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Isolation and culture of T cell clones for adoptive immunotherapy and epitope discovery

Daniel Verdon

A thesis submitted in complete fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences, The University of Auckland, 2016.
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I would like to thank my supervisor, Professor Rod Dunbar, for the opportunity he granted me in undertaking this research in his lab, and for the new opportunities and prospects that have followed. I will always have great memories of my time here and have been fortunate to make many lifelong friends.

I would also like to thank all the many people who have helped (and taught) me as I have progressed through this project – but particularly Drs Anna Brooks, John Taylor, James Ussher, James Dickson and Hilary Sheppard.

I am proud and grateful acknowledge the unconditional love and support of all my family, who have been there for me in so many ways whenever I have needed them.

Finally and most importantly I dedicate this thesis to my wonderful wife Christine, who has always believed in me and supported me unwaveringly through this often difficult journey. I could not have done this without you, and I hope that completing this thesis opens up new and exciting possibilities for our life together.
Abstract

This research project built on previous findings within the host laboratory that naïve CD4+ and CD8+ T cells can be stimulated and maintained *in vitro* under the influence of the common γc cytokine IL-7 and retain central memory characteristics. Further, this project aimed to expand these observations to the production of single-cell derived CD4+ and CD8+ T cell clones of known and unknown epitope specificity. We hypothesised that administration of cytokines IL-7 and IL-21 during T cell cloning would allow retention of ‘central memory’ characteristics and decouple proliferation from differentiation. The specific aims of this research project were to investigate the functional and phenotypic consequences of naïve and memory CD8+ T cell expansion and maintenance using various common γc cytokine treatments; to investigate the application of IL-7 and IL-21 to antigen-specific CD8+ T cell cloning via pentamer- and activation-marker-guided FACS, and to develop a protocol for the activation-marker-guided isolation and expansion of protein- or synthetic long peptide-specific CD4+ and CD8+ T cell clones without prior knowledge of their minimal peptide epitope. This study demonstrated that both naïve and memory T cells could be isolated and cloned with high efficiency under the influence of IL-7 and IL-21, and retained proliferative potential and memory phenotype associated with anti-tumour efficacy in clinical trials. This study further demonstrated that temporal detection of cell surface activation markers allowed isolation of T cells of unknown epitope specificity, and facilitated the definition of novel peptide:MHC restrictions in T cells specific for the therapeutically relevant cancer-testis antigen NY-ESO-1.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive cell therapy</td>
</tr>
<tr>
<td>AdV</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa Fluor®</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CCR</td>
<td>CC Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>TCM</td>
<td>Central memory</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CXCR</td>
<td>CX Chemokine receptor</td>
</tr>
<tr>
<td>d/D</td>
<td>Day(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunosorbent spot assay</td>
</tr>
<tr>
<td>TEM</td>
<td>Effector memory cells</td>
</tr>
<tr>
<td>TEMRA</td>
<td>Effector memory CD45RA+ cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter (light)</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational force</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Human serum</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ICFC</td>
<td>Intracellular flow cytometry</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T cell costimulator (CD278)</td>
</tr>
<tr>
<td>IFN-</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>K</td>
<td>Kilo</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor subfamily G member 1</td>
</tr>
<tr>
<td>LEAF</td>
<td>Low-endotoxin, azide-free</td>
</tr>
<tr>
<td>Lef-1</td>
<td>Lymphoid enhancer-binding factor 1</td>
</tr>
<tr>
<td>m</td>
<td>Milli</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MQ</td>
<td>MilliQ</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>Mono-DC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>T&lt;sub&gt;N&lt;/sub&gt;</td>
<td>Naïve cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
</tbody>
</table>
NKT  Natural killer T cells
NY-ESO-1  New York Esophageal (squamous cell carcinoma -1)
OD  Optical density
ON  Overnight
p  pico
PAGE  Polyacrylamide gel electrophoresis
PBMC  Peripheral blood mononuclear cell
PBS  Phosphate-buffered saline
PCR  Polymerase chain reaction
PMA  Phorbol-12-myristate-13-acetate (PMA)
PE  R-phycoerythin
PerCP  Peridinin chlorophyll protein
PE-TR  R-PE-Texas Red
pMHC  peptide:MHC
r  Recombinant
R  Receptor
rAAV  Recombinant Adeno-associated virus
RNA  Ribonucleic acid
RT  Room temperature
rpm  Revolutions per minute
T_{SCM}  Stem cell-like memory T cells
s  Second(s)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLP</td>
<td>Synthetic long peptide</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter (light)</td>
</tr>
<tr>
<td>SSP</td>
<td>Synthetic short peptide</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Tcf-1</td>
<td>T cell factor 1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;Rm&lt;/sub&gt;</td>
<td>Tissue-resident memory T cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>V&lt;sub&gt;gc&lt;/sub&gt;</td>
<td>Viral genome copies</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>β&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Common IL-2/15R beta chain (CD122)</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>γ&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Common gamma chain (CD132)</td>
</tr>
<tr>
<td>κ</td>
<td>Kappa</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
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Chapter 1. Introduction

1.1. T cell function and memory generation.

1.1.1. Introduction to T cell mediated immunity.

T cell precursors develop from haematopoietic stem cells in the bone marrow and traffic to the thymus as ‘cluster of differentiation’ (CD)4/CD8+ thymocytes. Here, the pre-T cell thymocytes express and rearrange the V, D and J segments of the TCRα and TCRβ genes that encode the two chains of their T cell receptor (TCR) to create a highly variable peptide-biding domain (Chou et al., 1987). These pre-T cells then express both CD4 and CD8 (designated double-positive) and undergo ‘positive selection’ in the thymic cortex, whereby pre-T are selected for survival based on the ability of their rearranged TCRα and β chains to form a functional heterodimer and to recognise self-Major Histocompatibility Complex (MHC) class I or II molecules through interaction with cortical thymic epithelial cells (cTEC) expressing a limited repertoire of peptides generated by a unique proteasome (Murata et al., 2007). Surviving pre-T cells (typically <10% of thymocytes) typically commit to single-positive CD8 or CD4 expression, based on their positive selection being mediated by MHC class I or II respectively. Positively selected pre-T cells finally undergo positive section mediated by medullary thymic epithelial cells (mTEC). mTEC collectively express the complete range of transcripts that can be presented by somatic cells, under the control of the transcriptional regulator AIRE (Anderson et al., 2002). Single-positive pre-T cells that recognise self-peptide presented by mTEC with high avidity are instructed to apoptose through a tumour-necrosis factor apoptosis-inducing ligand (TRAIL) and Bim-mediated pathway (Klein et al., 2014; Zheng and Chen, 2003). This negative selection kills any T cells that might strongly recognise epitopes derived from proteins expressed by somatic cells, and is the basis of ‘central tolerance’. Central tolerance is comprehensive, but many potentially self-reactive T cells of relatively low TCR:peptide-MHC (pMHC) affinity do emerge from the thymus (Zippelius et al., 2002). Self-peptide reactive T cells can play a role in the immune response to cancer, as will be discussed later in this review. Surviving cells emerge from the thymus as ‘naïve’ CD4+ or CD8+ T cells, and home to and become resident in lymph nodes. Interestingly, negative selection also provides a mechanism whereby both ‘conventional’ and ‘regulatory’ antigen specific T cells can be generated. T cells that recognise self pMHC with very low affinity survive and become conventional CD4+ and CD8+ cells, capable of producing ‘effector’ and ‘helper’ cytokines, and cytolytic granules on priming on reencounter with cognate peptide epitopes derived from pathogens (further discussed in section 1.1.2, below). By contrast, T cells bearing a TCR that recognises self pMHC with intermediate avidity (below the signalling threshold for induction of apoptosis) develop into ‘regulatory’ T cells (TREG) tasked with maintaining tolerance.
to self-antigens and temporally constraining effector T cell responses to limit organ and tissue damage (Apostolou et al., 2002; Modigliani et al., 1996). Naturally occurring T<sub>REG</sub> are typically CD4<sup>+</sup>. T<sub>REG</sub> development is instructed by the cytokine transforming growth factor-β (TGF-β) and T<sub>REG</sub> are characterised by constitutive expression of the transcription factor FOXP3, constitutive surface expression of the high affinity interleukin (IL)-2 receptor (R)α (CD25), a lack of expression of the IL-7Rα, and by constitutive expression of the immunoglobulin superfamily member protein Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Hori et al., 2003; Sakaguchi, 2005). T<sub>REG</sub> act to prevent the activation and priming of autoreactive conventional T cells in secondary lymphoid organs, or limit the activity of effector T cells in the periphery by several mechanisms. T<sub>REG</sub> can induce production of the enzyme indoleamine-2,3-dioxygenase (IDO) by myeloid cells through a direct CTLA-4:CD80/CD86 signalling axis. IDO perturbs conventional T cell activation and anabolism by degradation of the amino acid tryptophan (Munn et al., 2004). T<sub>REG</sub> are also able to limit the availability of IL-2, an important growth factor for recently activated conventional T cells, through their constitutively high level of surface CD25 (Furtado et al., 2002). Finally, T<sub>REG</sub> exhibit surface expression of the ectoenzymes CD39 and CD73, which act to sequentially degrade adenosine triphosphate to adenosine, which binds to inhibitory receptors on the surface of activated conventional T cells (Borsellino et al., 2007). The absolute necessity of T<sub>REG</sub> activity has been demonstrated by the phenotype of the FOXP3<sup>−/−</sup> mouse, whereby mice typically die within 2 weeks of birth due to overwhelming, T cell-mediated autoimmune organ and tissue damage (Brunkow et al., 2001; Wildin et al., 2001).

This review is primarily concerned with the major T cell subset in the body: αβ-T cells, so named because their TCR is comprised of a protein heterodimer encoded by the TCRα and TCRβ genes. Several other subsets of T cell exist, including mucosa-associated invariant T cells (MAIT), natural killer T cells (NKT) and γδ T cells, the TCR of which is comprised of subunits encoded by the TCRγ and TCRδ genes (Pang et al., 2012; Treiner et al., 2003). The role of these T cells and their participation in anti-tumour immunity is not discussed.

CD4<sup>+</sup> and CD8<sup>+</sup> T cells recognise peptide epitopes presented in the peptide-binding groove of highly polymorphic MHC I and II molecules. CD4<sup>+</sup> T cells express the MHC II-binding co-receptor CD4, and recognise the central regions of long peptide epitopes (up to 30 amino acids) within MHC II molecules (HLA-DR/DP/DQ) present on the surface of ‘professional’ antigen-presenting cells (APC) (Hemmer et al., 2000). CD8<sup>+</sup> T cells express the MHC I binding co-receptor CD8, and recognise short (8-11 amino acid) epitopes presented within MHC I molecules (HLA-A/B/C) present on the surface of all cells (Daniels and Jameson, 2000; Luescher et al., 1995). Peptide binding to MHC I and II molecules is mediated by the presence of particular ‘anchor’ amino acid residues that bind a peptide in place, while TCR:peptide engagement is typically mediated by central residues whose side chains project up towards the TCR peptide binding cleft (Cole et al., 2010). A classical view of peptide
antigen presentation holds that APC phagocytose exogenous antigens (for instance extracellular bacteria or dead tumour cell mass) which are broken down by pH and peptidase activity within an acidified endolysosome and loaded into MHC II molecules, while endogenously derived short peptides are continuously broken down within all normal cells by action of the proteasome, transferred into the endoplasmic reticulum via the TAP transporter, and there loaded into MHC I molecules which traffic to the surface. (Asano et al., 2011; Fehres et al., 2014; Mantegazza et al., 2008; Valmori et al., 2007) Through this process T cells can monitor the surface of normal somatic cells for the presence of their cognate antigen. If normal cells are infected with a virus, or begin to present an immunogenic mutant self-peptide, epitopes from viral proteins or mutated self-epitopes are also presented through this endogenous pathway and potentiate T cell recognition and killing of infected or abnormal cells (Rotzschke et al., 1990). However, as lymph-node resident naïve T cells are dependent on presentation of their cognate peptide epitope by professional, mature APC (as discussed below) for appropriate initial activation and expansion, APC are also able to ‘cross-present’ peptide epitopes. During cross-presentation exogenously derived antigens escape from the endolysosome and are re-targeted into the proteasome/TAP-mediated endogenous processing and presentation pathway (‘cytosolic pathway’), or transferred onto MHC I molecules in situ in the phagosome (‘vacuolar pathway’) (Pfeifer et al., 1993).

1.1.2. T cell activation and costimulation.

All naïve T cells constitutively express the plasma-membrane anchored proteins CD27 - a member of the tumour-necrosis factor receptor superfamily (TNFRSF), and CD28 – an immunoglobulin superfamily (V-type) protein (Chen and Flies, 2013; June et al., 1987). These ‘co-stimulatory’ molecules bind to the TNFSF member CD70 and the B7 protein family members CD80 (B7.1)/CD86 (B7.2), respectively, on the surface of mature, active APC (Frauwirth et al., 2000). APC are matured by stimulation of surface or intracellular pattern-recognition receptors (for instance the well-characterised Toll-like receptor), and produce cytokines such as type I interferons and interleukin (IL)-12 that are important in activating and programming naïve T cells. For appropriate initial activation (‘priming’) naïve T cells require three signals (Mempel et al., 2004):

- Signal 1: TCR:pMHC engagement of sufficient duration and strength to trigger TCR signalling through the TCR-associated CD3 signalling complex
- Signal 2: Co-stimulation though CD27 and CD28 engagement of CD70 and CD80/86.
- Signal 3: APC-derived programming cytokines that promote T cell division and acquisition of effector function.

Following reception of these three signals, naïve CD8+ T cells rapidly increase in volume, synthesise ‘effector granules’ containing the cytotoxic molecules perforin and granzymes A and B, undergo
mitochondrial biogenesis and begin to divide. CD28 co-stimulation is indispensable for naive T cell priming, as TCR-stimulation in isolation induces anergy and dysfunction (Yamamoto et al., 2007). Consequently, CD28 deficient mice display extremely poor T cell proliferation in response to general mitogens (Fraser et al., 1991). CD28 signalling induces production of IL-2, an important autocrine T cell growth factor; and also induces the expression of the high affinity receptor for IL-2 (CD25).

CD28 signalling induces expression of the anti-apoptotic proteins B-Cell-Lymphoma (Bcl)-2 and Bel-XL, and promotes the expression of the telomerase enzyme, vital for allowing T cell proliferation without erosion of telomeres (Wrighton, 2009; Wu et al., 2005). CD28 enhances glucose uptake by promoting the expression of glucose transporters, and facilitates a switch to glycolytic metabolism (although this is also controlled by IL-2 signalling), essential for ‘effector’ differentiation and rapid T cell expansion. CD28 and IL-2 signalling synergise to down-regulate p27kip, a cyclin-dependent kinase inhibitor, and thus allow entry into the cell cycle. The intracellular domain of CD28 contains an immunoreceptor tyrosine-based activation motif (ITAM) and CD28 signalling is primarily mediated by phosphorylation of Y170 and Y191 – this allows CD28 to interact with Lck kinase and prolong TCR signal transduction, while also signalling through the protein kinase C (PKC)-θ, phosphatidylinositol-3-kinase (PI3K) and Akt-mTOR pathways (Boise et al., 1995; Boomer et al., 2014; Fraser et al., 1991; Frauwirth et al., 2002).

CD27, a TNFRSF member, binds CD70. Unlike CD28, CD27 deficient mice do not exhibit a defect in initial acquisition of effector phenotype or primary proliferation, but they do exhibit a failure of effector cell survival and early contraction. Importantly, CD27 knockout mice show a profound defect in secondary expansion on antigen reencounter, partly due to poor formation of a memory T cell pool following effector contraction (Hendriks et al., 2000; Yamada et al., 2005). The intracellular domain of CD27 recruits TNF-receptor-associated factors (TRAF)-2, -3 and -5, which signal though mitogen-activated protein kinases (MAPK) and PI3K to promote both T cell proliferation and T cell survival (Chen and Flies, 2013; Hendriks et al., 2000; Hendriks et al., 2003).

1.1.3. T cell memory heterogeneity.

Following appropriate naïve T cell priming, effector T cell expansion and antigen clearance, a vast majority of effector T cells die, while a minority remain as ‘memory’ T cells either in peripheral tissues or within secondary lymphoid organs. These memory cells provide peripheral resistance to subsequent re-challenge by infectious agents, and facilitate more rapid effector expansion on subsequent engagement within antigen-presenting cells bearing cognate antigens.

Human CD4+ and CD8+ T cells that can be isolated from peripheral blood have typically been classified into four key subsets, based on differential expression of the chemokine receptor CCR7 and
the ‘RA’ and ‘RO’ alternatively spliced isoforms of the phosphatase CD45 (Sallusto et al., 2004b; Sallusto et al., 1999). In this schema naïve T cells express CD45RA and exhibit high levels of CCR7 expression. ‘Central memory’ T cells (T<sub>CM</sub>) express only the CD45RO isoform and are CCR7<sup>+</sup>, although their CCR7 surface density is lower than that of naïve T cells. ‘Effector memory’ T cells (T<sub>EM</sub>) express only the CD45RO isoform and are CCR7<sup>-</sup>, while ‘T<sub>EMRA</sub>’ exhibit re-expression of only the CD45RA isoform and are CCR7<sup>-</sup>. Differential expression of these markers correlates with subset function. Naïve and T<sub>CM</sub> are typically housed in the lymph node or bone marrow, and CCR7 expression dictates this homing based on responsiveness to lymph-node-stroma derived chemokines CCL19 and CCL21 (Stein and Nombela-Arrieta, 2005). Conversely, T<sub>EM</sub> and T<sub>EMRA</sub> are typically resident in the blood and peripheral tissues, and this tissue homing is mediated by alternative chemokine receptor and adhesion molecule expression (Masopust et al., 2001). Tissue-specific homing markers include cutaneous leukocyte antigen (CLA, directing homing to skin) and CD103 (directing homing to the lung or gut) (Fuhlbrigge et al., 1997; Zhou et al., 2008). CD45 is a tyrosine phosphatase that exists in multiple spliced isoforms, RA and RO representing the longest and shortest ectodomains respectively. Although the ligand for CD45 is undefined, CD45 is known to promote Lck kinase signalling and enhance TCR signal transduction by removal of an inhibitory phosphate group typically added to Lck by Src family kinases (Hermiston et al., 2003). As such CD45 expression acts to tune TCR sensitivity and enable more rapid effector function (Robinson et al., 1993).

