 following transplantation and was commenced on cidofovir 5mg/kg once weekly. Virus load obtained from the Virology Laboratory, Heart of England, UK are shown in Figure 5-10(b). Ad-specific T-cells were detected by CCS and tetramer staining in this donor according to protocol 2.5.2. Figure 5-9 shows examples of CSS and tetramer staining on day 43 and the collated data is shown in Figure 5-10 (a). Though the rise in the frequency of Ad-specific T-cells may correlate with a reduction in Ad copy numbers there are no data points for 30-40 days in between. This patient had problems with grade 3 GvHD following Ad clearance she is alive and well.

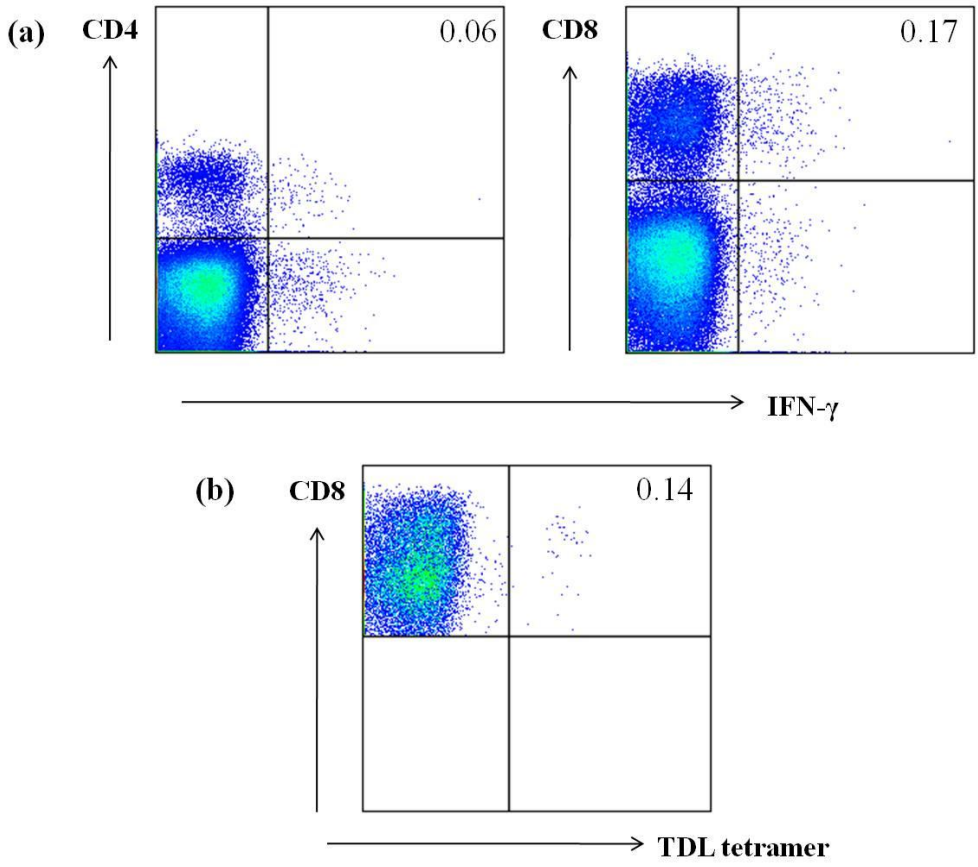

Figure 5-9 Ad-specific T-cells in patient PID 02 on day 43

PBMCs (10^6) from PID 02 were incubated with heat inactivated CTL102 ($2.5x10^4$ particles/cells, 16hr, 37°C) and CSS assay was performed according to protocol. PBMCs (10^5) were stained with TDL tetramer. Samples were labelled with antibodies to CD4, CD8, CD3 and PI. The number of (a) CD4 or CD8 T-cells releasing IFN-γ in response to Ad (b) TDL tetramer staining CD8 T-cells expressed as a percentage of the total.