Expression of CD62L (L-selectin) has also been proposed as a subset-defining marker – naïve T cells and T<sub>CM</sub> are CD62L<sup>+</sup> while T<sub>EM</sub> and T<sub>EMRA</sub> are CD62L<sup>-</sup> (Huster et al., 2004). Again, expression of this marker is correlated with function and homing, as CD62L facilitates binding to peripheral node addressin molecules on high endothelial venules in the lymph node and gut-associated lymphatic tissue to facilitate ingress (Brinkman et al., 2013a; Brinkman et al., 2013b). However, although CD62L is often used to delineate T<sub>CM</sub>/T<sub>EM</sub> in mice, this distinction is not absolute in humans (Schwendemann et al., 2005). Murine naïve and memory T cells are also delineated by differential expression of the adhesion molecule CD44, a schema which is not utilised when discussing human memory subsets (Mestas and Hughes, 2004). All memory T cells can be distinguished from naïve T cells by expression of CD122, allowing signalling by IL-15, and by expression of CD95 (Fas).

Expression of CD27 and CD28 is heterogeneous across naïve and memory T cell subsets. Although early in vitro work suggested that memory T cells were co-stimulation independent, this is no longer believed to be the case, and early co-stimulatory signals (mediated by CD27 and CD28) and later co-stimulatory signals, mediated by CD28 and TNFRSF members expressed 6-72h following T cell activation, are believed to be important for memory T cell proliferation and survival, although they maybe dispensable for effector granule synthesis and discharge. T<sub>CM</sub> retain expression of both CD27 and CD28 while T<sub>EM</sub> and effector T cells exhibit mixed expression. Retention or loss of co-
stimulatory markers has been used to define ‘early’ CD27+/CD28+, intermediate CD27+CD28− and terminally differentiated CD27−CD28− cells (Takata and Takiguchi, 2006). These differentiations are often observed among T_{EM} specific for acute or chronic viruses, respectively.

T cell subsets are exhibit differential proliferative capacity on stimulation, cytokine production profiles and effector granule expression. In a spectrum from naïve – T_{CM} – T_{EM} – T_{EMRA} proliferative potential is decreased from subset to subset. Naïve and central memory cells retain expression of IL-2 (an important autocrine growth factor) and Tumour necrosis factor α (TNFα) (capable of inducing target cell apoptosis through TNFR-ligation), but poorly produce interferon-γ (IFNγ), while T_{EM} and T_{EMRA} potently produce IFNγ and retain varied levels of TNFα-production capacity (on a cell-to-cell basis), but typically lose the ability to produce IL-2 (this is also associated with their poor proliferative potential) (Sallusto et al., 2004b; Sallustio et al., 1999). Further, while naïve and central memory T cells typically do not contain intracellular perforin or granzyme B, T_{EM} and T_{EMRA} do, (concomitant with their function as a poorly-proliferative but highly cytotoxic early peripheral defense system) and effector granule content is inversely correlated with CD27/CD28 expression. It should be noted however, that all effector cells derived from any resting subset rapidly express effector granule proteins, and this expression is subsequently differentially silenced as effector cells transition back to memory status.

Several recent studies have defined a novel, although rare, subset of memory T cells intermediate between naïve and T_{CM}, characterised as stem-cell memory T cells (T_{SCM}). These cells effectively exhibit a naïve phenotype with the exception of CD95 and CD122 expression, denoting them as antigen-experienced. T_{SCM} appear to exhibit the greatest proliferative and self-renewal potential of any T cell memory subset and have been defined in mice, humans and non-human primates (Gattinoni et al., 2011; Gattinoni et al., 2009b; Lugli et al., 2013). The relevance of T_{SCM} in anti-tumour immunity is further discussed in section 1.2.6.

The phenotypic, numerical and functional demarcation between naïve, effector and memory cells is relatively well-defined in an acute infectious setting. By contrast, conditions of chronic antigen exposure (and therefore chronic TCR stimulation) may lead to a state of T cell survival concomitant with a persistent and progressive failure of effector function. Under these circumstances T cells have been described as ‘exhausted’. T cell exhaustion was originally observed in murine models of chronic LCMV infection, wherein antigen-specific cells that failed to control viral titers were observed to progressively lose the ability to: produce IL-2; proliferate; synthesise de novo effector granules following degranulation; and produce TNF-α. CD8+ T cell exhaustion was exacerbated by a lack of CD4+ T cell ‘help’ and was correlated with duration of antigen exposure (Wherry et al., 2003; Wherry et al., 2007; Zajac et al., 1998). Exhausted T cells have subsequently been described in several human chronic viral illnesses and as a major component of tumour-infiltrating lymphocytes
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(TIL) in solid tumours (Ahmadzadeh et al., 2009b; Day et al., 2006; Kodumudi et al., 2016; Nazareth et al., 2007). Exhausted T cells express high levels of several ‘co-inhibitory’ surface receptors that act to counter TCR/CD3- and CD28-mediated phosphorylation signalling cascades, notably programmed cell death protein-1 (PD-1), T cell immunoglobulin and mucin-domain containing-3 (TIM-3) and Lymphocyte-activation gene 3 (LAG-3) (Bowen and Walker, 2005; Wherry et al., 2007). The role and clinical relevance of these co-inhibitory molecules is discussed in greater detail in section 1.2.5. Interestingly, although exhausted T cells appear functionally ‘anergic’, in a similar manner to naïve T cells that are stimulated in the absence of co-stimulatory signals, their in situ exhaustion is not permanently imprinted and can be alleviated by blockade of co-inhibitory receptor signalling (Barber et al., 2006; Freeman et al., 2006). Despite this, exhausted T cells survive poorly on antigen clearance, as they lose the ability to express receptors for the homeostatic cytokines interleukin-7 and interleukin-15 (further discussed in section 1.1.4, below) and instead are reliant on constant TCR-peptide:MHC ligation for their survival (Shin et al., 2007).

Although much work has focused on defining the phenotype and function of blood- and SLO-localised T cell subsets, the location and nature of T cell priming can also imprint both CD4+ and CD8+ T cells to take up semi-permanent residence in peripheral tissues. These cells are designated ‘tissue-resident memory’ T cells (TRM) and are derived from effector T cells that infiltrate inflamed tissues and do not undergo contraction (Schenkel and Masopust, 2014). TRM have been described in the gut epithelium, dermis, lung and brain (Jiang et al., 2012; Masopust et al., 2010; Teijaro et al., 2011). Like TEM, TRM are typically CCR7- and CD62L-, but unlike other memory cells they constitutively express the homotypic adhesion molecule CD69 (Casey et al., 2012; Schenkel and Masopust, 2014). Retention of TRM in peripheral tissues is mediated by their expression of several integrins, notably α1β1 and αEβ7 (CD103), allowing them to interact with basement membrane proteins and E-Cadherin expressed by stromal epithelial cells (Wakim et al., 2012). TRM constitutively express granzyme B and retain some proliferative potential, and as such provide an important primary defense against viral and bacterial infection in mucosal tissues (Jiang et al., 2012; Masopust et al., 2006).


The ‘common gamma chain’ (γc) cytokines are members of the larger four-α-helix-bundle cytokine family that also includes IL-10 and the interferons. Currently known γc cytokines include interleukin (IL)-2, -4, -7, -9, -15 and -21. These cytokines share the γc (CD132) as an essential part of their signalling receptor complex. Each γc cytokine binds to a cytokine-specific receptor-α chain, which may be capable (IL-4; -7; -9; 21) or incapable of signal transduction (IL-2; -15). IL-4, -7, -9 and -21 bind their Rα chains and then form a dimeric signalling complex with CD132 (Rochman et al., 2009). IL-2 and IL-15 both mediate their signalling through a shared intermediate affinity (10^9 M)
heterodimeric complex comprising CD122 and CD132, but require Rα binding for formation of a high affinity \(10^{-11}\) M complex (Roessler et al., 1994; Takeshita et al., 1992). IL-15 is unique in that it can also be presented \textit{in trans} by IL-15Rα-expressing cells to cells expressing the intermediate affinity receptor (Alves et al., 2007; Dubois et al., 2002; Lodolce et al., 2001).

IL-2, -4, -9 and -21 are typically produced by activated T cells within the lymph node paracortical or follicular zones. By contrast, IL-7 and IL-15 are produced by endothelial and stromal cells residing in the thymus, lymph node, bone marrow and liver. IL-15 is also produced and presented by myeloid cells, particularly DC (Schluns and Lefrancois, 2003; Stonier and Schluns). The site and source of production of each γc cytokine is broadly reflective of its function – IL-2, -4, -9 and -21 are typically involved in modulating and directing an ongoing immune response within a lymph node, while IL-7 and IL-15 are homeostatic in nature, and are primarily involved with maintenance and survival of T cells within a lymphatic or peripheral compartment (Alves et al., 2007).

γc cytokine signal transduction is mediated by Janus Kinases (JAK) noncovalently associated with the intracellular domains of CD132, CD122 and those Rα chains capable of signalling. CD132 associates with JAK3, while JAK1 is recruited to CD122 and the cytokine-specific Rα’s. (Jiang et al., 2004; Suzuki et al., 2000). Phosphorylation of JAK1/3 facilitates recruitment and phosphorylation of Signal Transducer and Activator of Transcription (STAT) transcription factors. Although the recruitment of JAK1/3 is conserved across γc cytokine receptors, the Rα’s exhibit different patterns of STAT recruitment: IL-2, -7 and -15 typically signal through STAT5; IL-4 signals through STAT6; and IL-21 signals through STAT1 and 3 (Alves et al., 2007). Although the ultimate consequences of γc cytokine signalling are dependent on cell type and differentiation status, signalling typically promotes T cell proliferation through PI3K/Akt/mTOR Ras and MAPK pathways, and survival through induction of Bcl family member expression (Rathmell et al., 2001). As an example, signalling via IL-7Rα results in: inhibitory serine-phosphorylation of the pro-apoptotic factor BAD via a JAK1/Akt dependent phosphorylation cascade; activating phosphorylation of the anti-apoptotic factor Bcl-2; and phosphorylation and cytoplasmic sequestration of the pro-apoptotic factor Bax via a cascade proceeding from JAK1 binding to the Box1 domain, with subsequent phosphorylation of Y449 (Palmer et al., 2008b). Site directed mutagenesis (Y449F) ablated this pro-survival signalling in a murine model (Jiang et al., 2004). IL-2 and IL-15 also have profound effects on T cell metabolism by promoting aerobic glycolysis and mitochondrial biogenesis (van der Windt et al., 2012).

The role of γc cytokines in the development, maintenance and function of lymphoid populations has been extensively investigated in mouse and human settings. Knockout of CD132, IL-7 or IL-7Rα in mice greatly diminishes T cell levels, and ablates NK and B cells. Similarly CD132 is mutated in patients with X-linked severe combined immunodeficiency, with a loss of T and NK cells, and functionally deficient B cells (Giliani et al., 2005). Loss of T cells in these settings is due to a failure
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of protection from apoptosis, as T cell survival in IL-7Rα−/− mice can be restored by overexpression of Bcl-2 (Akashi et al., 1997). Knockout of IL-15 or IL-15Rα does not ablate T-/B-/NK-cell development, but does impair the formation or survival of CD8+ memory T cells, suggesting that it may be important for memory homeostasis (Yajima et al., 2006). By contrast knockout of IL-2 does not affect initial T cell development, instead resulting in a loss of regulatory T cells and the rapid onset of fatal systemic autoimmunity (Isakson et al., 2012). Similarly, IL-4 and IL-21 knockout do not affect T cell development, but greatly impair B-cell class switching, suggesting the importance of these cytokines in the development of a humoral immune response (Rankin et al., 2011). As such, both murine and human data have demonstrated that IL-7 and IL-15 play a fundamental role in the development and maintenance of non- regulatory T cells, although the role of each cytokine in promoting homeostasis differs depending on the nature of the T cell population (Alves et al., 2007; Tan et al., 2002a).

CD4+ and CD8+ T cells express differing levels of γc cytokine receptors based on their differentiation status. Naïve T cells express high levels of IL-7Rα, but low or negligible levels of CD25, CD122, IL-15Rα and IL-21Rα (Berard and Tough, 2002). T cells that have entered a proliferative ‘effector’ phase following TCR ligation temporally upregulate CD25 and IL-21Rα, gain IL-15Rα and CD122 expression but lose IL-7Rα (Alves et al., 2008; Iezzi et al., 1998). This IL-7Rα loss is due to direct repression by IL-2, although interestingly, brief IL-2 exposure has been shown to be vital for subsequent IL-7Rα re-expression (Ramos et al., 2009; Xue et al., 2002). TCM and TEM typically exhibit continued expression of CD122 and IL-15Rα, may gain constitutive expression of CD25, and retain IL-7Rα expression - although at a lower surface density than naïve T cells - while TEMRA and exhausted T cells exhibiting a prolonged ‘effector’ phenotype in situations of chronic antigen exposure may entirely lose IL-7Rα expression (Bucks et al., 2009; Karl et al., 2005).

The role of γc cytokines in facilitating the survival and proliferation of naïve and memory T cells has typically been investigated in mice through the adoptive transfer of T cells into host animals deficient in production of one or more cytokines, sublethally irradiated to remove any existing lymphocytes. This is necessary as naïve and central memory T cells will not typically undergo homeostatic turnover in a full lymphatic compartment. The threshold of cytokine signalling needed to promote T cell survival in vitro is lower than that required to induce homeostatic division, suggesting that homeostatic turnover occurs in vivo only under lymphopaenic conditions, or when IL-7 has been deliberately over expressed (Goldrath et al., 2002). Survival and homeostatic proliferation of naïve CD4+ and CD8+ T cells is critically dependent on continued recognition of relevant restricting MHC molecules and on provision of IL-7. Although IL-4 and IL-15 are also able to stimulate homeostatic division of naïve T cells in vitro, only IL-7 was able to do so non-redundantly in vivo, as murine naïve T cells are unable to proliferate or persist on transfer into IL-7−/− host animals, but divide and persist normally on transfer into irradiated IL-4−/− or IL-15−/− hosts (Tan et al., 2002b). Several studies have
demonstrated that murine CD4$^+$ T$_{CM}$ and T$_{EM}$ are dependent on IL-7 for survival, and these cells fail to persist on transfer into IL-7$^{-}$ hosts, while IL-15 is dispensable for their survival (Geginat et al., 2003a; Geginat et al., 2003b; Sallusto et al., 2004b). These results have to a large extent been confirmed in human T cell subsets in vitro, whereby naïve and memory CD4$^+$ T cells have been shown to survive in IL-4, -7 and -15, while naïve and memory CD8$^+$ T cells both survive and proliferate in IL-7 and IL-15. In both human and murine studies, IL-21 has been shown to have no survival or mitogenic effect for naïve or memory T cells (Alves et al., 2005).

1.1.5. Memory T cell generation.

The acquisition of an effector phenotype and function and induction of rapid proliferation is governed by expression of the transcription factors T-Bet ($TBX21$), Blimp-1 ($PRDM1$) and Eomesodermin ($EOMES$), with reciprocal repression of the transcription factor Bcl-6 ($BCL6$) (Crotty et al.; Kallies et al., 2009; Rutishauser et al., 2009). Appropriate induction of these transcription factors is aided by ‘signal 3’ during T cell activation, in particular the provision of APC-derived IL-12 and IFN-$\alpha$ (Ramos et al., 2009). Several studies have used murine models to investigate the events that determine how a minority of effector T cells survive contraction and are directed to take on a memory phenotype. All stimulated naïve cells do enter an effector phase and express granzymes A and B. Kaech et al (2003) demonstrated that during naïve T cell expansion cells could be delineated into ‘short-lived effector cells’ (SLEC) and ‘memory precursor cells’ (MPEC) based on relative expression of IL-7R$\alpha$ and the inhibitory receptor KLRG1. Specifically, MPEC exhibited a CD27$^+$IL-7R$\alpha^+$KLRG1$^{lo}$ phenotype, with evidence of intracellular pro-survival molecules Bcl-2 and Bcl-XL, while SLEC were IL-7R$\alpha^-$KLRG1$^{hi}$ and CD27$. $Interestingly, subsequent studies suggested that while IL-7R$\alpha$ expression did definitively identify cells destined to become memory cells, it was not the causative factor in this transition, as in a conditional IL-7R$\alpha$ knockout model CD8$^+$ effector expansion and memory formation was normal. In this case the memory pool formed was dependent on IL-15 alone for its homeostasis (Buentke et al., 2006). Conversely, in a system where constitutive IL-7R$\alpha$ overexpression was utilised effector expansion was again unchanged, and cells still partitioned into KLRG1 and CD27$^{-/}$ subsets. Even in the presence of IL-7 signal transduction (indicated by STAT5 phosphorylation in this model) the KLRG1$^{hi}$ cells died and the KLRG1$^{-}$ cells went on to constitute the memory pool. This study found that KLRG1$^{hi}$ cells were constrained from proliferating by high levels of pkip27, a cyclin-dependent-kinase inhibitor. (Hand et al., 2007).

Several studies have demonstrated that signals during early T cell activation events determine whether expanding T cells take on SLEC or MPEC phenotype, including TCR signal strength and length of APC contact (Beuneu et al., 2010), duration of IL-2 exposure, with greater duration promoting SLEC phenotype (Kalia et al., 2008; Kalia et al., 2010) and exposure to IL-12, which facilitates a switch from T-bet to Bcl-6-guided transcriptional control beyond day 3 of expansion (Hinrichs et al., 2008;
Further studies have shown that the nature of priming APC-derived cytokines can dictate memory status even amongst those cells that do survive and form the memory pool. Both IL-12 and IFN-α act to prolong chromatin remodeling associated with T cell activation and facilitate EOMES expression (Agarwal et al., 2009), but IL-12 signalling subsequently promotes, and IFNα suppresses proliferation, guiding cells to rest as TEM or TCM respectively (Ramos et al., 2009). As such, early events in T cell priming determine long-term survival, but survival itself is mediated by continued homeostatic cytokine responsiveness.

Because T cell memory is retained through life in the absence of significant bone-marrow or thymus-derived renewal, and in the face of continuous antigenic pressure, it has long been proposed that there must be some a stem-cell-like element that enables both continuous expansion and self-renewal and maintenance (Fearon et al., 2001). In fact, naïve and central memory T cells exhibit many aspects of stem cells – they undergo asymmetric division, give rise to heterogeneous daughter cells that display varying stages of differentiation (i.e. exhibit multipotency), give rise to daughter cells that undergo a ‘transit amplification’ during effector expansion to terminal differentiation and death, remain in a defined niche (SLO) and exhibit expression of the enzyme telomerase to allow division without loss of telomere length (Akbar and Vukmanovic-Stejic, 2007). However, the exact identity of this stem-cell population, in particular whether it is TSCM or TCM (or both), and the nature by which fate decisions within dividing T cells are taken has remained contentious. Three competing models have been proposed for the differentiation of effector and memory T cell subsets. The first is the ‘linear differentiation model’, in which surviving effector T cells form a TEM memory pool post-contraction, a subset of which subsequently ‘back-differentiate’ into TCM and return to SLO. Evidence for this model is primarily based on observations that on adoptive transfer of T cell clones exhibiting a TEM phenotype, these cells can subsequently be observed in the host as TCM (Chapuis et al., 2012; Powell et al., 2005), although no study has put forward a mechanism for this apparent de-differentiation. The second model is the ‘bifurcative’ model; which suggests that a primed naïve T cell gives rise to daughter cells with alternative differentiation fates. This model is supported by evidence that on initial cell division transcription factors, notably T-bet, are differentially partitioned into the two immediate daughter cells, and by evidence that the daughter cell remaining in contact with the presenting APC may receive additional cytokine exposure and co-stimulatory signalling (Chang et al., 2011; Chang et al., 2007). The final model is the ‘progressive differentiation/decreasing potential model’, whereby primed naïve cells will differentially progress to TSCM, TCM, TEM, or terminal effector status depending on the nature of their early priming events, provision of IL-12, and local inflammatory milieu. This model is based on evidence of a spectrum of gene expression by which naïve cells, TSCM, TCM, and TEM exhibit a progressive directional overlap in acute and chronic viral diseases, and evidence that both TCR:pMHC affinity and interaction time with APC determine T cell memory fate decisions (Baumgartner et al., 2012; Busch and Pamer, 1999; Gattinoni, 2011; Holmes et
Irrespective of these controversies, several recent murine studies have demonstrated that single naïve T cells in fact give rise to a heterogeneous mix of T_{CM}, T_{EM}, and effector cells, and that the T_{CM} generated during these expansions can self-renew on serial antigen exposure, although these studies defined T_{CM} as being CD44^{+} CD62L^{+} and as such did not phenotypically distinguish T_{SCM} and T_{CM} (Gerlach et al., 2013; Gerlach et al., 2010; Graef et al., 2014; Stemberger et al., 2014).

1.2. T cells in the clinic.

1.2.1. Introduction to immunotherapy.

Immunotherapy seeks to harness one or more facets of the immune system to promote the eradication of, and lasting protection from, a disease causing agent. Many forms exist, including monoclonal antibodies, such as those used to target HER-2 positive breast cancers (Piccart-Gebhart et al., 2005); administration of immunomodulatory cytokines, commonly used in cancer therapy (Atkins et al., 1999); several vaccine strategies (Karbach et al., 2010; Karbach et al., 2011); or adoptive transfer of naturally occurring or genetically engineered virus- or tumour-reactive lymphocytes (Restifo et al., 2012). These therapies are often used in combination, and in conjunction with existing non-immune treatments, in patients refractory to standard chemo- and radiotherapeutic interventions (Jandus et al., 2009; Rosenberg et al., 2008).

Both IL-2 interferon (IFN)-α have been administered alone as treatment for melanoma and renal cell carcinoma. IFN-α mediates responses in pre-metastatic patients, and IL-2 is generally administered to patients with metastatic disease. Both show efficacy in a small minority of patients – with total response rates of 16%, and complete response rates (tumour regression) of 5 and 6% respectively. IL-2 is associated with potentially severe toxicities, including vascular leak syndrome which leads to systemic hypotension and possible organ failure, and thus its use is limited by patient tolerance (Dougan and Dranoff, 2009).

Cancer vaccines can utilise short peptides of known immunogenicity to stimulate a response, generally by CD8^{+} T cells, against a single immunogenic epitope. Peptides alone appear to be poorly immunogenic in humans, and the use of single peptides limits administration to patients that express the relevant HLA allele. In order to promote a broader immune response and to incorporate both CD4^{+} and CD8^{+} lymphocytes, whole recombinant proteins have been utilised. Both peptide and protein-based vaccines alone have met with generally disappointing results, but these can be significantly improved by incorporation of appropriate adjuvants.(Jandus et al., 2009). In one recent trial the peptide SLLMWITQC derived from ‘New York Esophageal squamous cell carcinoma-1’
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(NY-ESO-1), a protein expressed in many cancers, was co-administered with incomplete Freund’s adjuvant and CpG oligonucleotides (a toll-like receptor (TLR)-9 agonist) providing two potent “danger signals”. In this trial expansion of peptide-specific CD8+ T cells was observed (up to 0.16% of circulating lymphocytes in several patients) and maintained over 2 months. 6 of 14 patients exhibited ongoing responses lasting over 3 years. (Karbach et al., 2010). Similarly, in a trial in which whole recombinant NY-ESO-1 was administered using the same adjuvant regime, CD4+ and CD8+ T cell responses were induced in 17/18 and 9/18 patients respectively (Valmori et al., 2007). In order to overcome the generally poor immunogenicity of peptide and protein based vaccines several trials have attempted to use peptide-loaded, matured dendritic cells (DC)- the optimal antigen-presenting cell for priming and stimulating de novo T cell expansion (Alexiou et al., 2009; Banchereau and Steinman, 1998; Mempel et al., 2004; Palucka and Banchereau, 2012). Such trials have met with variable, but generally disappointing results, for instance one recent phase III trial comparing dacarbazin chemotherapy to peptide-loaded DC in treatment of metastatic melanoma found no advantage to the immunotherapy (Schadendorf et al., 2006). However it is possible that as our understanding of DC biology advances (in particular determining which DC subtypes are optimal for cancer vaccines) results may improve. At present however it seems that vaccines are a long way from fulfilling their therapeutic potential, especially given the recognition that there is often no correlation between the induction of a measurable vaccine-specific T cell pool and a clinically relevant anti-tumour response (Offringa, 2009). In fact, in many instances vaccines appear to propagate a ‘corrupt’ memory response characterised by poorly functional T cells (Klebanoff et al., 2006). It has also been recognised that in many melanoma patients lymphocytes able to infiltrate tumours are functionally impaired (Dunbar et al., 2000). In order to understand the nature of this immune dysfunction, it is important to consider the nature of the tumour microenvironment itself.

Early studies in mice involving chemical induction of large tumours demonstrated that while they were unable to control progression of primary, established tumours, an anti-tumour immune response did develop that was capable of preventing establishment and formation of secondary tumours. From these studies it became clear that despite the tumour cells themselves being immunogenic, the primary tumour was protected from the immune system and could be neither infiltrated nor eradicated (Klein et al., 1960). Similarly, during studies in which first cancer-testis antigen (MAGE-A1) was characterised, (van der Bruggen et al., 1991) were able to clone MAGE-A1 specific T-cells from a progressing melanoma patient - demonstrating the existence of a specific T-cell response that was unable to control the growth of an immunogenic tumour expressing their cognate peptide. As these tumour cells remained expression of MAGE-A1 and MHC I, loss of antigen presentation could not be the cause of immunosurveillance failure. Many subsequent studies have demonstrated the existence of primed and expanded tumour-infiltrating lymphocytes (TIL) or vaccination-induced T-cell responses.
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failing to control tumour growth despite localisation to the tumour environment (Ohtani, 2007; Rosenberg et al., 2005).

Established tumours are immunosuppressive for many reasons – some of these mechanisms are tumour cell-intrinsic, while others are determined by normal cells co-opted into the tumour stroma. Extracellular matrix deposition by cancer-associated fibroblasts limits the spatial infiltration of effector T cells, often arresting them at the tumour margin. T cells can easily migrate through areas of loose connective tissue but their motility is constrained in dense fibrotic areas around tumour cell “nests”. Degradation of the collagen in these areas facilitates normal T cell movement (Joyce and Fearon, 2015). T cells can express collagen cleaving enzymes (for example, the peptidase CD26) but typically only after a TCR stimulus (Morimoto and Schlossman, 1998).

The disorganised nature of tumour angiogenesis means that large tumours are often hypoxic, limiting access for blood-borne lymphocytes (Dougan and Dranoff, 2009). Interestingly, in a mouse model of cancer, deletion of rsg5, a master regulator of tumour vessel development, led to the growth of normal, organised vessels, which promoted lymphocyte infiltration and improved anti-tumour responses (Hamzah et al., 2008). Similarly, treatment of tumours with TNFα or LIGHT targeted to endothelium through RGD-conjugation kills disorganised vasculature, allows blood vessel reorganisation and restores T cell access (Johansson et al., 2012).

As immature monocytes and granulocytes migrate into tumours along a CCL2 chemotactic gradient, tumours are able to program their differentiation into myeloid-derived suppressor cells (MDSC). Similarly, resident tissue macrophages can be programmed into tumour-associated macrophages (TAM). TAM and MDSC are characterised by TGFβ, IL-10, arginase and IDO production (Rodriguez et al., 2004). IDO-dependent breakdown of tryptophan and production of kynurenine directly antagonises intratumoural T-cell expansion and can induce death of effector cells (Munn and Mellor, 2013). Interestingly, activation of TAM by CD40 agonism suppressed tumour growth in a mouse model of pancreatic cancer, reinforcing the active role that TAM can play in tumourigenesis or tumour suppression (Beatty et al., 2011; Buhtioarov et al., 2011). Established tumours are able to promote the chemotactic influx of CCR4+ T_{REG} through TAM- and fibroblast-derived CCL22, which then anergise cytotoxic TIL (Li et al., 2013).

Finally, tumour cells themselves can directly anergise or kill infiltrating effector T cells via PD-L1 and FasL expression (further discussed in section 1.2.5). Endothelial cells in tumour vasculature can express high levels of FasL, induced by vascular-endothelial growth factor (VEGF) and IL-10. This induces apoptosis of effector T cells, but not of T_{REG} which are protected by high levels of expression of c-FLIP, which binds to and deactivates caspase-8 and FADD. Attenuation of this VEGF-FasL axis promoted tumour killing in a murine model of ovarian cancer (Joyce and Fearon, 2015; Motz et al., 2014).
Despite the hazards of life inside a tumour, several reports have shown that over 80% of melanoma patients’ tumours contain infiltrating lymphocytes (Rosenberg et al., 2008). These lymphocytes often show anti-tumour function \textit{ex vivo}, indicating the presence of a potentially curative T cell pool in which failure to clear cancer is not due to any innate dysfunction (in contrast with the “exhausted” phenotype often seen in T cells in a chronic viral infection). Adoptive cell transfer (ACT) is the most effective current treatment for metastatic melanoma and for the treatment of viral reactivation after profound immunosuppression, as in cases of haematopoietic stem cell transplantation (Cobbold et al., 2005). In brief, ACT involves the isolation of T cells from the blood or excised tumour of a cancer patient, followed by the outgrowth, under specific culture conditions, of T cells able to recognise and kill cancer cells \textit{ex vivo}, and the reinfusion, \textit{en masse}, of the expanded cells in order to eradicate disseminated tumours. Much of the recent success of ACT has been built on the optimisation of patient pre-conditioning regimens to allow maximal T cell activity after reinfusion, further discussed in sections 1.2.3 - 1.2.4. Work in animal models demonstrated the synergy of ACT with: co-administration of IL-2, an important T cell growth factor; lymphodepleting chemotherapy; and total body irradiation, (Rosenberg et al., 2008) and the importance of these methodological advances, along with the advantages and limitations of the several ACT variations currently utilised in clinical trials, are discussed below.

\subsection*{1.2.2. Antigen target selection in cancer immunotherapy: safety and efficacy.}

Because of the potent cytotoxicity of effector T cells, target selection in cell-mediated immunotherapy is crucial for both safety and efficacy. There are four major classes of therapeutic target for T cell products: oncogenic or chronic virus-derived proteins, tumour-associated or overexpression antigens, cancer-testis antigens and mutant neoepitopes.

Viral epitopes can be targeted in two settings. Firstly, virus-specific T cells have been administered to patients following haematopoietic stem cell transplant, to prevent chronic viral re-emergence and disease. In this setting administration of small numbers of clonal CD8$^+$ T cells (Riddell et al., 1992) or polyclonal cell products directed against the latent viruses, Cytomegalovirus (CMV); Epstein-Barr Virus (EBV) and adenovirus prevented viraemia in a majority of patients (Gerdemann et al., 2011). Secondly, the potent immunogenicity of oncogenic viruses such as EBV, which can cause several types of lymphoma and carcinoma (Hsu and Glaser, 2013) has been exploited to target EBV-induced cancers, particularly lymphoma, by utilising T cells capable of recognising viral epitopes presented by cancerous cells (Rooney et al., 1998) inducing stable disease or remission.

Tumour-associated/differentiation antigens (TAA) are lineage-restricted self-proteins against which incomplete tolerance exists – allowing the escape of specific T cells from negative selection in the thymus (Zippelius et al., 2002). The best characterised of these for immunotherapy are the
melanocyte-lineage restricted proteins Melan-A/MART-1, gp100/pre-melanosome protein (gp100/pmel) and tyrosinase; and carcinoembryonic antigen (CEA) expressed in the developing gut. These proteins are known to be immunogenic, as T cells responsive to them and exhibiting a memory or effector phenotype can often be detected in the blood of cancer patients (Dunbar et al., 2000). TAA have been extensively targeted in murine models and in clinical trials utilising peptide vaccination, polyclonal T cell products, T cell clones, or T cells transduced with retroviral vectors encoding a de novo TCR specific for a TAA-derived peptide epitope (genetically engineered-TCR or geTCR) (Chapuis et al., 2014; Dudley et al., 2001; Khammari et al., 2009; Morgan et al., 2006b; Yee et al., 2002a). Targeting of these antigens can certainly induce objective responses but is associated with severe toxicity typically directed against healthy melanocytes in the skin, eye and gut (Palmer et al., 2008a). Similarly, in a colorectal trial where high affinity geTCR specific for CEA were utilised life-threatening colitis was observed (Parkhurst et al., 2011). These toxicities were also observed in murine models (Palmer 2008). These safety concerns, and observations that many cancer patients contain TAA-specific T cells that have patently failed to control tumour progression have limited the utility of TAA as therapeutic targets (Nagorsen et al., 2005).

Cancer-testis antigens (CTA) exhibit a highly restricted pattern of expression during gametogenesis, and are then epigenetically silenced in somatic tissues. Such proteins are absent from the normal thymic epithelial selection repertoire and as such T cells specific for them are not deleted (i.e. they are unaffected by central tolerance). In many cancers, CTA become re-expressed through global hypomethylation (Luca Sigalotti, 2008; Scanlan et al., 2004; Simpson et al., 2005). There are over 100 CTA family members, typically exhibiting no expression in adult tissues with the exception of the cerebellum and placenta, making them a much safer target than TAA. Further, although most CTA have not yet had a function defined, their expression appears to correlate with metastasis and de-differentiation, and as such they may be important in tumour function, making them less dispensable for growing cancer cells than lineage TAA (Ali O. Güre, 2000; Atanackovic et al., 2010; Pabst et al., 2010). The best characterised of the CTA are ‘New York Esophageal squamous cell carcinoma-1’ (NY-ESO-1) and the extensive Melanoma-associated-antigen (MAGE) family of proteins. CTA have been found to be highly expressed, although often with a heterogeneous pattern, across most known cancers, with particularly frequent expression (especially of NY-ESO-1) in melanoma and other epithelial cancers (Achim et al., 2002; Atanackovic et al., 2009; Chen et al., 2011).

The final, and most promising class of target are mutated neoepitopes/neoantigens. These are generated by non-synonymous single-nucleotide polymorphisms casing amino acid changes that create immunogenic epitopes typically either by increasing peptide:MHC binding affinity at an anchor residue (e.g. substitution for Leucine in an HLA-A2 context) or by altering epitope interaction with the TCR (Fritsch et al., 2014). Neoepitopes are typically donor-unique, and not shared. Neoepitope detection has been facilitated by improvements in sequencing technology and is typically performed

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by comparing whole exome sequences of tumour tissue against healthy tissue within a patient; predicting the ability of the neoepitopes generated to bind MHC class I and II molecules using proteasomal processing and peptide:MHC binding algorithms; and demonstrating the ability of epitope candidates to bind MHC via mass spectrometry or HLA-specific APC pulsing (Castle et al., 2012; Gubin et al., 2014a; Robbins et al., 2013; van Rooij et al., 2013). T cells can influence neoepitope expression in cancers by ‘immunoediting’ during development (by killing cells expressing immunogenic neoepitopes early in tumourigenesis). This immunoediting process may contribute to heterogeneity of neoepitope expression across a tumour mass (Adamson et al., 2009; Koebel et al., 2007; Schumacher and Schreiber, 2015; Shankaran et al., 2001). In recent years, T cell products targeted against neoepitopes have begun to be utilised on a patient-by-patient basis in the clinic. In one particularly successful recent example, Tran et al (2014) were able to sequence an immunogenic MHC II-restricted epitope within the Erbb2-interacting protein in a patient with metastatic cholangiocarcinoma. They were then able to isolate and expand a CD4+ T cell product enriched for cells able to recognise the mutation from this patient’s TIL. On administration, these T cells were able to mediate regression of metastases in this patient’s lung and liver, with regression continuing at a 1 year follow-up.

1.2.3. Adoptive cell transfer.

Several early ACT trials in humans utilised ex vivo expanded, IL-2 dependent CD8+ T cell clones, recognising single peptide epitopes from target tumour-associated antigens. T cell clones recognising these peptide epitopes with high avidity can be isolated from patients through fluorescence-assisted cell sorting (FACS) by utilising fluorophore-conjugated pentamers (Dunbar et al., 1999). Clonal T cells specific for a gp100/pmell epitope were infused into previously vaccinated melanoma patients, with co-administration of varying doses of IL-2. This protocol was clinically ineffective, and the infused T cells failed to persist in vivo beyond day 14 (Dudley et al., 2001). The failure of persistence in this trial may have been related to high IL-2 dose used to expand and maintain the T cell clones prior to infusion. The deleterious effects of long-term IL-2 culture are discussed in a later section. Interestingly, a subsequent phase I clinical trial using clones specific for gp100 and Melan-A, in conjunction with doses of IL-2 120-fold lower than those used in the failed trial, successfully induced disease stabilisation and minor tumour regressions in 8/10 patients. The infused clones were able to survive in vivo reaching up to 2.2% of circulating T cells, and were able to traffic to tumour sites. Interestingly, although in no patient were surviving blood-borne clonal cells detected past day 21, the mean response time was 11.5 months, with a maximum response duration of 21 months, potentially indicating the retention of an extravascular clonal memory population (Yee et al., 2002b). Importantly in 3/5 screened patients expression of Melan-A was lost in the tumour – this tumour target escape is one of the main limitations of clonal therapy, especially when the therapeutic target is
not essential for the survival of the cancer cells (Yee et al., 2002b). Nonetheless this study clearly demonstrated the therapeutic potential of clonal ACT. More recent trials have shown greater success.