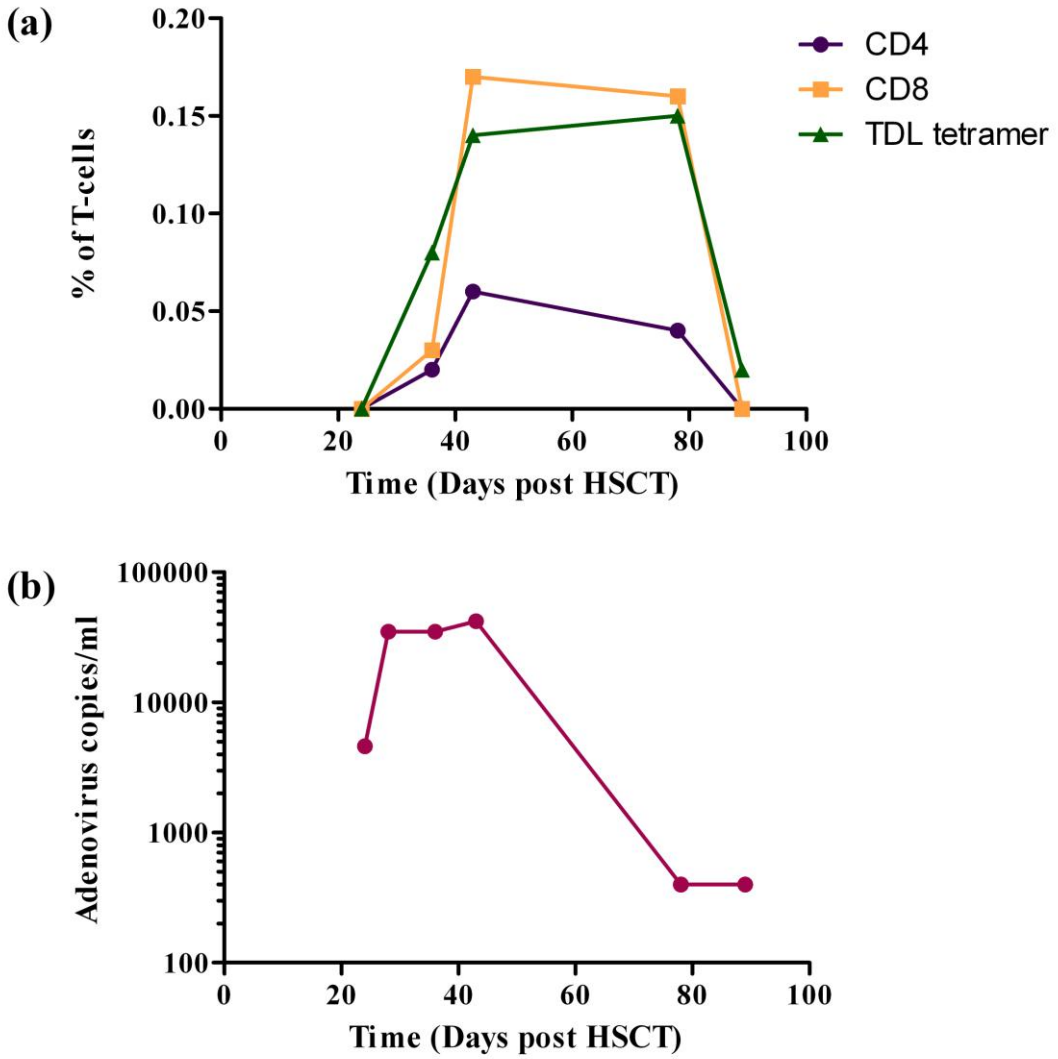

Figure 5-10 Collated data on PID 02

PBMCs (10^6) from PID 02 were incubated with heat inactivated CTL102 ($2.5x10^4$ particles/cells, 16hr, 37°C) and CSS assay was performed according to protocol. Remaining PBMCs (10^5) were stained with TDL tetramer. Samples were labelled with anti-CD4, anti-CD8, anti-CD3 and PI antibodies. (a) shows collated data on CD4/CD8 T-cells determined by either assay and (b) is the Ad DNA levels copies/ml of serum.

$1.2x10^3$ adenovirus genome copies/ml were detected in PID 03 on day 79, which reduced to < $4x10^2$/ml the subsequent week. On day 94 successful adenovirus-specific reconstitution could be demonstrated with a CD4 T-cell count of 0.05 and CD8 0.26% T-cells producing IFN-γ (data not shown). Tetramer-specific T-cells could not be stained as the patient was not HLA A*01. On day 402, 0.29% and 0.37% of CD4 and CD8 T-cells respectively were producing IFN-γ in response to heat inactivated CTL102 (Figure 5-11). In order to study the homing and effector phenotype of these cells, they were labelled with antibodies to CD45RA, CD45RO, CCR7, CD62L, LFA-1 or PI and analysed further by flow cytometry (LSRII). Ad-specific CD4 T-cells were $CD45RA^{low}$, $CD45RO^{high}$, $CCR7^{high}$, $CD62L^{high}$ and $LFA\text{-}1^{high}$ (Figure 5-12(a)) whereas CD8 T-cells were $CD45RA^{high}$, $CD45RO^{intermediate}$, $CCR7^{high}$, $CD62L^{intermediate}$ and $LFA\text{-}1^{high}$ (Figure 5-12(b)). This data needs to be interpreted with caution due to low number of data points.

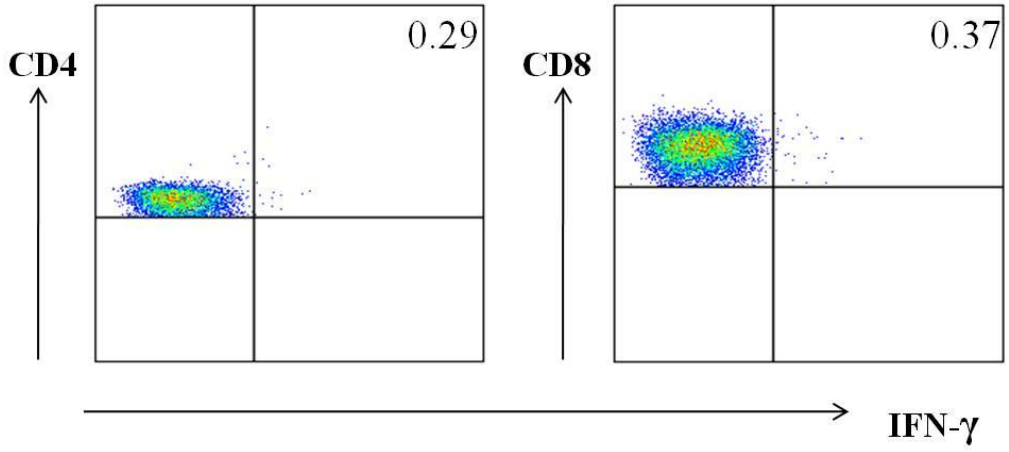

Figure 5-11 Frequency of Ad-specific IFN-γ secreting T-cells in PID 03

Ad-specific CD4 and CD8 T-cell frequency in Pat 03 after stimulation with heat inactivated CTL102 using CSS assay on day 402 following HSCT. Each FACS plot shows cells gated on CD3+, PI-ve cells. The numbers represent the frequency of T-cells calculated as percentage of total CD4 or CD8 T-cell.