One recent study used CD8+ clones specific for the Melan-A-derived peptide epitope ELAIGILTV, infused in conjunction with both IL-2 and IFN-α following a course of dacarbazine chemotherapy. In this study 6/14 patients showed evidence of tumour regression, with 2 patients exhibiting complete responses ongoing at 28 and >60 months. In this trial clinical responses were correlated with in vivo persistence and clonal expansion (Khammari et al., 2009). It is interesting to compare the relative efficacy of clonal ACT directed against tumour antigens with that used to prevent viral infection following stem-cell transplantation. Only small numbers of anti-viral clones (~8000 cells/kg) are required to mediate complete viral clearance in a majority of patients (Cobbold et al., 2005; Leen et al., 2006; Riddell et al., 1992), compared to the several hundred million or more clonal cells typically utilised in anti-tumour trials (Yee et al., 2002b). These observations provided important early evidence that the nature of T cell clone propagation ex vivo, and the subsequent phenotype, function and engraftment of the infused cell product could have a bearing on clinical efficacy in vivo.

While CD8+ T cell clones can mediate tumour regression or stabilisation in some patients, it is generally accepted that the development of an optimal anti-tumour immune response is dependent on the combinatorial activity of both CD4+ and CD8+ cells (Klebanoff et al., 2006). Unlike most CD8+ cells, CD4+ cells are also able to produce autocrine signals to sustain their own growth (Hunder et al., 2008). Recent reports have shown that CD4+ cells are capable of mediating tumour egression in human trial and in mouse models. In a melanoma patient refractory to IL-2 and IFN-α therapy, CD4+ clones specific for an HLA-DP4 restricted peptide from NY-ESO-1 caused complete tumour clearance in the absence of any supplementary therapy. The infused clone persisted for more than 80 days, and expanded to 3% of the total circulating T cell pool. Interestingly, complete tumour clearance occurred despite NY-ESO-1 only being heterogeneously expressed on the patients tumours – subsequent analysis showed that previously undetectable T cells directed against Melan-A and MAGA-A3 had also undergone de novo priming and expansion, a phenomenon known as “antigen spreading” (Hunder et al., 2008). The authors proposed that lysis of NY-ESO-1 expressing cells led to the release of other targetable proteins from the dying cancer cells, and release of antigen into a now immunostimulatory environment promoted expansion of Melan-A and MAGE-A3 specific cells following uptake by matured DC able to traffic to local lymph nodes. Two recent murine studies utilising adoptive transfer of tyrosinase-related protein 1 (TRP-1) specific CD4+ T cells demonstrated complete clearance of large, established and aggressive TRP-1+ B16-F10 and B16-BL6 melanoma. Interestingly in both studies TRP-1 CD4+ cells infused into hosts that had undergone lymphodepleting chemotherapy and sub-lethal irradiation took on a cytotoxic phenotype and could directly lyse target tumour cells (Quezada et al., 2010; Xie et al., 2010)
At present, the most effective ACT protocols are those utilising expanded populations of heterogeneous tumour-infiltrating lymphocytes (TIL), detectable in >80% of melanoma patients (Rosenberg and Restifo, 2015). The use of pre-existing TIL allows for the production of T cell products recognising a broad range of antigens, and comprising both CD4+ and CD8+ T cells, lessening the risk of tumour escape via target down-regulation or MHC allele silencing (as observed in clonal studies). TIL extracted from melanoma tissue can be expanded to massive numbers (typically up to 10^{10} T cells) after activation in vitro and continuous culture in extremely high doses of IL-2. Expanded TIL cultures are then typically screened for their ability to either produce kill or produce effector cytokines on co-culture with autologous melanoma cell lines (Gattinoni et al., 2006).

Early phase I TIL-based trials conducted through the National Cancer Institute (NCI; Bethesda, USA) in conjunction with IL-2 infusion and chemotherapy showed objective response rates of approximately 30-50%, but were characterised by poor TIL product persistence in vivo, and rapid relapse. Importantly, retrospective analyses suggested that in these trials clinical response rates were inversely correlated with the duration of ex vivo culture in IL-2 prior to infusion (Dudley et al., 2002; Rosenberg et al., 1988; Rosenberg et al., 1994). As such, a ‘rapid expansion protocol’ (REP) was developed, limiting TIL expansion time to 5-6 weeks, and utilising irradiated autologous PBMC as feeder cells to provide additional co-stimulatory ligand and cytokine support (Dudley et al., 2003; Dudley et al., 2002). A subsequent study utilising REP-TIL in conjunction with lymphodepleting, but nonmyeloablative fludarabine/cyclophosphamide chemotherapy and high-dose IL-2 infusion in previously IL-2-refractory metastatic melanoma was more successful – with 18/35 patients exhibiting an objective response (defined by >50% reduction in tumour burden); 4/35 patients undergoing complete remission (Dudley et al., 2005; Rosenberg and Dudley, 2004) and an overall mean response duration of 11.5 months. In this trial, TIL were able to expand to between 3% and 66% of the total circulating pool and persisted for >810 days in one patient. The lymphodepletive regimen led to increased production and serum availability of the homeostatic cytokines IL-7 and IL-15, enhancing TIL engraftment (Dudley et al., 2005; Gattinoni et al., 2005). More recent TIL-based trials have investigated patient preconditioning with lymphodepleting chemotherapy and total body irradiation (2 – 12Gy), and infusion of TIL with both high dose IL-2 and CD34+ haematopoetic stem cells (to enhance reconstitution of the myeloid compartment). In one exemplary study in metastatic melanoma conducted at the NCI, the addition of TBI was demonstrated to greatly enhance clinical efficacy, with reported ORR of 49%; 52% and 72%, and overall survival (at 1 year) of >20%, >40% and >50% in cohorts treated with chemotherapy and no TBI, 2Gy TBI and 12 Gy TBI, respectively. 22% of treated patients underwent complete remission with lymphodepleting chemotherapy and total body irradiation (2 – 12Gy), and infusion of TIL with both high dose IL-2 and CD34+ haematopoetic stem cells (to enhance reconstitution of the myeloid compartment). In one exemplary study in metastatic melanoma conducted at the NCI, the addition of TBI was demonstrated to greatly enhance clinical efficacy, with reported ORR of 49%; 52% and 72%, and overall survival (at 1 year) of >20%, >40% and >50% in cohorts treated with chemotherapy and no TBI, 2Gy TBI and 12 Gy TBI, respectively. 22% of treated patients underwent complete remission, and extensive TIL engraftment (up to 75% of total lymphocytes) and persistence (>64 months in one patient) were observed. Follow-up analysis demonstrated overall survival of 36% at 3 years and 29% at 5 years, but of those exhibiting complete remissions at the conclusion of the trial, 100% remained alive and in remission at 3 years, and 93% at 5 years, demonstrating an immune-mediated curative response (Dudley et al., 2008; Rosenberg and
Restifo, 2015; Rosenberg et al., 2011). Similar (although less marked) results have subsequently been consistently achieved across several centres in metastatic melanoma, with ORR of 38 – 50% (Besser, 2010; Pilon-Thomas et al., 2012; Radvanyi et al., 2012). In a phase II clinical trial, Besser et al. (2013) reported a median patient survival of 15.2 months, 40% ORR and ongoing complete remission in 5/49 patients (at a 28 month follow-up). Importantly, of those patients OR, 78% survived through to a 36 month follow-up, echoing results observed at the NCI.

Despite these successes, TIL based protocols still suffer from the reliance of the REP on extensive culture and rapid division under the continuous influence of high-dose IL-2. REP-grown TIL are often driven to terminal differentiation, losing expression of the co-stimulatory molecules CD27 and CD28, and are prone to activation induced cell death (AICD) (Crompton et al., 2014; Li et al., 2010; Restifo et al., 2012). Retrospective analyses of TIL in recent trials have shown that the key correlates of a clinical response are the CD27 expression level, mean telomere length of infused TIL and duration and extent of TIL persistence in vivo (Robbins, 2004; Rosenberg et al., 2011; Shen et al., 2007; Zhou et al., 2005). TIL able to persist in vivo had a mean telomere length of 6.2kb, while those unable to persist had a mean of 4.5k. Similarly, TIL associated with an objective response had a mean telomere length of 6.3kb, while the mean for clinically ineffective TIL was 4.9kb. (Zhou et al., 2005) Interestingly, persistent T cells isolated from patients exhibited a primarily CD27+ CD28+ CD45RA+ phenotype, suggesting that either persistent TIL are able to re-express co-stimulatory markers in vivo, or that a small minority of expanded TIL retaining CD27 and CD28 expression prior to infusion are able to repopulate the host, while the majority of the infused cells succumb to AICD (Rosenberg et al., 2008). Recent work aiming to optimise the REP has shown that substitution of IL-2 for a combination of the closely related cytokine IL-15, and IL-21, promotes retention of CD28 without sacrificing in vitro expansion. Interestingly, culture in IL-15 alone causes greater loss of CD28 than IL-2, while culture in IL-21 alone promotes CD28 retention but severely curtails expansion (Abbe, 2005; Alves et al., 2005; Li et al., 2010).

Melanoma exhibits the highest rate of mutation and neoepitope generation of all commonly occurring cancers, and induces the highest rate of lymphocyte infiltration - but this in not common to all cancers (Schumacher and Schreiber, 2015). As such, a potential lack of precursor cells may inhibit the application of TIL-based ACT to all cancers. Transduction of pooled T cells with a genetically engineered, retroviral vector-encoded TCR targeting TAA- or CTA- derived epitopes has been proposed as a means of avoiding this problem, in order to replicate the success observed in murine models in mediating B16 melanoma rejection using endogenously gp100/pmel-specific-TCR expressing T cells (Klebanoff et al., 2005; Overwijk et al., 2003), or splenocytes transduced to express a de novo gp100/pmel-specific TCR (Abad et al., 2008). TCR have been engineered, through substitution of amino acids in the peptide-binding domain, to exhibit peptide:MHC affinity 10^6-fold greater than naturally occurring TCR, guaranteeing a high avidity response (Li et al., 2005; Rosenberg
et al., 2008). In a recent phase I clinical trial, T cells transduced with TCR specific for epitopes derived from p53, NY-ESO-1, Melan-A and gp100 were able to persist in melanoma patients for up to 2 months at frequencies >10% of all lymphocytes. In 2 of 15 patients objective responses were seen, and in these 2 patients the infused T cells were still detectable in circulation after 12 months. Interestingly, the frequency of transgenic T cells determined by PCR and TCR Vβ staining was highly discordant (<1% and 8% respectively), suggesting that extensive mispairing of transduced and naturally encoded TCR domains was occurring (Morgan et al., 2006b). This is a potential safety risk, as the mismatching of TCR-α and β domains could potentially result in the formation of novel autoreactive TCR (Cohen et al., 2007). This issue was subsequently addressed through vector design - incorporation of an additional disulfide bridge between the engineered α and β domains resulted in greater TCR expression, minimal mismatching, and higher levels of transduced T cell effector function in a murine B16 melanoma model (Cohen et al., 2007; Frankel et al., 2010). The most recent phase I trial utilising an engineered TCR recognising NY-ESO-1 demonstrated clinical efficacy in patients with metastatic melanoma (5/11 patients), and with synovial sarcoma (4/6 patients). Three patients exhibited durable complete remissions, ongoing beyond 1 year (Robbins et al., 2011).

1.2.4. Supplementary therapies.

Some of the success of TIL-mediated ACT is attributable to a comprehensive patient conditioning regimen. Nonmyeloablative, lymphodepleting chemotherapy was first demonstrated to augment ACT in animal models, and is believed to promote T cell persistence by inducing increased host production of IL-7 and IL-15, important homeostatic survival signals, while concurrently removing competition for these signals by killing off dividing lymphocytes (Gattinoni et al., 2005; Rosenberg et al., 2008). Interestingly, as current protocols involve infusion of massive doses of IL-2, and since IL-2 is known to decrease expression of the IL-7 receptor (CD127), this method may fail to take full advantage of this increase in homeostatic survival signalling (Xue et al., 2002). Although lymphodepletion is also believed to remove regulatory T cells (TREG) from the body, the importance of this phenomenon in subsequent ACT this has been disputed. A recent study in mice suggested that removal of TREG is not an essential outcome of lymphodepletion, as equivalent augmentation of anti-tumour responses was observed in a TREG knockout mouse as in wild-type mice (Gattinoni et al., 2005). This study also suggested that the major effect of increasing IL-7 and IL-15 production was not only to promote T cell expansion, but also to increase the effector function of the infused cells (Gattinoni et al., 2005). Sub-lethal irradiation is believed to augment ACT by promoting death of dividing cancer cells, stimulating inflammation within the tumour and maturing resident DC as they take up released tumour antigens, with the effect of transforming the tumour microenvironment from immunosuppressive to immunostimulatory. Irradiation is also believed to damage the tumour...
vasculature leading to upregulation of cellular adhesion molecules (CAM) on the endothelium and thus promoting T cell migration into the tumour (Johansson et al., 2012; Xie et al., 2010).

At present the most potentially counter-productive of the supplementary therapies appears to be high-dose IL-2 infusion. IL-2 is poorly tolerated and often limiting as it induces vascular leak syndrome. Further, IL-2 is known to induce T cell apoptosis, inhibit memory formation, and drives the expansion of T\textsubscript{REG}, which constitutively express the high affinity IL-2 receptor (CD25) (Berger et al., 2008).

The γc cytokines IL-7, IL-15 and IL-21 have all been investigated as substitutes for IL-2 in ACT. IL-7 has been administered to lymphopaenic patients, and promotes the expansion of CD8\textsuperscript{+} and CD4\textsuperscript{+} lymphocytes without promoting the expansion of T\textsubscript{REG} (Rosenberg et al., 2006). Interestingly, IL-7 infusion in this context initially promotes an even more profound lymphopaenia followed by a rapid T cell reappearance and expansion. Investigation of the dynamics of this response in rhesus macaques (RM) revealed that IL-7 infusion coincides with expression of homing chemokines by the skin, lymph nodes and gut, and T cells migrate to these areas from the blood due to concomitant chemokine receptor upregulation. It is in these sites that T cell expansion is initiated, followed by release back into the periphery (Beq et al., 2009). In the murine ‘RIP-TAg’ model of pancreatic cancer, in which oncogenesis is driven by the SV40 large Antigen (TAg) under the control of the rat insulin promoter (RIP) IL-7 administration in conjunction with viral vaccination enhances tumour rejection and mouse survival. This study revealed that IL-7 exerts its effect by inhibiting cbl-b, a negative regulator of T cell activation (Pellegrini et al., 2009). IL-7 also enhances signalling though the ubiquitin ligase smurf2, which antagonises TGFβ signalling (Pellegrini et al., 2009). IL-7 is also well known to promote T cell survival by upregulating the expression of Bcl-2, an important anti-apoptotic molecule. T cells isolated from IL-7 conditioned mice in this study were not only protected from inhibition, but exhibited increased effector function and more rapid degranulation kinetics on restimulation (Pellegrini et al., 2009).

While IL-2 is believed to promote apoptosis, IL-15 has been shown to promote the survival of certain lymphocyte subsets, particularly CD8\textsuperscript{+} memory cells and natural killer cells, themselves important in tumour immunosurveillance and rejection. This subset-specific expansion has also been demonstrated in a non-human primate model, and IL-15 in this study was not associated with any serious toxicity (Berger et al., 2009). IL-15 infusion may be particularly appropriate for ACT models in which CD8\textsuperscript{+} T cell clones are infused. IL-21 has also shown an ability to augment anti-tumour responses in animal models. When combined with ACT and chemotherapy in a mouse model of B16 melanoma, infusion of IL-21 promoted an increase in circulating tumour-specific T cells detectable at days 4 and 7. Interestingly IL-21 did not stimulate an absolute increase in TIL numbers, but did improve and prolong TIL persistence \textit{in vivo}, enabling tumour rejection (Petersen et al., 2010).

IL-21 is known to promote the expression of both the IL-7 receptor and the lymph node homing molecule CD62L, which characterises naïve and central memory cells in mice (Hinrichs et al., 2008). Finally, IL-21 conditioned cells have been shown to directly out-perform IL-2
conditioned cells in ACT in mice, despite inhibiting acquisition of an effector phenotype and full in
vitro effector function (Hinrichs et al., 2008). Thus, each of these γc cytokines could rationally be
used as a replacement for IL-2 in ACT, and an investigation of their efficacy in a full human ACT
trial would certainly be exciting.

1.2.5. Checkpoint blockade – restoring T cell functionality in situ.

As described in section 1.1.2, the immune system incorporates multiple molecular checks and
balances to control the specificity, timing and extent of effector T cell expansion, longevity and
function. Co-stimulatory ligand:receptor interactions provided to T cells by matured, ‘licenced’ APC
are essential to allow naïve T cell activation, expansion and acquisition of effector function (Chen and
Flies, 2013). The immune system also exhibits parallel, and often temporally regulated, co-inhibitory
ligand:receptor interactions designed to control the specificity and extent of effector T cell activation
and expansion. These co-inhibitory receptors are typically expressed on T cells as natural
consequence of TCR/CD3-mediated signalling, but can be co-opted in a cancer setting to inhibit anti-
tumour responses. Because they act at defined stages during the initiation or propagation of an
immune response, co-stimulatory and co-inhibitory signals are typically referred to as immunological
‘checkpoints’, and several strategies to block these ‘checkpoints’ using neutralising monoclonal
antibodies to allow T cells to bypass or ignore negative regulation have been developed (Sharma and
Allison, 2015).

The best understood and characterised inhibitory checkpoint systems are the surface receptors
Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4/CD152) and programmed Cell Death Protein
1 (PD-1/CD279). Both CTLA-4 and PD-1 are, like CD28, members of the Immunoglobulin
Superfamily (V-type) (Agata et al., 1996; Allison, 1996). CTLA-4 is expressed only on T cells,
under the transcriptional control of Forkhead Box P3 (FoxP3) (Wing et al., 2008). FoxP3 and CTLA-
4 are both constitutively expressed on T
REG cells. By contrast naïve CD4+ and CD8+ T cells express
negligible CTLA-4 mRNA and no surface protein prior to activation and priming. Following an
initial TCR/CD3-mediated stimulus, de novo CTLA-4 is rapidly synthesised and stored in intracellular
clathrin-coated vesicles that can be rapidly shuttled to an immunological synapse on subsequent,
prolonged TCR/CD3 stimulation (typically 48-72h post initial stimulus) (Zhang and Allison, 1997).
CTLA-4 binds the same B7 family ligands as CD28, CD80 and CD86, expressed specifically on APC,
but binds them at a ten-fold higher affinity (Pardoll, 2012).

CTLA-4 appears to be an important control mechanism in T
REG-mediated control of inappropriate
activation of T cells that escape central tolerance, and in limiting the duration of an ongoing
inflammatory immune response. This is reinforced by the rapidly lethal phenotype of CTLA4
knockout mice, which typically die within two weeks from autoimmune inflammatory organ and
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tissue damage, characterised by massive infiltration of activated T cells. This organ damage can be ameliorated by administration of a blocking α-CD28 antibody or by concurrent knockout of B7 molecules (Khattri et al., 1999).