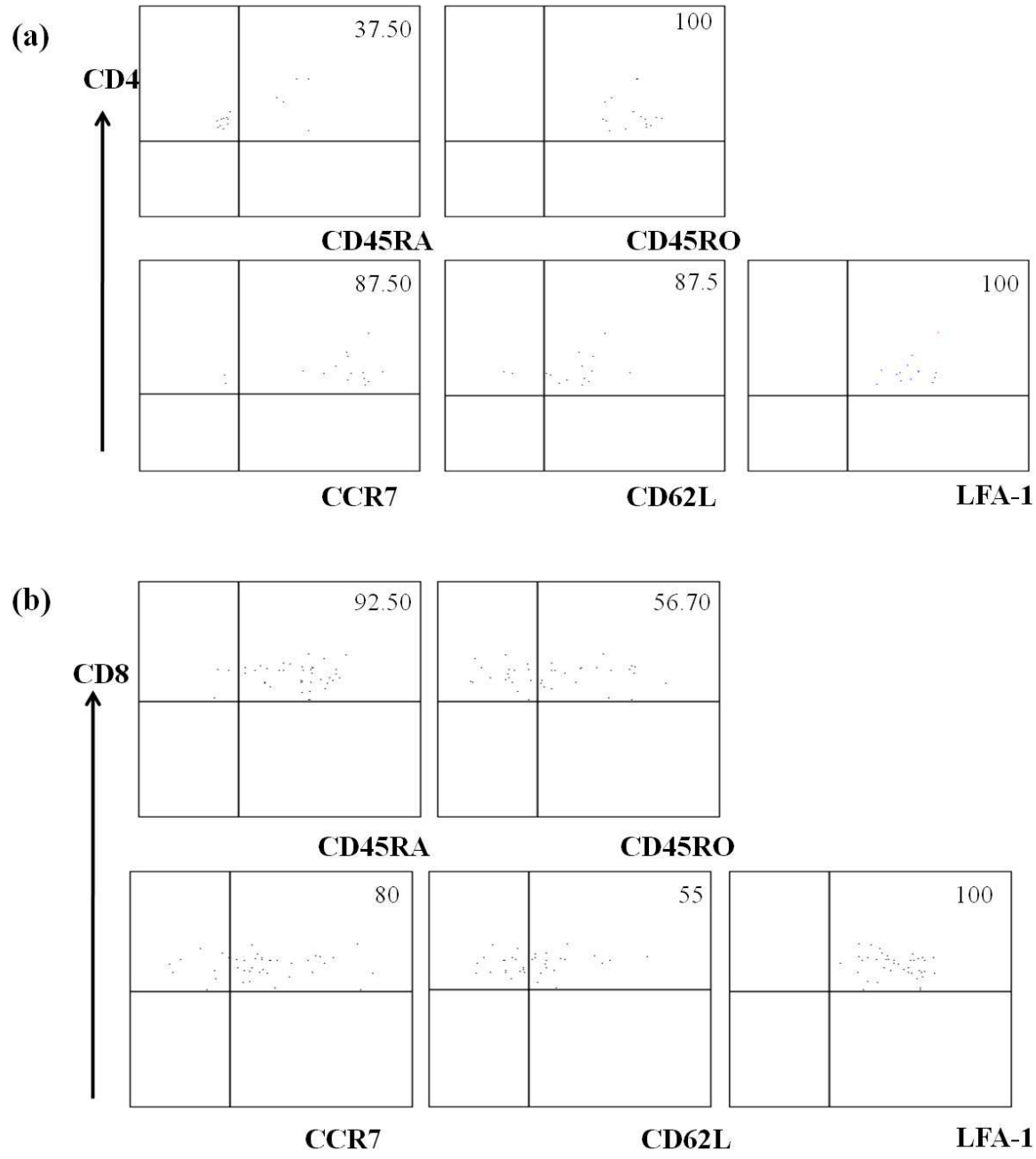

Figure 5-12 Phenotype of CD4/8 Ad-specific T-cells in PID 03

Ad-specific CD4/8 T-cell frequency in PID 03 after stimulation with heat inactivated CTL102 using CSS assay on day 402 following HSCT. Cells were labelled with antibodies to CD3, CD4, CD8, CD45RA, CD45RO, CCR7, CD62L and LFA-1 or PI and analysed by flow cytometry. Percentage of IFN-γ secreting CD4 T-cells (a) and CD8 T-cells (b) expressing the respective molecules. This data needs to be interpreted with caution due to low number of data points.

5.6 Discussion

Although not reproducible on fresh PBMCs, Streptactin PE staining was comparable to tetramer staining on polyclonal T-cell lines (Figure 5-3). Streptamer magnetic bead enrichment resulted in a significantly lower purity in comparison to the tetramer stained and enriched cells. The low enrichment could be attributed to the low frequency of these cells in peripheral blood. The streptamer protocol in comparison to the tetramer enriched sample involves multiple washing techniques which resulted in a significant loss (10 fold) of cells.

Recently Wang *et al* (Wang et al, 2010) identified (Wilms tumour suppressor gene-1) WT-1 derived HLA*A02 peptide-specific CD8 T-cells using streptactin staining in the peripheral blood of 40/40 healthy donors and 10/10 AML patients with a frequency of (0.08%-1.61%) and (0.13%-3.65%) respectively. They optimised the enrichment procedure by incubating the monomer, streptamer magnetic beads in a final volume of 90µl Buffer IS rather than 150µl as recommended by the manufacturer. On enrichment with streptamer magnetic beads Wang *et al* showed that the WT-1-specific CD8 T-cell frequency increased from (0.28-1.04%) to (4.3-26.0%), resulting in a 20-fold increase in purity. Cell frequency increased only by 10 fold in peptide stimulated polyclonal T-cell lines. Their staining pattern of the WT-1-specific T-cells showed CD8+ve and CD8-veT-cell population of CD3 gated cells staining for WT-1. The authors referred to these as high and low frequency double positive WT-1-specific T-cells but these could also be due to non-specific staining of the streptamer. As the frequency of the TDL-specific T-cell is comparable to WT-1 peptide-specific T-cell frequency it may be possible to use streptamers to enrich Ad peptide-specific T-cells, with some modifications of the manufacturer's protocol.

Recently a Phase III randomised controlled clinical trial, (Cytomegalovirus immunoprophylactic adoptive cellular therapy study-CMV IMPACT) comparing the

prophylactic infusion of CMV-specific T-cells selected by CSS (35 patients) or streptamers (35 patients) to standard practice of antiviral drug therapy (40 patients) was launched (ISRCTN74928896 URL: http://isrctn.org/ISRCTN74928896). Though CMV-specific T-cells occur at a 10 fold higher frequency than Ad-specific T-cells, experiences with respect to streptamer selection may help in further optimising staining and isolation techniques. Biotin labelled pentamers or dextramers are currently available (ProImmune Limited, Oxford, United Kingdom). T-cells stained by these pMHC multimers can be enriched by anti-biotin magnetic bead labelling (Miltenyi Biotech, Bergish Gladbach, Germany). Both these reagents are available to GMP standards allowing use in a clinical trial.

pMHC multimers are useful for isolation and enrichment of antigen-specific T-cells, but knowledge of epitopes restricts the MHC types that can be offered therapy. Functionally inert cells may be identified by this method. CSS assays allow the detection and enrichment of viable antigen-specific T-cells but the multistep procedure is laborious. In this setting rapid identification and isolation of the full repertoire of CD4 and CD8 antigen-specific T-cells would be advantageous. Activation markers identified as possible targets include CD69, CD25, CD137, CD38 and HLA-DR. A thorough investigation by Wolfl *et al* (Wolfl et al, 2007) found that CD69, CD25 and CD137 were the only markers upregulated sufficiently to differentiate antigen-specific T-cells from unstimulated T-cells. CD69 and CD25 were increased on all cells as non-specific activation markers. They concluded that CD137 was a good marker but the selection procedure was even longer (3 days) and retained a significant proportion of alloreactive T-cells.

The Ad-specific T-cells selected using LD20 met the criteria for the AdIT trial for potential adoptive transfer. Although 4/5 trial runs implemented for clinical grade enrichment of Ad-specific T-cells by CCS was not successful, a standard operating procedure for selection to

GMP grade standards could be set up. Further work will be required to ensure that the technical aspects are fully controlled to avoid loss of precious donor sample. Two other groups have recently reported success on this selection method for the purposes of adoptive transfer (Aissi-Rothe et al, 2010, Zandvliet, 2010 #976).

Data from the 3 patients with active Ad infection highlights the usefulness of both CCS assay and tetramer staining to study Ad-specific immune reconstitution. Patient PID01 was able to clear Ad before Ad-specific T-cells could be detected by CCS, probably as a result of the antiadenoviral effect of cidofovir. Patient PID 02 had a higher percentage of CD8 T-cells than CD4 T-cells at the time of Ad clearance (Figure 5-10). Similar observations have been documented in 3 patients who received adenovirus directed gene therapy as part of a trial to treat prostate cancer (Onion et al, 2009). A recent study on HSCT recipients with Ad infection, although Ad hexon-specific CD8 T-cells could not be detected in 13/13 patients, at the time of Ad viraemia, they were demonstrated after virus clearance in 2/13 by intracellular IFN-γ staining and 7/13 of the patients had Ad hexon-specific CD8 T-cells after maintaining PBMCs in culture following antigen stimulation (Zandvliet et al, 2010). They also observed higher frequencies of Ad hexon-specific T-cells after virus clearance.