CTLA-4 inhibits T cell activation by several mechanisms. The intracellular domain of CTLA-4 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits inhibitory SRC-Homology Region Domain-Containing Protein (SHP)-2 and Protein Phosphatase 2A (PP2A) phosphatases that antagonise CD3- and CD28-mediated phosphorylation cascades. When recruited into the immunological synapse CTLA-4 is able to sequester B7 ligands away from CD28 based on its higher relative affinity, especially for the more potent CD80 ligand, and thus prevent co-stimulatory signalling from occurring (Egen and Allison, 2002; Linsley et al., 1994). Finally, CTLA-4 is able to bind and actively remove CD80 and CD86 from APC plasma membranes, internalising them inside the T cell (Qureshi et al., 2011). Given the timing and T cell/APC-specific expression of CTLA-4 and its ligands, our current understanding of CTLA-4 function suggests that it acts to prevent improper priming and activation of autoreactive or low-affinity T cells in secondary lymphoid organs.

Like CTLA-4, PD-1 binds two ligands of the B7 family: PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-1 is also absent from naïve CD4\(^+\) and CD8\(^+\) T cells, and is transiently upregulated following TCR stimulation (Agata et al., 1996). Unlike the CTLA-4/CD80/CD86 axis, expressed only on T cells and APC, PD-1 is expressed on T-, B-, NK-, and myeloid cells, while its ligands PD-L1/2 are expressed not only on APC, but also on somatic stromal cells and transformed cells in various cancers (Blank et al., 2004; Topalian et al., 2012). PD-1, like CTLA-4 contains an ITIM and exerts its inhibitory function by recruiting SHP-1 and SHP-2 to counteract both TCR/CD3- and CD28-mediated phosphorylation cascades, with particularly strong inhibitory effects on T cell proliferation and IL-2 production through negative regulation of the PI3K and PKC-θ pathways (Chennnitz et al., 2004; Parry et al., 2005). Conversely, PD-1-mediated signalling promotes the expansion and regulatory function of regulatory T cells, and may play an important role in creating an ‘immunosuppressive’ tumour microenvironment (Francisco et al., 2009).

Transcriptional control of the *PDCD1* (PD-1) locus is complex with upstream cis-regulatory elements containing binding sites for the transcription factors AP-1, NFATc1, FOXO1, NFκB, and Interferon-Stimulated Response Elements (ISRE) (Cho et al., 2008; Xiao et al., 2012). Interestingly, *PDCD1* transcription can be perturbed during effector cell expansion by competitive binding of BLIMP-1 to the NFATc1 binding site, and by antagonism of *FOXO1* transcription by T-bet (Lu et al., 2014a). The *PDCD1* locus is also subject to epigenetic regulation via methylation of CpG islands upstream of the gene. *PDCD1* is typically epigenetically silenced in naïve T cells and transiently demethylated following TCR/CD3 stimulation (Youngblood et al., 2013a). Constitutive PD-1 re-expression is typically observed on dysfunctional or ‘exhausted’ T cells under conditions of chronic antigenic
stimulation, typically during chronic viral infections, on tumour-infiltrating lymphocytes in human cancers, or in murine models of either. Under these circumstances, several studies have shown that the regulatory region of the PDCD1 locus remains demethylated, facilitating continued transcription (Barber et al., 2006; Blank et al., 2004; Day et al., 2006; Topalian et al., 2012). Interestingly, despite its role as a negative inhibitor of T cell activation, expression of PD-1 during the late stages of effector T cell expansion may also be important in promoting memory T cell formation. In acute viral infection models PDCD1 knockout mice generate an effector pool of equivalent size and functionality to that seen in WT mice, but fail to form a memory T cell pool, due to T cell hyporesponsiveness to homeostatic cytokines (Yuzefpolskiy et al., 2016). In this setting it is plausible that PD-1 may act to impair ongoing TCR/CD3-mediated stimulation at a timepoint that allows expanding T cells to ‘rest’ and undergo transition to a memory phenotype. The expression of PD-L1/2 is induced on exposure to IFN-γ signalling, providing a mechanism whereby non-transformed stromal cells can protect themselves from off-target T cell attack during an immune response in organs and tissues – however this mechanism of induction can also be co-opted by cancer cells to evade intratumoural T cell and NK cell-mediated destruction (Dong et al., 2002; Nazareth et al., 2007; Wilke et al., 2011). Further, in some tumours constitutive PD-1 expression has been described to be driven by Anaplastic Lymphoma kinase (ALK) – STAT3 signalling, or due to deletion of the tumour suppressor gene PTEN (Marzec et al., 2008).

Early work in mouse models demonstrated that administration of α-CTLA-4 enabled rejection of the immunogenic colon carcinoma 51BLim10, and that α-CTLA-4 in combination with an irradiated tumour cell vaccine engineered to overexpress GM-CSF allowed control or rejection of aggressive, poorly immunogenic B16-BL6 melanoma (Allison, 1996; Krummel and Allison, 1995). Importantly, these studies showed that the immune response generated against the primary tumour was also protective against a secondary tumour challenge. In the B16-BL6 melanoma model, animals exhibited profound depigmentation, suggesting de novo priming of T cells specific for shared melanoma/melanocyte lineage antigens. Subsequent phase I/II trials in human melanoma and: prostate; urothelial; ovarian and renal cell carcinomas demonstrated antigen-specific T cell expansion, infiltration of activated T cells into tumour beds, and partial responses (primarily stabilisation of disease) in up to 40% of patients. However α-CTLA-4 therapy was also associated with a high incidence (25-33% of enrolees) of grade 3-4 autoimmune or inflammatory toxicities, typically manifesting as hypophysitis or severe gastrointestinal and enteric inflammation (Carthon et al., 2010; Hodi et al., 2010; van den Eertwegh et al., 2012; Yang et al., 2007). Interestingly in these studies anti-tumour responses and autoimmune toxicity were strongly correlated, suggesting the priming of autoreactive T cells targeting tumour-associated lineage antigens.

Two α-CTLA-4 monoclonal antibodies, tremelimunab and ipilimumamb progressed to phase III trials in melanoma. Tremelimunab failed to demonstrate enhanced survival when co-administered with the
chemotherapeutic agent dacarbazine as compared to dacarbazine alone, and exhibited objective response rates (ORR – as determined by RECIST criteria) of only ~10% (Ribas, 2010). In a melanoma vaccination trial using an immunogenic gp100 peptide ipilimumab demonstrated a survival enhancement of 3.5 months alone or in combination with vaccine, compared to vaccination alone (Hodi et al., 2010), and in a subsequent trial demonstrated a 2 month increase in survival time in advanced melanoma compared to dacarbazine, with ~20% or patients exhibiting some tumour regression and surviving at 1 year post-therapy (Robert et al., 2011). Importantly, at a five year follow-up ~90% of these responders remained alive, demonstrating for the first time an immune-mediated curative response (Schadendorf et al., 2015). Based on the results of these trials ipilimumab was licenced for the treatment of melanoma.

Murine models demonstrated that α-PD1 therapy enabled rejection of immunogenic tumours, and, conversely, that overexpression of PD-L1 on normally rejectable tumours abrogated anti-tumour responses by inducing T cell apoptosis in a Fas- and TRAIL-independent manner (Dong et al., 2002). Subsequent phase I/II human trials targeting PD-1 or PD-L1 demonstrated clinical efficacy. Administration of α-PD-L1 antibodies resulted in ORR of ~30% in bladder cancer; ~20% in melanoma and ~10% in renal and lung carcinoma (Brahmer et al., 2012; Powles et al., 2014). α-PD-1 therapy achieved even more marked results – in two trials the antibody pembrolizumab exhibited ORR of ~40% in advanced melanoma, and ORR of ~26% in advanced melanoma refractory to previous ipilimumab therapy (Hamid et al., 2013; Robert et al., 2014), while the antibody nivolumab gave ORR of 18%, 28% and 27% in lung, melanoma and renal cell malignancies respectively (Robert et al., 2015; Topalian et al., 2012). Interestingly, in a phase I trial of Hodgkin’s (HL) Lymphoma refractory to stem cell transplantation, nivolumab administration resulted in an 87% OR rate, and an 86% overall survival rate at 24 weeks. HL is an excellent candidate for checkpoint blockade therapy as HL Reed-Sternberg cells constitutively express both PD-L1 and PD-L2 due to copy number gain (Ansell et al., 2015). All early α-PD-L1 and α-PD-1 trials exhibited some instances of complete responses and regressions were typically ongoing at 1 year post-study. In a subsequent Phase III trial, again in ipilimumab-refractory melanoma, nivolumab alone demonstrated an overall survival rate of 76% to and ORR of 40%, compared to 43% survival and 14% ORR observed in dacarbazine therapy (Robert et al., 2015). The success of these therapies following ipilimumab treatment failure suggests either that de novo priming is dispensable in tumour rejection, and that in many patients existing TIL are able to mediate rejection following alleviation of PD-1/PD-L1 negative regulation, or that T cells primed and expanded during ipilimumab therapy fail to mediate tumour regression due to PD-1/PD-L1 negative regulation. Importantly, α-PD-L1 and α-PD-1 studies were associated with much lower rates of grade 3-4 toxicity than observed with Ipilimumab, and even lower toxicity than those observed with dacarbazine treatment alone, although in one study 3/296 patients were killed by an inflammatory pulmonary toxicity (Topalian et al., 2012).
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As α-CTLA-4 and α-PD-1/PD-L1 operate at different stages in a T cell response, enabling T cell priming and peripheral effector activity, respectively, it was theorised that combinatorial therapy would be synergistic. This was borne out in mouse models, where co-administration facilitated rejection of poorly immunogenic B16 melanoma (which could not be controlled using either monotherapy) and promoted prominent intratumoural effector T cell infiltration (Curran et al., 2010). In a phase I melanoma trial co-administration of nivolumab and ipilimumab resulted in OR in 53% of enrollees, and importantly in 36 of 47 patients with observable tumour regressions, >80% of solid tumour burden was lost and several complete remissions, ongoing 6 months post-therapy, were observed (Wolchok et al., 2013).

In order to determine the utility of PD-L1 as a prognostic biomarker for clinical responses, several α-PD-1 and α-PD-L1 trials included immunohistochemical analysis of patient samples for evidence of PD-L1 expression on tumour cells and on tumour-infiltrating myeloid cells. Several studies reported a correlation between graded intratumoural PD-L1 expression and clinical responses to α-PD-L1 and α-PD1 therapy – however other studies have reported no correlation, in most instances some OR were observed in patients designated PD-L1-negative. The predictive power of PD-L1 expression may also vary according to cancer type and the varying thresholds of ‘PD-L1-positivity’ used across trials (Brahmer et al., 2012; Hamid et al., 2013; Herbst et al., 2014; Powles et al., 2014; Topalian et al., 2012). Interestingly, studies delineating intratumoural cancer cell and myeloid cell PD-L1 expression have suggested that expression of PD-L1 on myeloid cells and the frequency of PD-1+ T cells located at the invasive tumour margin are more accurate predictors of clinical response than PD-L1 expression on cancer cells themselves, speaking to the important role played by intratumoural APC in facilitating continued effector T cell activity and the inhibitory role played by PD-L1+ MDSC (Herbst et al., 2014; Teng et al., 2015).

Although it has been difficult to establish a prognostic immunohistochemical marker for checkpoint blockade therapy, several studies have shown that clinical response rates are correlated with the ‘mutational burden’ of a patient – that is, the frequency of immunogenic mutant neoepitopes expressed by their cancer cells. The presence and efficacy of neoepitope-specific T cells has been demonstrated in murine models using the 3-methylcholanthene-induced sarcoma cell lines d42m1-T3 and F244. In one recent study, profiling of the d42m1-T3 sarcoma exome revealed H2-Kb- and H2-Dd-restricted mutant neoepitopes in the Asparagine-linked glycosylation 8 (Alg8) and Laminin-α subunit 4 (Lama4) proteins. Expanded T cell populations specific for these epitopes (via tetramer binding) could be detected in TIL, but treatment with either α-CTLA-4 or α-PD-1 was necessary to facilitate rejection of tumours (Gubin et al., 2014b). Further, prophylactic serial vaccination using synthetic long peptides containing the mutant epitopes with adjuvant poly I:C allowed mice to reject subsequently implanted d42m1-T3 tumours. Rejection was abrogated in Batf3−/− mice, demonstrating that processing and presentation by CD8α+ CD103+ dendritic cells was essential for the anti-tumour
response. In humans trials tumour exome sequencing has demonstrated that patient mutational burden and clinical responses are correlated during treatment with either α-CTLA-4 (both ipilimumab and tremelimumab) (Snyder et al., 2014) or α-PD-1 (pembrolizumab) (Rizvi et al., 2015a), and that mutant neoepitope-specific T cells can be isolated from the blood of clinical responders (Rizvi et al., 2015b; van Rooij et al., 2013). Interestingly, however, in melanoma previously unobserved vitiligo is also a significant correlate of clinical outcome in trials utilising α-CTLA-4 and α-PD-1, suggesting that T cells targeting mutant neoepitopes and de novo responses primed against melanocyte lineage antigens may co-operate in tumour clearance in both therapies (Lo et al., 2015; Sanlorenzo et al., 2015).

1.2.6. Room for improvement: the optimal T cell subset for adoptive immunotherapy.

While effector function in vitro is not necessarily a correlate of clinical efficacy, cellular characteristics such as homing ability, proliferative capacity (mediated in part by telomerase expression, itself dependent on co-stimulatory signalling), in vivo survival (mediated by responsiveness to γc cytokines and up-regulation of survival molecules), and multipotent cytokine production (expression of IL-2, IFN-γ and tumour necrosis factor-α) are (Jandus et al., 2009). Interestingly, recent work has demonstrated that invasive melanoma are capable of expressing the CCR7 ligands CCL19 and CCL21, indicating that the same chemokine receptors that mediate homing to secondary lymphoid organs can also promote homing to tumours (Harlin et al., 2009; Shields et al.) As such, several studies have attempted to define which T cell subset is the most effective for ACT, by comparing effector cells derived from pure naïve (T_N), T_scm, T_cm, T_em populations or the effectors derived from these. A comparison of Pmel-1 effector cells derived from T_cm and T_em transduced with a TCR recognising a gp100/pmel epitope in a murine B16 melanoma model demonstrated that, in conjunction with a gp100 peptide vaccination, T_cm-derived effectors provided complete protection from B16 tumour growth and mortality at day 28, while T_em-derived cells showed much poorer control of tumour growth and no protection from mortality at a similar time point. On phenotypic analysis, T_cm-derived cells were found to express higher levels of CD62L, CCR7 and the anti-apoptotic protein Mc1-1. By contrast, while T_em-derived effectors showed greater cytotoxic efficacy ex vivo, they also expressed higher levels of the pro-apoptotic proteins Bid and Bad, consistent with their poor in vivo survival. (Klebanoff et al., 2005)

In a non-human primate model CD8+ clones derived from T_cm, but not T_em, were able to survive and persist in vivo, home to both lymph nodes and bone marrow, and went on to repopulate both central and effector memory niches. T_em-derived cells were unable to exit the periphery, and none persisted in the blood after day 5. The homing exhibited by T_cm-derived cells is interesting as both T_cm and T_em-derived effector cells became CD62L-, CD28- and CD27- on in vitro stimulation, indicating a
rapid re-expression of homing molecules \textit{in vivo}. T_{CM}-derived effector cells could be maintained \textit{in vitro} by IL-15, but T_{EM}-derived cells could not, indicating they had lost the ability to express the receptor for this homeostatic cytokine. These reports indicate that T_{CM}-derived cells are clearly superior to T_{EM}-derived cells in an ACT context, especially one reliant on memory T cell proliferation following vaccination (Berger et al., 2008).

More recent studies have compared T_{CM}-derived effector cells with T_{N}-derived effector cells in the same pmel mouse model. In this case, T_{N}-derived cells mediated significantly better anti-tumour response that T_{CM}-derived effectors following vaccination and IL-2 infusion. Effectors from both cell populations took on very similar phenotypes \textit{in vitro}, but a subset of T_{CM}-derived cells maintained CD62L expression, consistent with previous studies. T_{N}-derived cells appeared to have greater proliferative potential, and no surface expression of the exhaustion marker KLRG1 was evident over three stimulation events, while approximately 30\% of T_{CM}-derived cells expressed KLRG1 after a third expansion. Similarly, T_{CM} derived cells produced less IL-2 than T_{N}-derived cells, indicating a more differentiated phenotype. Both sets of effectors expressed T-bet, as expected, but T_{CM}-derived cells expressed \textit{EOMES} at 100-fold greater levels. Interestingly, on adoptive transfer T_{CM}-derived effectors mediated equivalent protection until day 20, at which time tumour relapse occurred in T_{CM}-infused mice, but not T_{N}-infused mice. Intriguingly, these results do not correspond well with previous reports in which T_{CM}-derived cells in the same mouse model were completely protective beyond day 28 (Hinrichs et al., 2009).

Finally, one recent study investigated the use of T_{SCM} - these cells did not acquire an effector phenotype on \textit{in vitro} restimulation, yet outperformed T_{CM} and T_{N}-derived effector cells on reinfusion into a b16 melanoma mouse model (Gattinoni et al., 2009a). This report did correspond well with Hinrichs \textit{et al}, as effector cells derived from Tem restrained tumour growth only until day 10, those derived from T_{CM} were protective until 30, while T_{SCM} derived effectors were completely protective beyond day 30. The \beta-catenin pathway activated in this model was shown to exert an effect activating the transcription factors Lef and Tcf1, both of which promote memory formation. Interestingly, both of these transcription factors have been shown to be up regulated by IL -21 signalling,(Gattinoni et al., 2009b; Koehn and Schoenberger, 2009). This group subsequently identified T_{SCM} in humans, and demonstrated that in a humanised mouse model they exerted superior protection to both T_{CM} and T_{EM} to melanoma challenge, and exhibited superior expansion and engraftment (Gattinoni et al., 2011).

Taken together, these reports indicate that the less differentiated the T cell product used for adoptive cell therapy is the more effective the anti-tumour protection and memory formation this product is able to exert. As such, an opportunity exists to develop a protocol for the large-scale \textit{in vitro} culture of antigen-specific T cells with retention of T_{CM} or T_{SCM} characteristics, to replicate the success observed by gene-engineered naïve T cells in murine models and human trials. Recent studies have had success
in this area, with two recent papers describing the rational application of IL-7, IL-15 and IL-21 to the expansion of naïve antigen-specific T cell products directed against Melan-A and against leukemic blasts (Albrecht et al., 2011; Wolfl et al., 2011). Finally, as our understanding of both checkpoint blockade inhibition and adoptive T cell therapy progress, a combinatorial approach in which the continued anti-tumour activity of an adoptively transferred product of optimal phenotype is mediated by targeted alleviation of intratumoural immunosuppressive mechanisms appears to hold great promise. Such an approach has already proven highly successful in murine models of melanoma (Mahvi et al., 2015) and colorectal cancer (Kodumudi et al., 2016).