The phenotype of Ad-specific T-cells in patient PID03 was compared with the mean values obtained for 10 healthy volunteers using the same experiment (Figure 5-13). The CD4 T-cells had CD45RA and CD62L expression levels similar to that observed in healthy donors ten days after antigen stimulation (Figure 4-27). CD8 T-cells on the other hand had a phenotype similar to that observed in donors immediately *ex vivo*. Zandvliet *et al* reported change in CD62L expression on Ad hexon-specific CD4 and CD8 T-cells on proliferation following antigen stimulation. The phenotype switch of Ad-specific CD4 T-cells in PID 03 may indicate a functional role of these cells. Though the phenotype switched observed in Ad-

specific CD8 T-cells following antigen stimulation and proliferation in healthy donors (section 4.3.2.1) it was not evident in PID 03 after virus clearance though the proportion of these cells was higher than that observed in healthy volunteers (Figure 3-17). A higher proportion of Ad-specific CD4 and CD8T-cells was observed following virus clearance by other groups (Feuchtinger et al, 2005; Zandvliet et al, 2010). This highlights that these CD8 as well as CD4 T-cells are proliferating and required in the event of Ad infection for virus clearance.

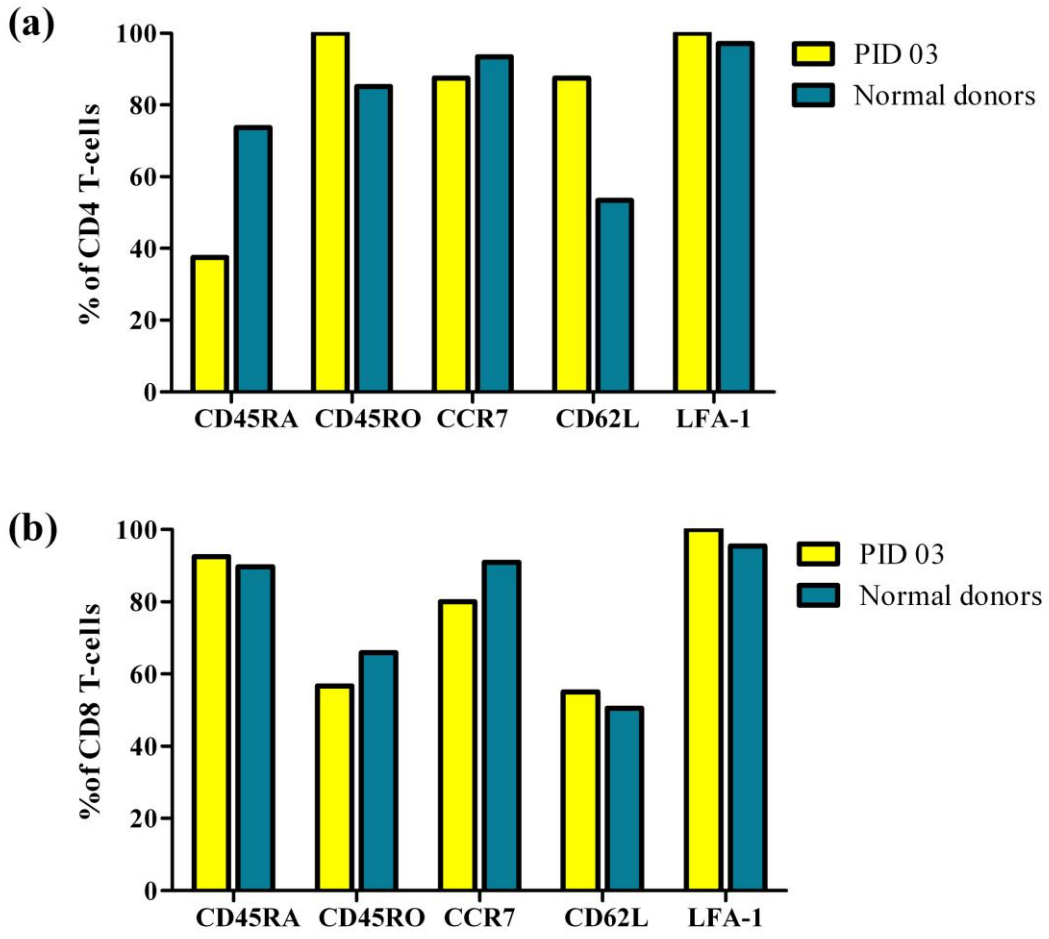

Figure 5-13 Data comparing the phenotype of patient PID 03 and healthy volunteers

CCS was performed on PBMCs from PID 03 and expression of CD45RA, CD45RO, CCR7, CD62L and LFA-1 was determined on CD4 and CD8 T-cells by flow cytometry Figure 5-12. This was compared with the mean values of the same parameters obtained on 10 healthy volunteers (Figure 4-23 and Figure 4-24).

6 Summary and Future work

Work described in this thesis supports the use of Ad-specific T-cells selected by CCS or tetramer selection for adoptive transfer purposes for HSCT patients with active Ad infection. These cells have good proliferative capacity, a minimally differentiated phenotype, cytotoxic ability across Ad species and can limit virus replication. Both methods can be used to monitor Ad-specific immune reconstitution in patients with active infection following HSCT or adoptive transfer.

6.1 AdIT trial

After establishing that the clinical grade selection of Ad-specific T-cells was feasible by CCS and tetramer, a trial was designed to study the safety and tolerability of adoptive immunotherapy of Ad-specific T-cells selected by either method for Ad infections in the HSCT setting. The phase I trial-'Adoptive immunotherapy for adenovirus infection in stem cell transplant recipients (AdIT)'- was to include three groups of patients: five patients whose donors have Ad-specific T-cells (detected by HLA multimers) to receive multimer-selected T-cells (multimer - Group I); five other patients to receive antigen stimulated, IFN-γ selected T-cells (CSS - Group II). Once five patients were treated in each Group I and II, provided safety criteria have been met, a further 5 patients eligible for multimer treatment will be offered multimer plus CCS selected T-cells (combination - Group III) (Figure 6-1). As Ad-specific tetramer staining cells are predominantly HLA A*01 there may be an HLA bias on comparing adoptive transfer by these two methods. No statistical power was required (as per advice from statistician) at this stage to avoid HLA bias as the aim of the trial was to study the safety of adoptive transfer. The aim of the trial was to study the safety and tolerability of each arm, compare outcomes of each treatment groups, measure virus load before and after adoptive transfer and adenovirus specific immune reconstitution following adoptive transfer.

Primary objectives

1. To determine the safety and tolerability of administration of Ad specific donor T-cells to SCT patients
2. To evaluate Ad-specific T-cell expansion and survival of Ad antigen stimulated T-cells and/or multimer selected CD8 T-cells
3. To measure the effect of treatment on virus load

Secondary objectives

1. Assessment of Ad-specific immune recovery following adoptive transfer
2. To compare single and dual treatment modalities

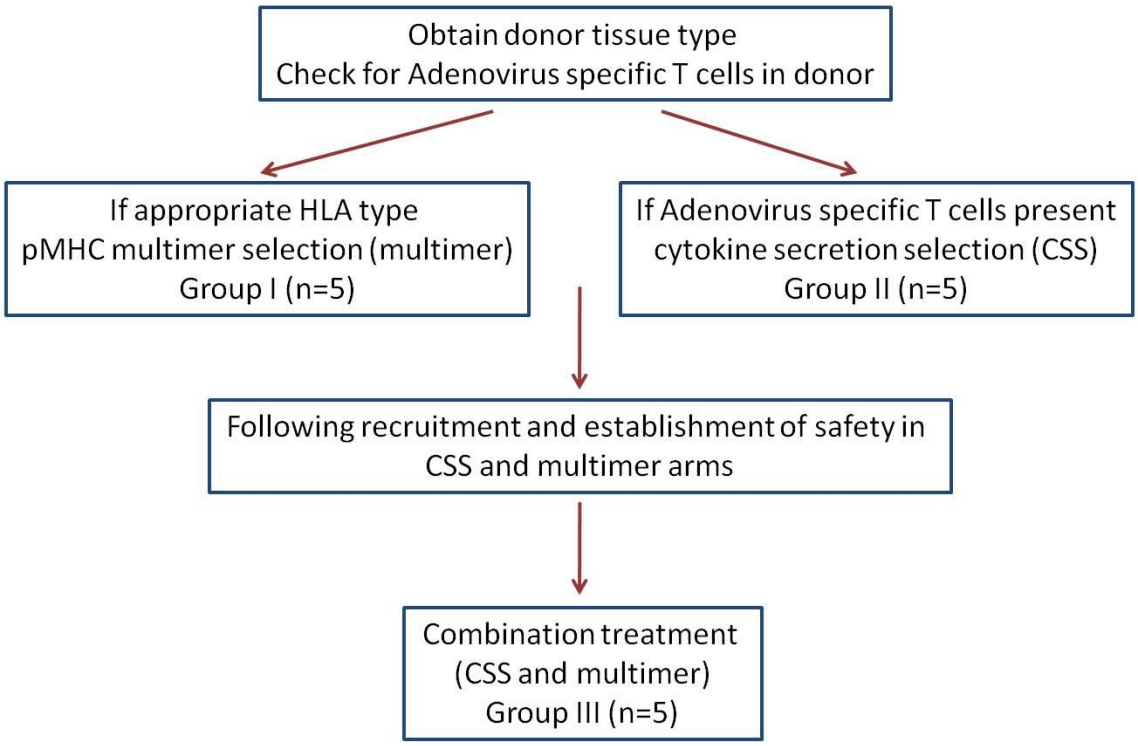

Figure 6-1 AdIT Trial design

Adoptive immunotherapy for adenovirus infections in HSCT recipients (AdIT)

Criteria to identify eligible patients were defined

Inclusion criteria

1. Allogeneic SCT recipient
2. UK donor >16 years old
3. Patient or guardian able to give consent if HSCT recipient younger than 16
4. Evidence of adenovirus infection, defined as rising or elevated titre of Adenovirus measured in whole blood/serum by QPCR on 2 consecutive occasions

   Or

5. Persistence or recurrence of Adenovirus DNA in peripheral blood after 2 weeks of antiviral therapy.

Exclusion criteria

1. Grade 3 GvHD
2. Previous adoptive transfer

The patients will be recruited from the West Midlands transplant centres, Birmingham Children's Hospital, Birmingham Heart of England Hospital and University Hospitals Birmingham. The local transplant unit will contact the donor and request a minimum of $1x10^8$ PBMCs (500ml of blood). Cell selection is to be carried out at West Midlands National Blood service, stem cell laboratory according to Good Manufacturing Practice (GMP), under the supervision of Clinical Scientist, Head of Specialist Procedures, NBS, Birmingham. A single dose of selected adenovirus-specific T-cells was to be infused to the HSCT recipient who meets the above criteria, within 24 hours of donor harvest. Each participant will be followed up for a period of one year after adoptive transfer to assess response in terms of viral load and adenovirus-specific immune reconstitution. The estimated recruitment period is 3 years.

The yield of Ad-specific T-cell obtained by each selection method will depend on the number of cells available for selection and the response to the antigen. In order to limit the risk of GvHD from the infused cells, limits will be placed on the number of infused cells:

1. $\leq 10^6$/kg Ad-specific T-cells will be infused
2. $< 10^5$/kg non-specific T-cells will be infused ($< 5 \times 10^4$/kg for haploidentical transplant recipients)

Primary end points for systemic safety and tolerability will be clinical examination and laboratory investigations, using local standard transplant guidelines.

Ad-specific T-cell reconstitution will be evaluated by the following:

1. Cytokine capture assay to evaluate overall Ad T-cell response (CD4 and CD8)
2. Adenovirus derived pMHC multimer staining to quantitate epitope-specific CD8 T-cell response
3. Contribution of donor T-cells by chimerism/clonotype analysis
4. Humoral immune responses - virus neutralising activity

Information on virus load, measured by PCR, will be provided by the participating centres. Virus isolation and characterisation will be carried out at the School for Cancer Sciences laboratory. This trial protocol was given approval by the Gene Therapy Advisory Committee (GTAC) in April 2008, provided Medicine and Health Regulatory Authority offered authorisation.

The role for adoptive transfer of Ad-specific T-cells selected by CSS in HSCT recipients has been shown in the phase I trial by Feuchtinger et al (Feuchtinger et al, 2006) but that of multimer selected T-cells remains to be demonstrated. The proposed randomised control trial comparing the 2 selection methods, AdIT described in section 6.1, would offer more information into the role of CD8 T-cells in Ad-specific immune reconstitution as well as Ad clearance, as well as aid in studying the impact of combining the selection methods (currently

class II tetramers for known Ad epitopes are unavailable), thereby increasing the CD8 T-cells. As more class II tetramers become commercially available both class I and class II tetramers could be combined for cell selection and adoptive transfer. Despite the poor yield of antigen-specific T-cells with streptamers alternative agents such as biotin labelled pentamers or dextramers (ProImmune Limited, Oxford, United Kingdom) can be used to enrich antigen specific T-cells using GMP-grade anti-biotin magnetic beads (Miltenyi Biotech, Bergish Gladbach, Germany) enabling the launch of the proposed trial. Another hurdle for the future of the above trial is the availability of antigen as the use of CTL102 is no longer possible due to withdrawal of permission by the manufacturer. The comparable antigen responses obtained using hexon as antigen Figure 3-18 as well as experiences by other groups (Aissi-Rothe et al, 2010; Feuchtinger et al, 2006) support use of hexon which is commercially available.