1.3. Research aims and objectives.

This research project builds on previous findings within the host laboratory that naïve CD4+ and CD8+ T cells can be stimulated and maintained in vitro under the influence of the common γc cytokine IL-7 and retain some central memory characteristics (Brooks, 2007; Ho, 2012). Further, this project aims to expand these observations to the production of single-cell derived CD4+ and CD8+ T cell clones of known and unknown epitope specificity. We hypothesise that timed administration of the cytokines IL-7 and IL-21 during T cell cloning will allow retention of central memory characteristics and decouple proliferation from differentiation. The specific aims of this research project are to:

- Investigate the functional and phenotypic consequences of naïve and memory CD8+ T cell expansion and maintenance using various common γc cytokine treatments. In particular we are interested in determining the effect of IL-7 and IL-21 on T cell phenotype post-expansion. Subsequently we intend to investigate the application of IL-7 and IL-21 to antigen-specific CD8+ T cell cloning via pentamer- and activation-marker-guided FACS.

- Define the specificity and kinetics of TCR/CD3-dependent surface ‘activation marker’ expression by CD4+ and CD8+ T cells, in order to define specific proteins that can be targeted to isolate epitope-specific activated T cells. T cell activation is associated with dynamic changes in metabolism, cell size and morphology, and plasma membrane expression of adhesion molecules, nutrient receptors, and co-stimulatory and co-inhibitory receptors (Smith-Garvin et al., 2009). Several studies have demonstrated that it is possible to isolate and expand virus- or TAA-specific T cell from PBMC using fluorophore-conjugated tetramers or pentamers (Dunbar et al., 1999; Dunbar et al., 2000). However this method requires a priori knowledge of the specific peptide epitope and HLA restriction required by the T cells, and as such is not amenable to novel epitope discovery. Other studies have investigated several methods to detect antigen responsive T cells in the absence of knowledge of their cognate epitope of HLA restriction. Mannering et al. (2005) have
described a method for antigen-specific cloning by dilution of cell labelling dyes such as CFSE. This technique has the benefit of allowing several days for T cell activation and expansion to occur, and as such is not constrained by a temporal window of specific protein expression. Cloning efficiency in this study was low, however, in part due to cytokine-mediated and bystander proliferation, and CFSE is associated with significant toxicity and with TCR-independent modulation of adhesion molecules (Last'ovicka et al., 2009). Other studies have utilised surface capture of effector cytokines to detect antigen-specific T cells, notably IFNγ and TNFα (Campbell et al., 2011). This technique is also prone to bystander labeling due to incomplete surface capture, and one recent study has shown that the use of IFNγ to isolate CMV-specific clones exhibits a two-fold lower specificity and efficiency than the use of specific activation marker expression (Wehler et al., 2008). Finally, Wolfl et al. (2007) have pioneered the use of CD137 expression as a means of isolating activated virus- and TAA-specific CD8+ T cells. Interestingly this study showed that CD137 expression and pentamer binding (at a population percentage level) were highly correlated following activation with short synthetic peptides. Further, they showed that while IFNγ production and CD137 expression were highly correlated for memory populations, this did not hold true for primed naïve T cells, suggesting that activation marker expression may allow capture of a broader spectrum of antigen-specific T cell phenotypes. Similarly, Wehler et al. (2008) found that both CD4+ and CD8+ T cells specific for CMV and EBV isolated by CD137 did not universally express IFNγ on peptide re-challenge. Although CD137 appears to be a solely TCR/CD3-dependent activation marker, this specificity has not been determined for several other proteins known to be expressed following TCR/CD3 stimulation, (discussed in further detail in section 6.1) nor has the effect of γc cytokine provision in culture been assessed (Matthias Wölfl, 2008).

- Develop a protocol for the activation-marker-guided isolation and expansion of protein- or synthetic long peptide (SLP)-specific CD4+ and CD8+ T cell clones without prior knowledge of their minimal peptide epitope or HLA restriction. Protein and SLP have different internalisation and processing requirements to those of short peptide epitopes, necessitating investigation of the in vitro processing and presentation kinetics of synthetic peptides and recombinant proteins by professional and non-professional antigen-presenting-cells (Rosalia et al., 2013; Zandvliet et al., 2012). MoDC generation is time-consuming, laborious, and often limiting when working with patient samples of low cell number. Further, MoDC may be dispensable for antigen-specific T cell activation in scenarios where de novo T cell priming is not required. As such, we aim to develop a MoDC-independent system for expanding antigen-specific memory T cells.
Finally, based on a synthesis of the aims listed above, we aim to isolate and propagate CD4+ and CD8+ T cell clones specific for the model cancer-testis antigen NY-ESO-1. We aim to characterise the epitope specificities and TCR:pMHC restrictions for these clones by investigating their response patterns to overlapping synthetic peptide libraries, in order to define novel therapeutic targets and expand our understanding of NY-ESO-1 immunogenicity. NY-ESO-1 was first characterised in 1997 via antibody screening of tumour-derived cDNA products. NY-ESO-1 is an X-chromosome encoded CTA of approximately 180 amino acids, and is cytoplasmically localised. NY-ESO-1 is not expressed in any somatic tissue with the exception of the cerebellum (at trace levels). However NY-ESO-1 is widely re-expressed in solid tumours, particularly melanoma, ovarian cancer and synovial sarcoma, although expression is often heterogeneous across a single tumour mass. Re-expression is caused by chromatin hypomethylation (Simpson et al., 2005). NY-ESO-1 expression is associated with metastatic potential and poor prognosis, although no specific function has as yet been determined for NY-ESO-1 (Pastorcic-Grgic et al., 2010; van Rhee et al., 2005; Velazquez et al., 2007b). NY-ESO-1-specific cells typically compromise up to 20% of TIL in melanoma, and NY-ESO-1 has been a major focus of immunotherapeutic interventions. Several vaccination trials have targeted NY-ESO-1, utilising recombinant protein or synthetic peptides, typically administered in combination with TLR agonists. Evidence of durable clinical responses in these trials has been rare, although in one recent study 6 of 14 melanoma patients with NY-ESO-1+ tumours exhibited remissions ongoing over 3 years. NY-ESO-1 is highly immunogenic, and in most vaccination studies robust CD4+ and CD8+ T-cell responses are generated (indicating that T-cell dysfunction, potentially due to PD-1 expression, rather than T-cell expansion failure is the cause of poor clinical response). This has facilitated mapping of MHCI and MHCII restricted epitopes for a variety of HLA specificities across much of the protein. NY-ESO-1 has also been targeted using \textit{ex vivo} expanded T cell clones, and through adoptive transfer of T cells retrovirally transduced with a TCR construct recognising an HLA-A2-restricted peptide epitope, as discussed in section 1.2.3. As such, any novel peptide epitopes or peptide:MHC restrictions discovered through the production and characterisation of T cell clones in this study would expand the availability of these therapies to a wider, HLA-disparate patient pool, and would have immediate clinical relevance.
Chapter 2. Materials and methods

2.1. Materials.

2.1.1. General Buffers and Solutions.

Aqueous solutions were sterilised by autoclaving at 121°C and 15 psi pressure for 20 min. Heat sensitive solutions were sterilised by filtration through a 0.22 μm membrane.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>2.7mM KCl, 1.8mM KH₂PO₄, 137mM NaCl, 10mM Na₂HPO₄, pH 7.4</td>
</tr>
<tr>
<td>PBS-T</td>
<td>1 x PBS, 0.1% v/v Tween-20</td>
</tr>
<tr>
<td>TBS</td>
<td>50mM Tris HCl, 150mM NaCl pH 7.6</td>
</tr>
<tr>
<td>TBS-T</td>
<td>1 x TBS, 0.1% v/v Tween-20</td>
</tr>
<tr>
<td>FACS Wash</td>
<td>1 x PBS, 1% v/v FBS</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris HCl, 1mM EDTA pH 8.0</td>
</tr>
<tr>
<td>TAE</td>
<td>40mM Tris, 20mM glacial Acetic Acid, 2mM EDTA pH 8.0</td>
</tr>
<tr>
<td>IPTG</td>
<td>600mM isopropyl-β-D-thiogalactopyranoside.</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>

2.1.2. Alkaline-Lysis Miniprep Solutions.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>25mM Tris HCl pH 8.0, 50mM glucose, 10mM EDTA</td>
</tr>
<tr>
<td>II</td>
<td>200mM NaOH, 1% w/v SDS (prepared fresh)</td>
</tr>
<tr>
<td>III</td>
<td>3M potassium acetate, 11.5% v/v glacial acetic acid</td>
</tr>
</tbody>
</table>

2.1.3. Solutions for making competent *E.coli*.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSB/TSS buffer</td>
<td>LB broth pH 6.5, 10% w/v PEG 6000, 50mM MgCl₂, 5% v/v DMSO</td>
</tr>
<tr>
<td></td>
<td>DMSO added after autoclaving. TSB/TSS aliquots flash frozen in LiN.</td>
</tr>
</tbody>
</table>
Chapter 2 – Materials and methods

2.1.4. Western Blotting Solutions.

Transfer Buffer 25mM Tris, 200mM Glycine, 20% v/v methanol.

Membrane Blocking /Antibody staining solution

PBS-T, 5% w/v non-fat milk powder

Wash buffer 1 x PBS-T

Detection ECL™ detection Kit (Amersham Biosciences)

2.1.5. SDS-PAGE and protein visualisation.

Laemmli Loading Buffer (5x) 300mM Tris HCl pH 6.8, 10% w/v SDS, 50% v/v glycerol, 0.05% bromophenol blue. Reducing samples supplemented with 5% 2-βM.

SDS-PAGE solution A 30% w/v acrylamide/0.8% w/v bisacrylamide (Bio-Rad)

SDS-PAGE solution B 1.5M Tris HCl pH 8.0, 0.4% w/v SDS

SDS-PAGE solution C 0.5M Tris HCl pH 6.8

Polymerisation reagents 10% APS; TEMED

SDS-PAGE running buffer 25mM Tris, 200mM Glycine, and 0.1% w/v SDS pH 8.0

Coomassie Brilliant Blue 0.06% w/v Brilliant Blue R-250 (Sigma-Aldrich; MO, USA), 45% v/v methanol, 10% v/v glacial acetic acid

Coomassie Destain 45% v/v methanol, 10% v/v glacial acetic acid

Silver stain Fix 30% v/v ethanol, 10% v/v glacial acetic acid

Silver stain Farmer’s Reagent 12mM NaS₂O₃, 2.5mM K₃Fe(CN)₆, 4.5mM NaCO₃

Silver stain Developer 235mM NaCO₃, 0.02% v/v formaldehyde

Silver Nitrate 0.1% w/v AgNO₃
### 2.1.6. Primers.

#### Table 2-1: Primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’ restriction site underlined)</th>
<th>Restriction</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NYESO1_HTB_F</td>
<td>CGCCGCCGATCCATGCAGGCCGAAG GCCGGGGCACAGGCCGGGGC</td>
<td>BamHI</td>
<td>60°C</td>
</tr>
<tr>
<td>NYESO1_HTB_R</td>
<td>CGCCGCAAGCTTTTATGCCTGTGCCTGCC CTGAGGGAGGCTGAGCCAAA</td>
<td>HindIII</td>
<td>60°C</td>
</tr>
<tr>
<td>NYESO1_AAV-</td>
<td>CGCCGCTTAATTTAAATGCAGGCCG AAGGCCGCGGACAG</td>
<td>PacI</td>
<td>65°C</td>
</tr>
<tr>
<td>NYESO1_AAV_R</td>
<td>CGCCGCATCGATTTAGCGCCTCTGCC CTGAGGGAGGCTGAGCCAAA</td>
<td>ClaI</td>
<td>65°C</td>
</tr>
<tr>
<td>NYESO1_seq_1F</td>
<td>TCTACCTCGCCATGCTTTC</td>
<td>NA</td>
<td>56°C</td>
</tr>
<tr>
<td>NYESO1_seq_RC</td>
<td>GAAAGGCATGCGAGGTAGA</td>
<td>NA</td>
<td>56°C</td>
</tr>
<tr>
<td>β-actin sense</td>
<td>GCTCGTCTCGACGGCTGAGCTC</td>
<td>NA</td>
<td>55-60°C</td>
</tr>
<tr>
<td>β-actin antisense</td>
<td>CAAACATGATCGGTCATCTTCTC</td>
<td>NA</td>
<td>55-60°C</td>
</tr>
<tr>
<td>HLA-C3 sense</td>
<td>CACAGACTGACGGGTAGGAG</td>
<td>NA</td>
<td>67°C</td>
</tr>
<tr>
<td>HLA-C3 antisense</td>
<td>GCGTCTCTGCTCTCCGTTCTT</td>
<td>NA</td>
<td>67°C</td>
</tr>
</tbody>
</table>
2.1.7. Cell culture media.

DMEM + L-glutamine (Gibco®)

RPMI 1640 (Life Technologies; Carlsbad, CA, USA) +/- Phenol Red.

Complete RPMI RPMI 1640 (Life Technologies) supplemented with 1 x Pen/Strep (Life Technologies) and 1 x GlutaMax™-I (Life Technologies).

Fetal Bovine Serum (FBS) Fetal Bovine Serum (Life Technologies), heat inactivated at 56°C and filter-sterilised.

Human Serum (HS) Human Serum (Life Technologies), heat inactivated at 56°C and filter-sterilised.

DF10 DMEM + L-glutamine, 10% FBS

RF10 Complete RPMI, 10% v/v FBS

RS5 Complete RPMI, 5% v/v HS

Freezing Medium 80% v/v FBS, 20% v/v DMSO (Sigma-Aldrich).

Sf900™ III SFM Medium (Life Technologies)

Sf9 Growth Medium SF900™ III, 1% v/v FBS.
2.1.8. Cell lines.

Table 2-2: Human cell lines used

<table>
<thead>
<tr>
<th>Name</th>
<th>Cell Type and details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK 293 (T)</td>
<td>Human, embryonic kidney</td>
<td>Molecular Virology Group, SBS</td>
</tr>
<tr>
<td>LG2</td>
<td>HLA-A2+ EBV-transformed B-Lymphoblast cell line (B-LCL)</td>
<td>Dunbar group, SBS</td>
</tr>
<tr>
<td>T2</td>
<td>HLA-A2+ T-B Lymphoblast Hybrid</td>
<td>Commercially available (ATCC®, CRL-1922™)</td>
</tr>
<tr>
<td>DAGI</td>
<td>Melanoma cell line</td>
<td>Dunbar group, SBS</td>
</tr>
<tr>
<td>Me275</td>
<td>Melanoma cell line</td>
<td>Dunbar group, SBS</td>
</tr>
<tr>
<td>MZ2</td>
<td>Melanoma cell line</td>
<td>Dunbar group, SBS</td>
</tr>
<tr>
<td>Na8</td>
<td>Melanoma cell line</td>
<td>Dunbar group, SBS</td>
</tr>
<tr>
<td>SK-mel-23</td>
<td>Melanoma cell line</td>
<td>Memorial Sloan-Kettering Cancer Centre</td>
</tr>
<tr>
<td>SK-mel-29</td>
<td>Melanoma cell line</td>
<td>Memorial Sloan-Kettering Cancer Centre</td>
</tr>
<tr>
<td>Trombelli</td>
<td>Melanoma cell line</td>
<td>Dunbar group, SBS</td>
</tr>
</tbody>
</table>

Table 2-3: Non-human cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Species origin and details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B95-8</td>
<td><em>Sanguinus oedipus</em> cell line. Releases infectious EBV.</td>
<td>Dr See-Tan Woo, ADHB</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugifera</em> cell line. Permissive for Baculovirus infection and protein expression</td>
<td>Dr James Dickson, SBS</td>
</tr>
</tbody>
</table>
2.1.9. **Bacterial growth media.**

Luria-Bertani (LB) Broth  
1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0.

LB-Agar  
2% w/v agar in LB broth, supplemented with selective antibiotics as desired.

2.1.10. **Antibiotics and selection reagents.**

**Table 2-4: Antibiotics and selection reagents.**

<table>
<thead>
<tr>
<th>Antibiotic/Reagent</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Sigma-Aldrich)</td>
<td>50mg/ml stock filter sterilised and stored at -20°C. Used at a final concentration of 100µg/ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>50mg/ml stock in 100% ethanol stored at -20°C. Used at a final concentration of 30µg/ml</td>
</tr>
<tr>
<td>Gentamicin (Serva GmBH; Heidelberg, Germany)</td>
<td>34mg/ml stock filter sterilised and stored at 4°C. Used at a final concentration of 34µg/ml</td>
</tr>
<tr>
<td>Kanamycin (Gibco®)</td>
<td>50mg/ml stock filter sterilised and stored at -20°C. Used at a final concentration of 50µg/ml</td>
</tr>
<tr>
<td>100x Penicillin/Streptomycin (Life Technologies)</td>
<td>Added to RPMI at 1:100</td>
</tr>
<tr>
<td>Tetracycline (Sigma-Aldrich)</td>
<td>10mg/ml stock in 70% ethanol stored at -20°C. Used at a final concentration of 10µg/ml</td>
</tr>
<tr>
<td>IPTG (Sigma-Aldrich)</td>
<td>Used at a final concentration of 40µg/ml, powder added directly to medium.</td>
</tr>
<tr>
<td>X-gal (Sigma-Aldrich)</td>
<td>Used at a final concentration of 150µg/ml, powder added directly to medium.</td>
</tr>
</tbody>
</table>
### 2.1.11. Antibodies and Pentamers.