Cytolytic CD4 T-cells specific to CMV (Crompton et al, 2008), EBV (Khanolkar et al, 2001) and HSV (Yasukawa et al, 1999) have been described. In the context of the ability of Ad to down regulate MHC class I expression (Ackrill et al, 1991) it is possible that CD4 T-cells play a predominant role in cellular immunity. As Ad-specific T-cells share a phenotype similar to influenza virus-specific T-cells it is possible that these cells are maintained at very low levels with minimal antigen stimulation. They are capable of rapidly switching to an effector phenotype, enabling virus clearance, which may explain the phenotypic differences in CD4 T-cells observed in the patient recovering from Ad. In addition to analysing the Ad-specific T-cells in HSCT recipients by CCS or multimers, chimerism studies may be valuable in providing information on host or recipient DNA following HSCT. In view of the phenotypic similarity of Ad-specific T-cells to virus-specific memory T-cells resistant to chemotherapy (Turtle et al, 2009), it will be of interest to study the origin of these cells, *i.e.* whether they are of donor or recipient origin or both.

Ad infection is a significant problem in allograft recipients. A risk stratified approach whereby high risk patients (mainly cord, haploidentical and unrelated donor recipients) are identified upfront and treated with cidofovir on intial detection of the virus may allow identification of patients who may benefit from adoptive therapy (Lindemans et al). Cytokine secreted T-cells have shown to be beneficial in this setting (Feuchtinger et al, 2006) and maybe the first choice for adoptive transfer according current knowledge. The role of Ad-specific tetramer selected T-cells cannot be refuted and they may well be sufficient in virus clearance based on encouraging results for allograft recipients with CMV infection (Cobbold et al, 2005). In summary this thesis has improved the understanding of Ad-specific immune response and supports a clinical trial which will benefit HSCT patients with Ad infection.

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8 Appendix

8.1 Appendix I

Standard Operating procedure for the Enrichment and Isolation of Adenovirus specific T-cells using the CliniMACS Cytokine Capture System

SAFETY PRECAUTIONS AND HAZARDS

All preparation steps should be performed using aseptic technique in a clean area. All leukapheresis products and tubing sets in contact with body fluids should be treated as biohazards.

The CliniMACS Cytokine capture system is not for use with patients known to have sensitivity to mouse immunoglobulins or iron-dextran.

INSTRUCTIONS

The maximum capacity of the CliniMACS cytokine capture system is 1 x 10^9 total cells. Remove the appropriate volume of cellular starting product donated by a responsive donor who has previously been screened for all mandatory virology markers.

Cellular starting products should not be older than 24 hours when starting the labelling or separation procedure.

The super-paramagnetic iron-dextran magnetic particles covalently conjugated to antibody are 50nm in diameter. The Column is an ion-sphere with matrix 0.3μm and is magnetised by a strong magnet, the pre-column is the same matrix as the separation column but it is not magnetised, it acts as a filter.

Maximum loading Routine set 6 X 10^{10} TNC (Total Nucleated Cells)

Column sample should be approx 65 ml

DAY 1

Buffer and medium preparation for use throughout

1. Dress and enter the clean room
2. Spray IPA and wipe all consumable items required for the selection procedure into the hatch
3. Spray IPA and wipe then pass the plastic container containing the harvest bags into the hatch
4. Spray IPA and wipe all items from the hatch. Place on stainless steel trolley
5. Wipe the laminar flow hood with Klericide and lay out 2 TSA agar settle plates on either side of the work area and a SABC plate at the centre back.
6. Monitor the air by using the particle counter whilst using the hood.
7. Monitor the surface by touching contact plates to the surface at the end of the laminar hood use.
8. Monitor operator by performing finger dabs onto settle plates at the end of the procedure.
9. Check all bags and paperwork within the clean room and sign the data processing sheet

10. To each of 5 litre packs of CliniMACS PBS/EDTA add 25 ml of 20% HAS (Human Albumin solution) (final 0.5%). Keep one at room temp and the other four at 4^{O}C.
11. Supplement each of 5 x 500ml bottles of RPMI medium with 25ml AB serum (final 5%).
12. Transfer two of the bottles (1 litre) into two 600ml transfer packs, using mixing needle and syringe after attaching sampling site couplers, and retain at room temp.
13. Transfer 1 litre into a 2 litre transfer pack and warm to 37^{O}C.

14. Transfer the remaining 500ml into a 600ml transfer pack and place in the fridge at 4^{o}C.**(The final volume of RPMI needed depends on the cell count. The above is for 1x10^{9} cells. For 1x10^{8} cells you will need 10 times less. The important part is to keep equal amounts @ 4^{o} C and 37^{O} C**
15. Prepare a 600ml transfer pack with 600ml water to act as centrifuge balance.

Mononuclear cell preparation

16. Isolate a section of side tube from the collection bag and perform a cell count on initial donation/s to estimate total lymphocyte content (outside clean room).
17. If the donation is blood perform a mononuclear cell preparation using lymphoprep to isolate lymphocytes. Transfer to a 600ml transfer pack – dock on a waste bag but do not open seal. Centrifuge RT 10mins, 200g acceleration 7 no brake.
18. Remove carefully from centrifuge, place into a plasma press and open heat seal. Press off supernatant into waste bag and discard. Re-suspend cells by rolling on edge of bench.
19. First wash with CliniMACS buffer/ RPMI at RT: Dock on buffer bag via transfer set, add vol to 600ml. Dock on a waste bag but do not open seal. Centrifuge RT 10mins, 200g acceleration 7 no brake.
20. Second wash use RPMI/serum medium at RT. Spin as above, press off supernatant.
21. Mix bag, seal off a length of side tube and perform a cell count on this sample.
22. Resuspend cells in warm RPMI at a vol to give approx 1 x 10^{7}/ml max volume = 100ml (e.g. if 0.5 x 10^{9} lymph then resuspend in 50ml).

Stimulation with antigen at appropriate concentration

23. **Put 1ml of the cell suspension into a plastic well of a 24 well plate or 0.5ml into 2 plastic wells of a 48 well plate, to act as the un-stimulated control as a measure of spontaneous cytokine secretion**.
24. Inject the remaining volume into a Miltenyi culture bag. The Ideal conc. is 50ml to 50cm^{2} – hence the 100ml bag is a little large. Bend over the bottom 1/3 and support with blue plastic spencer wells to reduce surface area. For volumes of 60ml and above leave the bag unfolded. Do not write on the bag plastic since the ink may permeate the bag.
25. Put bags and plate into the 37^{o}c incubator.
26. As late as possible on day 1 inject the heat inactivated viral antigen at a minimum 1000 virus particles /cell.(on first expt 2 x 10^{12} particles were used for the total lymphocyte count of 1.1 x 10^{9} (0.5 x 10^{9} (buffy 1) and 0.6 x 10^{9} (buffy 2))
27. Set the centrifuge to 4^{O}C, 300g, acceleration 7 and brake 3.Start spinning to attain temp. Close lid between uses to maintain 4^{o}C. (Program 7)
28. Retrieve bag/s and the control plate from incubator
29. Retrieve the cold RPMI/serum medium bag from fridge
30. Transfer the contents of the wells to a 50ml tube (needed to hold 20ml) and top up with a little of the cold RPMI medium via the side tube.
31. Transfer the contents of the culture cell bag/s to a 600ml transfer pack, rinse the cell bag with the cold RPMI medium bag and transfer washings into the transfer pack without leaving a port. (Never spin bag with port)
32. Dock an empty transfer pack onto the cell pack **but do not open the heat seal.**
33. Place the attached bags and centrifuge balance bag into the centrifuge and leave to cool for 10 minutes.
34. Let the 50ML tube with un-stimulated control cells (neg control) cool in the fridge for 10 mins.
35. Spin the 50ML tube (neg control) in the lab centrifuge and the cells in the bag centrifuge as before

36. Remove the cell bag carefully from the centrifuge and place in the plasma press. Weigh the waste bag so that the final volume for the cells is at little as possible ~30-40ml**.**
37. Release the heat seal and allow the supernatant to be expressed into the waste bag until a cell pellet remains. Re-suspend pellet over edge of bench.
38. Remove supernatant from cell pellet in 50ML tube (neg control) and re-suspend.

Catch reagent – to be used directly from fridge

39. Take up 7.5 ml of the cold catch reagent into a 10ml syringe, using an **airway** to help flow.
40. Take up the dregs from the bottle (about 0.1ml) into a 1 ml syringe.
41. Add the large vol to the cell bag and the smaller vol to the neg control.
42. Incubate bag and 50ML tube for 5 mins at $4^{o}C$.
43. For catch phase need to dilute the cells to 1 x 10^{6}/ml with warm medium – use the pre-warmed litre of medium in the 2 litre pack. If cell count is less then do not need 1L, e.g. for 1x10^{8} cells only 100ml is needed and hence can use 600ml transfer pack.
44. First, make neg control cells up to 10ml with warm medium then dock the transfer pack with warm medium to the cell pellet pack (600ml/ 2L).
45. Add medium to cells, rinse and return to empty 2 litre pack. (For 1 x 10^{9} cells, 1 litre is required to achieve 1 x 10^{6}/ml.) The larger vessel is needed to allow room for the buffer following the secretion phase
46. Incubate for precisely 45 minutes at $37^{O}C$, mix a little during this time

Stop reaction and wash

47. Following the 45 minute incubation add 10ml of cold CliniMACS buffer (taken by syringe from the buffer bag) to the neg control (total now 20ml) then dock on and add equal amount of (1 litre if it was 1L of warm medium) of cold buffer swiftly to the cell bag using a wide bore connector.
48. Incubate cell bag and 50ML tube of control cells at $4^{o}C$ (or on ice) for 10 mins.

Volume reduction (This step is more relevant when the starting cell count is >1x10^{9})

49. Dock the 2 litre pack containing cells and buffer to a 600ml pack and transfer 500ml across. Dock this transfer pack to another to act as the waste bag but do not open seal. Place in centrifuge to keep cold.
50. Repeat the above step to create a balance for the first.
51. Prepare another two packs in the same way, allowing weight to accommodate the neg control. Centrifuge on same setting as before.
52. Remove bags and press off supernatant then re-suspend the cell pellets. Sterile dock the cell containing bags one by one and combine into one bag.
53. Spin down control cells to one pellet.

Wash then incubate with magnetic label

54. Add cold CliniMACS buffer to fill the transfer pack, then centrifuge against the original balance on the previous setting.
55. Re-suspend cell pellet in cold buffer and spin the neg control with the bag
56. To the bag cell pellet add 7.5ml of magnetically labelled antibody again use 0.1ml of the dregs to add to the re-suspended neg control pellet.
57. Incubate 15 mins at $4^{o}c$ or on ice.
58. Top up neg control to 10 ml with cold CliniMACS buffer then dock on the bag of CliniMACS buffer to the cell bag. Top up to match the weight of the centrifuge balance. Dock on a waste bag but do not release seal.
59. Centrifuge bag as before at $4^{o}C$ and the 50ML tube in the lab centrifuge.
60. Press off the supernatant and re-suspend the cell pellet in approximately 65ml of CliniMACS buffer.
61. Take a 1ml start "pre-sort" sample and keep at $4\ ^{O}C$ for analysis.
62. Resuspend the Neg control pellet in about 200ul Facsflow and keep at $4^{o}C$ until analysis.

Automated Selection of Labelled cells

Preparation of the CliniMACS instrument

63. Switch on the machine - when the CliniMACS cartoon appears press 'ENT'.
64. Programme menu appears - the option required is Enrichment 3.1 or **3.2**
65. Enter tubing set ref: If a routine size set is being used the code is 161-01, if a research set is used code is 166-01. Press 'ENT'

Preparing tubing set

66. Make up 2 ice packs using plastic bags, ice and the Multivac in the stem cell room. Seal the bag at ~ 300 so that enough vacuum remains in the bag
67. Open the CliniMACS tubing set, record lot number on worksheet. **Make sure luer connections are tightly closed on columns. Check that the blue connectors are tight.**
68. Remove the tubing set from its packaging and lay out in the laminar hood. Arrange the tubes so that on the upper end of the tubing set, buffer bag, labelled cells and priming bag are arranged in the order from left to right.
69. Take a small collection bag (150ml) and heat seal its loose end. Then attach it to the **only loose end** at the lower port of the tubing set using a luer connector. The lower end of the tubing set will have enriched sample, negative fraction bag and the waste bag
70. Use an airway to pierce the rubber seal on an unused buffer bag. Attach the buffer (PBS/EDTA/0.5% HSA) bag to the top left port of the tubing set and use blue plastic **spencer wells (1)** to close the line. Attach the ice packs to the front and back of the buffer bag.
71. Attach the Pall filter to the bubble trap, pushing spike very firmly into the filter using a screwing motion - may feel click. **This is a possible leakage point.** *Immediately* put blue plastic **spencer wells (2)** on the line just below bubble trap and filter.
72. Attach the labelled cell bag to the Pall filter. Record lot number of filter on worksheet.
73. Carefully remove the set from the laminar hood, holding the three bags (buffer bag, labelled cells and priming waste bag from left to right) high for attachment to the hangers on the CliniMACS machine.