**Table 2-5: Antibodies and pentamers for flow cytometric detection of cell surface antigens**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Supplier</th>
<th>Clone</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>FITC</td>
<td>Serotec (Oxford, UK)</td>
<td>NA1/34-HLK</td>
<td>2.5</td>
</tr>
<tr>
<td>CD3</td>
<td>unconjugated</td>
<td>Biolegend (CA, USA)</td>
<td>OKT3</td>
<td>Various</td>
</tr>
<tr>
<td>CD3</td>
<td>BV510</td>
<td>Biolegend</td>
<td>UCHT1</td>
<td>0.625</td>
</tr>
<tr>
<td>CD3</td>
<td>BV510</td>
<td>Biolegend</td>
<td>UCHT1</td>
<td>0.625</td>
</tr>
<tr>
<td>CD3</td>
<td>BV510</td>
<td>Biolegend</td>
<td>UCHT1</td>
<td>0.625</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC</td>
<td>Biolegend</td>
<td>RPA-T4</td>
<td>0.5</td>
</tr>
<tr>
<td>CD4</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>RPA-T4</td>
<td>2.5</td>
</tr>
<tr>
<td>CD8</td>
<td>FITC</td>
<td>Biolegend</td>
<td>RPA-T8</td>
<td>0.5</td>
</tr>
<tr>
<td>CD8</td>
<td>AF700</td>
<td>Biolegend</td>
<td>RPA-T8</td>
<td>0.5</td>
</tr>
<tr>
<td>CD14</td>
<td>FITC</td>
<td>Biolegend</td>
<td>HCD14</td>
<td>5</td>
</tr>
<tr>
<td>CD14</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>HCD14</td>
<td>5</td>
</tr>
<tr>
<td>CD16</td>
<td>FITC</td>
<td>Biolegend</td>
<td>3G8</td>
<td>5</td>
</tr>
<tr>
<td>CD19</td>
<td>FITC</td>
<td>Biolegend</td>
<td>HIB19</td>
<td>5</td>
</tr>
<tr>
<td>CD20</td>
<td>PE</td>
<td>Biolegend</td>
<td>2H7</td>
<td>1.25</td>
</tr>
<tr>
<td>CD20</td>
<td>PE-CF594</td>
<td>Biolegend</td>
<td>2H7</td>
<td>1.25</td>
</tr>
<tr>
<td>CD21</td>
<td>APC</td>
<td>BD Biosciences (NJ, USA)</td>
<td>BLY4</td>
<td>10</td>
</tr>
<tr>
<td>CD25</td>
<td>APC-Cy7</td>
<td>Biolegend</td>
<td>BC96</td>
<td>5</td>
</tr>
<tr>
<td>CD27</td>
<td>APC-Cy7</td>
<td>Biolegend</td>
<td>O323</td>
<td>10</td>
</tr>
<tr>
<td>CD28</td>
<td>unconjugated</td>
<td>Biolegend</td>
<td>CD28.2</td>
<td>Various</td>
</tr>
<tr>
<td>CD28</td>
<td>APC</td>
<td>Biolegend</td>
<td>CD28.2</td>
<td>2.5</td>
</tr>
</tbody>
</table>
### Chapter 2 – Materials and methods

<table>
<thead>
<tr>
<th>CD</th>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Code</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD38</td>
<td>PE-Texas Red</td>
<td>Molecular Probes (OR, USA)</td>
<td>HIT2</td>
<td>2.5</td>
</tr>
<tr>
<td>CD45RA</td>
<td>PE-CF594</td>
<td>BD Biosciences</td>
<td>HI100</td>
<td>0.3</td>
</tr>
<tr>
<td>CD45RO</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>UCHL1</td>
<td>2.5</td>
</tr>
<tr>
<td>CD56</td>
<td>FITC</td>
<td>Biolegend</td>
<td>HCD56</td>
<td>5</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>HCD56</td>
<td>1.25</td>
</tr>
<tr>
<td>CD69</td>
<td>PerCP</td>
<td>BD Biosciences</td>
<td>FN50</td>
<td>5</td>
</tr>
<tr>
<td>CD69</td>
<td>AF700</td>
<td>Biolegend</td>
<td>FN50</td>
<td>0.25</td>
</tr>
<tr>
<td>CD62L</td>
<td>PerCp-Cy5.5</td>
<td>Biolegend</td>
<td>DREG-56</td>
<td>1.25</td>
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</table>

**L-Selectin**

<table>
<thead>
<tr>
<th>CD</th>
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<th>Code</th>
<th>Amount</th>
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<tr>
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<td>PE</td>
<td>BD Biosciences</td>
<td>MA712</td>
<td>20</td>
</tr>
<tr>
<td>CD95 (Fas)</td>
<td>APC</td>
<td>BD Biosciences</td>
<td>DX2</td>
<td>5</td>
</tr>
<tr>
<td>CD98</td>
<td>FITC</td>
<td>BD Biosciences</td>
<td>UM7F8</td>
<td>5</td>
</tr>
<tr>
<td>CD127</td>
<td>PE</td>
<td>Beckman Coulter (CA, USA)</td>
<td>R34.34</td>
<td>10</td>
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</table>

**IL-7Ra**

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<tr>
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<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>CD127</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>hIL7R-M21</td>
<td>1.25</td>
</tr>
<tr>
<td>CD134</td>
<td>PE</td>
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<td>ACT35</td>
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<tr>
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<td>4B4.1</td>
<td>10</td>
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<tr>
<td>CD154</td>
<td>APC</td>
<td>Biolegend</td>
<td>TRAP1</td>
<td>5</td>
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<tr>
<td>CD197/CCR7</td>
<td>FITC</td>
<td>Biolegend</td>
<td>G043H7</td>
<td>2.5</td>
</tr>
<tr>
<td>CD197/CCR7</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>G043H7</td>
<td>2.5</td>
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</table>
Table 2-6: Antibodies and isotype controls for intracellular flow cytometry (ICFC)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Supplier/Brand</th>
<th>Clone</th>
<th>Dose</th>
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<tbody>
<tr>
<td>IL-2</td>
<td>PE</td>
<td>Biolegend</td>
<td>MQ1-17H12</td>
<td>0.3</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>FITC</td>
<td>BD</td>
<td>4S.B3</td>
<td>0.4</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>AF647</td>
<td>Biolegend</td>
<td>4S.B3</td>
<td>1.25</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>FITC</td>
<td>BD</td>
<td>GB11</td>
<td>10</td>
</tr>
<tr>
<td>Perforin</td>
<td>PE</td>
<td>Biolegend</td>
<td>B-D48</td>
<td>0.625</td>
</tr>
<tr>
<td>TNF-α</td>
<td>A488</td>
<td>Biolegend</td>
<td>Mab11</td>
<td>1.25</td>
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</table>

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Fluorophore</th>
<th>Supplier</th>
<th>Clone</th>
<th>Dose</th>
<th>Primary antibody controlled for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1, κ</td>
<td>AF488</td>
<td>Biolegend</td>
<td>MOPC21</td>
<td>1.25</td>
<td>TNFα</td>
</tr>
<tr>
<td>IgG1, κ</td>
<td>AF647</td>
<td>Biolegend</td>
<td>MOPC21</td>
<td>1.25</td>
<td>IFNγ</td>
</tr>
<tr>
<td>IgG1, κ</td>
<td>FITC</td>
<td>Biolegend</td>
<td>MOPC21</td>
<td>1.25</td>
<td>IFNγ, GrzB</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Clone</th>
<th>Dose</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1, κ</td>
<td>PE</td>
<td>Biolegend</td>
<td>MOPC21</td>
<td>1.25</td>
<td>Perforin</td>
<td></td>
</tr>
<tr>
<td>IgG2a, κ</td>
<td>PE</td>
<td>Biolegend</td>
<td>MOPC173</td>
<td>1.25</td>
<td>IL-2</td>
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</table>

Table 2-7: Antibodies for western blot and immunocytochemistry

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Clone</th>
<th>Dose</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-NY-ESO-1, IgG1 (mouse)</td>
<td>Unconjugated</td>
<td>Ludwig Institute</td>
<td>ESI21</td>
<td>1:15000</td>
<td>WB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat-α-mouse-IgG1</td>
<td>HRP</td>
<td>Santa Cruz Biotechnology (CA, USA)</td>
<td>Polyclonal</td>
<td>1:5000</td>
<td>WB</td>
</tr>
<tr>
<td>α-Melan-A, IgG2b (mouse)</td>
<td>Unconjugated</td>
<td>Sigma-Aldrich (Cell Marque)</td>
<td>M2-7C10</td>
<td>1:250</td>
<td>ICC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat-α-mouse-IgG2b</td>
<td>AF488</td>
<td>Molecular Probes</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>ICC</td>
</tr>
</tbody>
</table>

2.1.12. ELISPOT kits and reagents.

Human IFN-γ ELISPOT kit (BD Biosciences, catalogue #551849)

Capture antibody: NA/LE™ α-human-IFNγ (used at 1:200; 5µg/ml)

Detection antibody: Biotinylated α-human-IFNγ (used at 1:250; 2µg/ml)

100x Streptavidin-Horseradish Peroxidase (BD)

Dilution buffer: PBS, 10% FBS v/v

AEC solution: AEC (3-amino-9-ethyl-carbazole, Sigma-Aldrich) dissolved in DMF (N,N-Dimethylformamide, Sigma-Aldrich) at 10mg/ml.
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0.1M Acetate solution: 148ml 0.2M glacial acetic acid, 352ml 0.2M sodium acetate, milliQ water to 1L, pH 5.0

H₂O₂ (30%)

2.1.13. Recombinant human cytokines.

Recombinant human interleukin-2; -4; -7; -12; -15; -21, interferon-gamma, and GM-CSF were purchased from PeproTech Inc. (NJ, USA) and prepared according to the manufacturer’s recommendations. Final concentrations used are listed in text.


Sf9 lysis buffer 25mM Sodium Phosphate pH 7.4, 500mM NaCl, 20mM imidazole, 6M urea, 0.5% v/v Nonidet-P-40 (Merck-Millipore; Calbiochem). Supplemented with Complete™ mini EDTA-free protease inhibitor tablets (Roche; Basel, Switzerland).

Nuclear solubilisation Buffer 25mM Sodium Phosphate pH 7.4, 300mM NaCl, 5% v/v glycerol, 1% w/v SDS

IMAC Wash Buffer I 25mM Sodium Phosphate pH 7.4, 500mM NaCl, 20mM imidazole, 6M urea, 5% v/v glycerol

IMAC Wash Buffer II 25mM Sodium Phosphate pH 7.4, 500mM NaCl, 40mM imidazole, 6M urea, 5% v/v glycerol

IMAC Elution Buffer 25mM Sodium Phosphate pH 7.4, 500mM NaCl, 500mM imidazole, 6M urea, 5% v/v glycerol

Protein Storage buffer 25mM Sodium Phosphate pH 7.4, 500mM NaCl, 6M urea, 5% v/v glycerol

2.1.15. rAAV Heparin affinity chromatography (HAC).

HEK293 lysis buffer 50mM Tris-HCl pH 8.0, 150mM NaCl

HAC Buffer A 20mM Tris-HCl pH 8.0, 100mM NaCl

HAC Buffer B 20mM Tris-HCl pH 8.0, 1M NaCl
2.1.16. Restriction enzymes.

Restriction enzymes BamHI and HindIII were purchased from Roche. Restriction enzymes Clal and PacI were purchased from New England Biolabs (MA, USA). Restriction endonuclease digestion as carried out following manufacturer’s instructions as described in text.

2.1.17. Plasmids.

Table 2-8: Plasmids used or produced in this study

<table>
<thead>
<tr>
<th>Vector Name</th>
<th>Selection Marker</th>
<th>Source</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFastbacHTb</td>
<td>Ampicillin</td>
<td>Dr James Dickson, School of Biological</td>
<td>Cloning vector</td>
</tr>
<tr>
<td>(Invitrogen, Life</td>
<td></td>
<td>Sciences, University of Auckland</td>
<td></td>
</tr>
<tr>
<td>Technologies™)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFastbacHTb-</td>
<td>Ampicillin</td>
<td>Produced in this study</td>
<td>Cloning vector encoding NY-ESO-1</td>
</tr>
<tr>
<td>NYESO1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAM/CAG-NYESO1-WPRE-bGhpA</td>
<td>Ampicillin</td>
<td>Produced in this study</td>
<td>rAAV vector genome encoding NY-ESO-1</td>
</tr>
<tr>
<td>pAM/CAG-eGFP-WPRE-bGhpA</td>
<td>Ampicillin</td>
<td>Dr James Ussher, School of Biological</td>
<td>rAAV vector genome encoding eGFP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sciences, University of Auckland</td>
<td></td>
</tr>
<tr>
<td>pFdelta6</td>
<td>Ampicillin</td>
<td></td>
<td>Encodes Adenoviral helper functions for</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>production of rAAV</td>
</tr>
<tr>
<td>pNLRep</td>
<td>Ampicillin</td>
<td></td>
<td>Packaging plasmid for AAV2</td>
</tr>
<tr>
<td>pAAV2/6</td>
<td>Ampicillin</td>
<td></td>
<td>Packaging plasmid for AAV6</td>
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2.1.18. Bacterial strains.

Table 2-9: Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>E.coli DH5α</td>
<td>F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1</td>
<td>Molecular Virology Group, School of Biological Sciences, University of Auckland</td>
</tr>
<tr>
<td>E.coli DH10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC)</td>
<td>Dr James Dickson, School of Biological Sciences, University of Auckland</td>
</tr>
<tr>
<td>Multibac</td>
<td>φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ (ara, leu)7697 galU galK λ-rpsL nupG/ pMON14272 Δ (chiA, v-cath) / pMON7124</td>
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</tr>
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</table>

2.1.19. Proteins and synthetic peptides.

Table 2-10: Proteins and synthetic peptides used or produced in this study

<table>
<thead>
<tr>
<th>Protein/Peptide Designation</th>
<th>Protein of origin</th>
<th>Sequence</th>
<th>Supplier</th>
<th>Diluent</th>
<th>Stock Concentration</th>
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</thead>
<tbody>
<tr>
<td>rNY-ESO-1</td>
<td>NY-ESO-1</td>
<td>Full sequence</td>
<td>Produced in this study</td>
<td>6M Urea</td>
<td>500µM</td>
</tr>
<tr>
<td>GLC_{280-288} (EBV)</td>
<td>BMLF1</td>
<td>GLCTLVAML</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>10mM</td>
</tr>
<tr>
<td>NLV_{495-503} (CMV)</td>
<td>pp65</td>
<td>NLVPMVATV</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>10mM</td>
</tr>
<tr>
<td>ELA_{26-35(A27L)}</td>
<td>Melan-A</td>
<td>ELAGIGLTV</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>10mM</td>
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referred to as 26-35 in-text
<table>
<thead>
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<th>Peptide</th>
<th>Antigen</th>
<th>Source</th>
<th>Secondary Proteins</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GILGFVFTL</td>
<td>Matrix (Influenza)</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>10mM</td>
</tr>
<tr>
<td>CLGGLLTMV</td>
<td>LMP2 (EBV)</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>10mM</td>
</tr>
<tr>
<td>CAGILARNLV</td>
<td>pp65 (CMV)</td>
<td>Dr Geoff Williams, University of Auckland</td>
<td>DMSO</td>
<td>10mM</td>
</tr>
<tr>
<td>ILARNLVPMVATVQGQNLKYQEFFWDANDIYRI</td>
<td>ILA491-522</td>
<td>Dr Geoff Williams, University of Auckland</td>
<td>DMSO</td>
<td>10mM</td>
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<tr>
<td>LKEFTVSGNIL</td>
<td>NY-ESO-1</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>KEFTVSGNILT</td>
<td>NY-ESO-1</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>EFTVSGNILTI</td>
<td>NY-ESO-1</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>FTVSGNILTIR</td>
<td>NY-ESO-1</td>
<td>Sigma-Aldrich</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>GARGPESRLLEF</td>
<td>p79-116</td>
<td>Dr Geoff Williams, University of Auckland</td>
<td>DMSO</td>
<td>3mM</td>
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<tr>
<td>EFYLAMPFAFTPMEAEILARRS</td>
<td>p118-143</td>
<td>as above</td>
<td>DMSO</td>
<td>3mM</td>
</tr>
<tr>
<td>GARGPESRLLEF</td>
<td>p153-180</td>
<td>as above</td>
<td>DMSO</td>
<td>3mM</td>
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<tr>
<td>NGCCRCGAGPESRLLEF</td>
<td>p73-90</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
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</table>
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<table>
<thead>
<tr>
<th>Page</th>
<th>NY-ESO-1 Peptide Sequence</th>
<th>Institute</th>
<th>DMSO</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>p79-96</td>
<td>GARGPESRL EFYLAMPF</td>
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</tr>
<tr>
<td>p85-102</td>
<td>SRLLEFYLM PFATPMEA</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
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<tr>
<td>p91-108</td>
<td>YLAMPFATPM EAEARRS</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p97-116</td>
<td>ATPMEAELAR RSLAQDAPPL</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p103-120</td>
<td>ELARRSLAQD APPLPVG</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
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<tr>
<td>p109-126</td>
<td>LAQDAPPLPV PGVLLKEF</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p115-132</td>
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<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p121-138</td>
<td>VLLKEFTVSG NILTIRLT</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p127-144</td>
<td>TVSGNILTIRL TAADHRQ</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p133-150</td>
<td>LTIRLTAADH RQLQLSIS</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p139-156</td>
<td>AADHRQLQLS ISSCLQQL</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p145-162</td>
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<td>Ludwig Institute</td>
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<td>15mM</td>
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<td>p151-169</td>
<td>SCLQQSSLLM WITQCFLPV</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
<tr>
<td>p157-174</td>
<td>SLLMWITQCF LPVFLAQP</td>
<td>Ludwig Institute</td>
<td>DMSO</td>
<td>15mM</td>
</tr>
</tbody>
</table>
2.2. Molecular biology methods.

2.2.1. Bacterial culture.

2.2.1.1. Plate culture.

Bacteria were either streaked (using a sterile wire loop or pipette tip) or spread (using a sterile glass spreader) onto LB-agar plates supplemented with appropriate antibiotics. Plates were inverted and incubated at 37°C overnight. Unused plates were stored at 4°C wrapped in parafilm for up to 1 month.

2.2.1.2. Broth Culture.

Appropriate volumes of LB broth supplemented with appropriate antibiotics were inoculated with single colonies from agar plates. Cultures were grown overnight at 37°C shaking at 200rpm. For plasmid DNA ‘maxipreps’, starter cultures were grown for 6h as described, then diluted 1:100 and cultured overnight. Culture density was assessed via measuring absorbance at 600nm (OD$_{600}$).

2.2.2. Agarose Gel Electrophoresis (AGE).

0.8% w/v agarose gels were prepared by dissolving agarose powder in TAE buffer. SYBR®Safe DNA stain (Life Technologies) was incorporated into the gel to allow DNA visualisation. PCR or DNA products were electrophoresed for 35 min at 100V in a HE33 mini horizontal submarine unit (Amersham) and gels imaged using a GelDoc 2000 and Quantity One Software (Bio-Rad). DNA band size was assessed using a 1 Kb Plus DNA ladder (Invitrogen).