CliniMACS Procedure:

74. Following instructions on screen; tuck the filter behind the stainless steel bracket.
75. Load pre column if present - wings of plastic on top of column should project forwards - 'ENT'.
76. Insert selection column and adjust tubing into valve 5 press 'ENT';
77. Load tubing into upper pinch valves (1, 2, 3, 4,)
78. Insert tubing into liquid sensor at top of instrument - 'ENT'.
79. Open pump door, insert tubing into peristaltic pump, turning pump mechanism clockwise to guide tubing in and turning anti-clockwise to check tubing is seated correctly. Close pump door - 'ENT'.
80. Load tubing into lower pinch valves as indicated (7, 8) - 'ENT'.
81. Load tubing into lower pinch valves (6, 9, 10, 11) - 'ENT'.
82. Insert waste/ negative fraction bags into compartment.
83. Seating valves – wait.
84. Check valves 'ENT'.
85. Press 'ENT' for attachment of selection buffer bag and Pall filter.
86. Recheck tubing and attachments - 'ENT'
87. **Remove spencer wells (1).** Start prime (1-2min) by pressing 'RUN' - check for leaks and check tubing at end. (N.B. Cannot return to instrument set-up procedure after this step.)
88. Inspect buffer waste bag and priming waste bag - should be some fluid in each. Should be no fluid in negative fraction bag, cell collection bag, bubble trap or Pall filter 'ENT'.

89. **Remove spencer wells (2).** Press 'ENT' for attachment of cells to Pall filter. **Make sure sterile docking seal is broken between tubing set and product bag, if it exists, or undo clamp on adaptor.**
90. Check liquid sensor - 'ENT'
91. Mix cells in sample bag. Press 'RUN' to start procedure - 45 mins to completion. Open the luer connector to the enriched sample bag.
92. When screen reads 'selection completed', seal and disconnect collection bag. Weigh and record weight on worksheet. Transfer bag to the laminar flow hood
93. Remove the tubing set and bags from the CliniMACS press 'ENT'. Enter **process code** on worksheet, shut down.
94. Disinfect the CliniMACS instrument with Klericide A/B.
95. Helpline : Technical advice 0049-2204-8306-80
Jason Jones 01483 799800

Trouble-shooting problems associated with the CliniMACS procedure

96. May get column clogging if
- sample too old
- not equilibrated to room temperature
- not stored at < 200 x 10^6/ml
- overloaded at > 4 x 10^8/ml

97. If column is blocked the tubing below the column will be kinked due to a vacuum in the line. Press 'Stop'.
98. Choose '2' and the process will continue with the cells already loaded on the column. Change to a new tubing set for remaining cells. ***(Always have spare tubing set to hand)***
99. Emergency programme '4' elutes all cells in tubing set in 100ml volume.
100. If there is a short break in the power supply, clamp off cells, buffer, waste bag, column, below 9, 10 and 11 pinch valves. Remove pump tubing.
101. When power is re-instated, programme reverts to beginning of run.
102. Key through to stage where procedure ceased due to power cut, press 'Stop' and have 10 min in which to re-install pump tubing and remove clamps.

Sampling

103. Within the laminar hood sample 2ml of neg fraction and transfer 1ml into each aerobic/ anaerobic BacTalert bottles.
104. When starting with 1 x 10^9 lymphocytes a 1ml sample is sufficient from the enriched fraction for lower starting cell numbers use a higher volume for analysis i.e. for a starting range 1 – 5 x 10^8 sample 10ml, for 5 – 9 x 10^8 sample 5 ml spin cells down and resuspend.
105. Take a 1 ml from the neg fraction for FACS analysis
106. Remove the neg control sample and the pre-sort sample from the fridge and pass all samples out in a rack via the hatch.

Clean room exit

107. Pass clinical items and equipment out of the clean room via the hatch
108. Spray and wipe the hatch with Klericide A/B solution
109. Spray the clean room floor with Klericide A or B and using a mop with a clean mop head wipe the floor as moving towards the door. Also mop the area of the inner change room nearest the clean room.
110. Remove garments and place outer blue garments in box near clean room door for laundering.
111. Retain undergarments in a garment bag if the clean room is to be entered again within the week, if not place for laundering
112. Place clinical waste plastic bag in a yellow clinical waste container in the research lab and waste paper in the waste bin

113. Take culture bottles to the Vat room, complete a CliniPac with the details of the bottle and date. Send culture bottles to the Microbiology dept of the QEH for analysis.

FACS Analysis

114. Unstimulated fraction, start "pre-sort" sample, product and negative fraction are all analysed.
115. Eight Falcon tubes are required to allow each fraction to be assessed for CD4 and CD8.
116. Use unstimulated control as resuspended from fridge.
117. Use neg fraction.
118. Set up assay as follows :
119. To CD4 tubes add 10µl CD4-FITC, 10µl CD14 PerCP (optional), 10µl IFN gamma –PE (sent with kit).
    To CD8 Tubes substitute the CD4-FITC with CD8-FITC all other antibodies the same.
120. Incubate at RT for 10 mins in the dark.
121. Add 5µl 7AAD and 1ml pharmlyse to neg control, pre-sort and neg fraction, add only 250µl pharmlyse
122. Incubate for 5 to 10 mins
123. Set up cytokine template on the FACS – instrument settings Cytokine capture in Cell quest Experiments folder.
124. Analyse 100,000 events for all tubes except product where count as many as possible.
125. Allow file count to increase and change name on parameter description and CD4 /CD8 status.
126. R1 region should be around lymphocytes, R2 region is to include cells that are negative for CD14 (i.e. monocytes) and 7AAD (i.e. live).
127. The FL3 vs. IFN dot plot serves to show dead cells that are IFN neg but also any on the diagonal low right are true IFN-gamma live events
128. The quadrant stats are based on the gate G2 where R1 and R2 apply i.e. live lymphocytes with no monocyte contamination.
129. Neg control of unstimulated cells should have a CD4 or CD8 pos population but no/few IFN-gamma events.
130. Start sample should contain IFN-γ pos events if the stimulation has worked – present in MR quadrant. % = MR/ µr+µl.
131. Product should show increased numbers of IFN-γ cells if the enrichment has been successful.
132. Neg fraction should have no/few pos events for IFN-gamma – i.e. nothing going through the system and not being loaded.

8.2 Appendix II

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Adenovirus vector-specific T cells demonstrate a unique memory phenotype with high proliferative potential and co-expression of CCR5 and integrin $\alpha_4\beta_7$

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Running title: Ad5-specific T cells express CCR5 and integrin $\alpha_4\beta$

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Brief Communication

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