2.2.3. DNA amplification by Polymerase chain reaction (PCR).

PCRs were performed in a Nexus Gradient Mastercycler (Eppendorf). Template DNA was amplified using Platinum® Pfx DNA polymerase (Life Technologies) because of its proof-reading 3’ to 5’ exonuclease activity. Reactions were performed in a total volume of 50-100µl, following manufacturer’s recommendations. Typically a 50µl reaction contained: 1x Pfx Amplification Buffer; 0.3mM dNTP mixture; 1mM MgSO$_4$; 0.3µM forward and reverse primer; template DNA (10pg - 200 ng) and 1U Platinum® Pfx DNA Polymerase. In cases where standard amplification conditions proved unsuitable, 2x Pfx Amplification Buffer or 1-2x PCR Enhancer solution were included.
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The mixture was heated to 94°C for 5 min to denature template DNA, followed by 35 cycles of: denaturation (94°C, 15 s); annealing (temperature dependent on primer, as described in Table 2-1, 30s); and extension (68°C, 1 min per kb of product). A final extension period (68°C, 7 min) was performed and reactions cooled to 15°C. Completed reactions were analysed by AGE and stored at 4°C or -20°C. Where desired, PCR products were cleaned using the PureLink™ PCR purification kit (Life Technologies) according to manufacturer’s specifications.

2.2.3.1. HLA-C*03 typing by Polymerase chain reaction (PCR).

PCR’s were performed in a Nexus Gradient Mastercycler (Eppendorf). HLA-C*03 or β-Actin products were amplified using KAPA2G Robust polymerase (KAPA Biosystems). Reactions were performed in a total volume of 10µl including: 1x KAPA2G Robust HotStart Readymix (polymerase, dNTP and 2mM MgCl2 included); 0.5µM forward and reverse primer; and 10ng genomic DNA template.

Optimal annealing temperature and cycle number were determined empirically. For amplification of a 562bp HLA-C*03 product, samples were heated to 95°C for 3 min to denature template DNA, followed by 40 cycles of: denaturation (95°C, 15 s); annealing (67°C, 15s); and extension (72°C, 15s). A final extension period (72°C, 1 min) was performed and reactions cooled to 15°C. For amplification of a 353bp β-Actin product the annealing temperature was decrease to 60°C, but all other conditions remained the same. Completed reactions were analysed by AGE.

2.2.4. Genomic DNA extraction.

Genomic DNA was extracted from 1-10M cultured cells using the PureLink® Genomic DNA Mini Kit (Invitrogen) following manufacturer’s instructions. Genomic DNA was eluted in 100µl molecular biology grade water. Genomic DNA products were analysed on a 1% agarose gel, and typically exhibited a single band at >12kb with no visible degradation products.

2.2.5. Nucleic acid quantification.

DNA and RNA were quantified by measuring absorbance at 260nm on a Nanodrop-1000 (Nanodrop Technologies Inc.)

2.2.6. RNA extraction.

RNA was isolated from cultured melanoma cell lines or human fibroblasts using the RNeasy™ mini kit (Qiagen) according to manufacturer’s instructions. Briefly, 1-3 million cells were harvested using TrypLE™ Express (Life Technologies), washed once with PBS, resuspended in lysis buffer RLT
supplemented with 1% v/v β-ME and passed through a 20-gauge needle. An equivalent volume of 70% ethanol was added, and the lysate mixed by pipette and transferred to an RNeasy spin column. The lysate was centrifuged at >10,000 x g for 30 s and the flow-through discarded. The column was washed once with buffer RW1, and a 15 min on-column DNase I digestion performed. The column was then washed once with buffer RW1, twice with buffer RPE and spun until completely dry. 50µl of molecular biology grade water was applied to the column, and allowed to incubate for 1 min. The column was then transferred to a clean 1.5 ml microfuge tube and spun at > 10,000 x g for 60 s to elute RNA. This step was repeated to maximise RNA elution. Eluted RNA was stored at -80°C.

2.2.7. Reverse transcription and cDNA synthesis.

RNA isolated from cultured cells as described above, or Ambion® Human Testes Total RNA (Life Technologies™), was used as a template for reverse transcription/ first-strand cDNA synthesis. Reactions were performed in DNA Mastercycler (Eppendorf) using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies™) according to manufacturer’s recommendations. Briefly, for each reaction, ≤5µg template RNA, 1µl 50µM oligo(DT)20 primer, 1µl 10µM dNTP and DEPC-treated water were combined in a reaction volume of 10µl, heated to 65°C for 5 min and then cooled on ice for. 10µl cDNA synthesis mixture (per reaction) was prepared by combining: 2µl 10x RT buffer; 4µl 25mM MgCl2; 1µl (40U) RNaseOUT™ and 1µl (200U) SuperScript™ III RT. The template and cDNA synthesis mixtures were combined and heated to 50°C for 10 min to allow reverse transcription, then to 85°C for 5 min to terminate the RT reaction. 1µl RNase H was added to remove any remaining RNA, and tubes were incubated at 37°C for 20 min. First-strand cDNA was stored at -20°C, or 2µl used as the template for PCR.

2.2.8. Restriction endonuclease digestion.

Double digestion of purified PCR products and plasmid vectors was performed according to manufacturer’s recommendations in appropriate buffers. Briefly, HindIII and BamHI double digestion of NY-ESO-1 amplicons or plasmid pFastBacHTB was performed in SuRE/Cut Buffer B (all Roche) for 1 h at 37°C. PacI and ClaI double digestion of NY-ESO-1 amplicons or plasmid pAM/CAG-WPRE-bGHpA was performed in NEBuffer 4 (all New England BioLabs Inc.) for 1 at 37°C. Digested products were analysed by AGE and gel-purified as described below.

2.2.9. Gel extraction and purification.

Gel bands were visualised using a UV gel box and excised using a sterile scalpel. Excised products were recovered using the Zymoclean™ gel DNA recovery kit (Zymo Research Corp.) following manufacturer’s instructions. Briefly, the gel fragment was weighed and > 3 volumes ADB buffer was added. The gel was incubated at 50oC with periodic vortexing, until dissolved. This solution was
transferred to a column and spun at > 10,000 x g for 60 s. The flow-through was discarded and the column washed twice with 200 µl wash buffer. 15µl of molecular biology grade water pre-heated to 50°C was applied to the column, and allowed to incubate for 3 min. The column was then transferred to a clean 1.5 ml microfuge tube and spun at > 10,000 x g for 60 s to elute DNA.

2.2.10. Dephosphorylation.

In order to decrease ligation background, digested plasmid backbones were dephosphorylated using rAPid alkaline phosphatase (Roche) according to manufacturer’s instructions. Briefly, ≤ 1µg vector DNA, 1µl (1U) enzyme and 2µl 10x rAPid alkaline phosphatase buffer were combined in a total of 20µl reaction volume (made up with molecular biology grade water) and incubated for 10 min at 37°C. The enzyme was then inactivated by incubation at 75°C for 2 min, and the dephosphorylated plasmid backbone used in subsequent ligation reactions.

2.2.11. Ligation.

Following gel-extraction, restriction endonuclease-digested PCR products and dephosphorylated plasmid backbones were ligated using T4 DNA ligase (Invitrogen) according to manufacturer’s instructions. Typically a molar ratio of 1:3 or 1:6 (vector:insert) were combined with 1U of T4 ligase and 4µl 5x ligation buffer in a total volume of 20µl. Ligation reactions were performed for 5 min at room temperature, and then analysed by AGE. Successful ligations were used to transform *E. coli* as described below.

2.2.12. Preparation of Chemically competent *E. coli* DH5α cells.

*E. coli* DH5α were thawed from -80°C and streak-plated on LB-Agar overnight. A single colony was selected and grown in 10ml LB overnight, shaking. 250µl of the starter culture was transferred to 25ml fresh LB broth in a 100 ml conical flask at 37 °C shaking at 200 rpm, and the bacteria were allowed to grow to an O.D 600 of 0.2-0.4. The media was then transferred to a 50 ml polypropylene tube (Falcon) and chilled on ice for 15 min. Once chilled, the tubes were centrifuged at 4000 rpm at 4°C for 10 min to pellet the bacteria. The supernatant was discarded and the bacterial pellet resuspended in 10ml 0.1M CaCl₂. This was then transferred into pre-chilled microfuge tubes as 1ml aliquots, and centrifuged at 5000 rpm, 4°C to re-pellet the bacteria. The supernatant was discarded and the bacteria resuspended in 100µl ice-cold 0.1M CaCl₂ *via* gentle vortexing. The competent cells were used for transformation immediately or were mixed with an equal volume of glycerol and stored at -80°C.
2.2.13. Transformation of chemically competent E. coli DH5α cells.

Transformations were performed in pre-chilled microfuge tubes. Competent DH5α cells were thawed on ice, gently mixed and distributed as 50µl aliquots. Ligation reactions were diluted 1:5 in sterile distilled water. 5µl was added to 50µl bacteria and incubated on ice for 30 min. Bacteria were then heat-shocked by immersion in a 42°C water bath for 90 s, followed by incubation on ice for 2 min. 945µl of room temperature SOC medium was then added and bacteria incubated at 37°C shaking at 200 rpm for 1 h. Tubes were spun for 5 min at 5000 rpm to pellet bacteria. 900 µl supernatant was discarded and pellet resuspended and spread-plated overnight on LB-agar containing appropriate antibiotics (typically LB-agar supplemented with 100µg/ml ampicillin). Successfully transformed colonies were streak-plated on appropriate plates to confirm transformation, and used to inoculate overnight cultures to prepare glycerol stocks for long-term storage.

2.2.14. Isolation of plasmid DNA from DH5α.

Individual bacterial colonies were picked using a sterile pipette tip, inoculated into 2ml of LB broth continuing appropriate antibiotics in a round-bottom test tube, and incubated at 37°C shaking at 200rpm overnight. Cells were then pelleted at 5000 rpm, and plasmid DNA extracted using a PureLink® HiPure Plasmid Miniprep kit (Invitrogen, Life Technologies™) following manufacturer’s instructions. Plasmids were resuspended in molecular biology grade water or TE buffer and stored at -20°C.

To produce plasmids on a large scale for HEK293T cell transfection, 5ml starter cultures from single colonies were grown for 6 h as described above, and then seeded into 250-500 ml fresh pre-warmed LB broth containing appropriate antibiotics in 1-2L conical flasks, and incubated at 37°C shaking at 200rpm overnight. Cells were then pelleted at 4000 rpm, and plasmid DNA extracted using a PureLink® HiPure Plasmid Maxiprep kit (Invitrogen, Life Technologies™) following manufacturer’s specifications. Plasmids were resuspended in molecular biology grade water or TE buffer and stored at -20°C.

2.2.15. Preparation of Chemically competent E. coli DH10 Multibac cells.

E.coli DH10MultiBac were thawed from -80°C and streak-plated on LB-Agar supplemented with Tetracycline (10µg/ml) and Kanamycin (50µg/ml) overnight. A single colony was inoculated into 3ml LB broth supplemented with Tetracycline (10µg/ml) and Kanamycin (50µg/ml). Bacteria were grown overnight at 37°C with agitation. The following morning 20ml of prewarmed LB broth Agar supplemented with Tetracycline (10µg/ml) and Kanamycin (50µg/ml) was inoculated with 200µl of the overnight culture and incubated with shaking at 37°C until OD600 was approximately 1.0. Cells were chilled on ice for 30 min and then centrifuged at 3000xg for 10 min. Cells were resuspended in
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2ml TSB/TSS buffer. Aliquots of 200µl were prepared, flash frozen in liquid nitrogen and stored at -80°C

2.2.16. Transformation of chemically competent *E. coli* DH10MultiBac cells.

Transformations were performed in pre-chilled 1.7ml microfuge tubes and 14ml polypropylene tubes. Competent DH10MultiBac cells were thawed on ice, gently mixed and distributed as 100µl aliquots. Plasmid pFastbacHTB-NYESO1 was diluted to 20ng/µl in sterile distilled water. 5µl (100ng) was added to 100µl bacteria and incubated on ice for 30 min. Bacteria were then heat-shocked by immersion in a 42°C water bath for 60 s, followed by incubation on ice for 2 min. 900µl of room temperature SOC medium was then added and bacteria incubated at 37°C shaking at 225 rpm for 4 h. Tubes were spun for 5 min at 4000 rpm to pellet bacteria. 900 µl supernatant was discarded and pellet resuspended and spread- plated on LB-agar containing appropriate antibiotics and selection reagents (typically LB-agar supplemented with 50µg/ml Kanamycin sulphate, 34ug/ml Gentamicin sulphate, 10ug/ml tetracycline, 150µg/ml X-gal and 40µg/ml IPTG – ‘KGTIX’). Bacteria on KGTIX plates were cultured for 48 h at 37°C, then overnight at room temperature. Large white colonies were selected as positive transformants/transposants, streak-plated on fresh KGTIX plates to confirm transformation and transposition, and used to inoculate overnight cultures of LB broth supplemented with 50µg/ml Kanamycin sulphate, 34ug/ml Gentamicin sulphate and 10ug/ml tetracycline to prepare glycerol stocks for long-term storage.

2.2.17. Isolation of bacmid DNA from DH10 Multibac by alkaline lysis miniprep.

Bacmids are large DNA molecules and are susceptible to shearing from mechanical and freeze/thaw stress. As such, recombinant bacmids containing the NY-ESO-1 tranogene were isolated from *E.coli* DH10Multibac using a gentle alkaline lysis protocol, all solutions containing naked DNA were mixed by inversion rather than by pipette, and bacmids were stored at 4°C as described below.

Individual bacterial colonies from KGTIX plates were picked using a sterile pipette tip, inoculated into 1.5ml LB broth supplemented with 50µg/ml Kanamycin sulphate, 34µg/ml Gentamicin sulphate and 10µg/ml tetracycline and incubated at 37°C shaking at 200rpm overnight. Cells were then transferred to sterile microfuge tubes, pelleted at 5000 rpm and resuspended in 100µl Solution I supplemented with 50µg/ml RNase A. 200µl Solution II was added and tubes mixed by inversion and incubated on ice for 5 min. 150µl solution III was added and tubes mixed by inversion and incubated on ice for 10 min. Tubes were then centrifuged at maximum speed at 4°C for 5 min to pellet genomic DNA and cell fragments, and 400µl supernatant was transferred to a fresh tube. Bacmid DNA was precipitated by addition of 400µl isopropanol on ice for 15 min, and pelleted by centrifugation at maximum speed at 4°C for 5 min. The bacmid DNA was cleaned and pelleted twice (as above) with
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750µl 700% ethanol and then allowed to air dry. Bacmid DNA was resuspended in 50µl molecular biology grade water or TE buffer and stored at 4°C prior to use.

2.2.18. Sf9 transfection with bacmid-NY-ESO-1 and recombinant baculovirus production.

Sf9 cells were cultured to exponential growth phase in Sf900 III SFM and seeded into 6-well plates in 2ml media at a density of 4 x10^5 cells/ml and allowed to adhere. 1µg NY-ESO-1-encoding bacmid DNA was diluted in 100µl media, and combined with 3µl GeneJuice™ transfection reagent (Novagen) diluted in a further 100µl media. Solutions were gently mixed and DNA:lipid complexes allowed to form for 1h and then applied to the Sf9 cells for 5h at 28°C. Media was then removed and replaced with 2ml fresh Sf900 III SFM, and cells incubated for a further 72h, until evidence of cell lysis could be observed. Baculovirus-containing supernatant was collected and gently clarified. This low-titre solution was designated ‘P1’ stock. To produce a higher-titre P2 stock, Sf9 cells were seeded at 2 x10^6 cells/ml and incubated with a 1:5 dilution of P1 for a further 72h. This P2 supernatant was collected and gently clarified. A larger scale, high-titre P3 stock was then generated by seeding Sf9 cells at 2 x10^6 cells/ml in 10ml Sf900 III SFM in a 50ml capped conical flask, and incubating these shaking with a 1:100 dilution of P2 stock for a further 72h. This P3 stock was collected and clarified, and used for subsequent protein expression optimisation and large-scale protein production.

2.2.19. Sf9 infection and Protein Expression.

Sf9 cell culture, production of bacmid and baculoviral vectors and rNY-ESO-1-His6 was carried out with the help of Dr James Dickson (School of Biological Sciences).

2.2.19.1. Sf9 infection with recombinant baculovirus.

Sf9 cells were counted and seeded at 2.5 x 10^6 cells per ml in 200ml Sf900 III medium with 1% FBS, and were infected with P3 Baculovirus expressing rNY-ESO-1 at 1:5000 v/v in 1L Erlenmeyer flasks. Cells were incubated at 28°C shaking at 130rpm for 72 h. Cell number and morphology were monitored daily for signs of viral infection, and at 48 h medium was supplemented with 5mM sterile L-glutamine and 5mM sterile D-glucose to maximise protein expression. At 72 h cells were transferred to 50ml Falcon tubes and harvested by centrifugation at 600 x g for 30 minutes. Cell pellets were washed once with PBS, pooled and collected again by centrifugation. Pellets were snap-frozen in liquid nitrogen and stored at -80°C until required.

2.2.19.2. rNY-ESO-1 purification by Immobilised metal ion affinity chromatography (IMAC).

Sf9 cell pellets were thawed on ice and resuspended in 20ml Sf9 lysis buffer. This solution was kept on ice for 10 min to allow for complete lysis, and then clarified by centrifugation at >13,000 x g for
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30 min at 4°C in a Sorvall SS34 rotor. The supernatant was collected and the clarified lysate loaded through a 0.22µm filter (Millipore; MA, USA) onto a HiTrap™ Chelating HP column (GE Life Sciences; Buckinghamshire, UK) which had been charged with 100mM NiCl₂ and equilibrated with 20CV IMAC wash buffer I. The column was then washed with 20CV IMAC wash buffer II to remove weakly bound contaminants. Column loading and wash steps were performed using a peristaltic pump, at a flow rate of 3ml/min, and all flow-through fractions collected and stored on ice.

The loaded column was then attached to an AKTA Express workstation (GE Life Sciences) and bound rNY-ESO-1-His₆ eluted by applying a gradient of 0-100% IMAC elution buffer over 5CV. 1ml fractions were collected, and protein elution was visualised in situ by measuring eluate absorbance at 280nm. Typically, rNY-ESO-1-His₆ eluted from column at ≥300mM imidazole. Purity and specificity were assessed via SDS-PAGE and Western Blot. NY-ESO-1- containing fractions were pooled and concentrated to ≤1 ml in a Vivaspin®-20 5K MWCO centrifugal concentrator (Sartorius-Stedman; Gottingen, Germany) and dialysed into protein storage buffer overnight in a Slide-A-Lyzer® 10K MWCO Dialysis cassette (Thermo Fisher Scientific; MA, USA). Typically 5-10mg of NY-ESO-1-His₆ was harvested from a 200ml Sf9 culture.

2.2.20. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.2.20.1. Gel and sample preparation.

12% SDS-PAGE gels (12.5% resolving; 4% stacking) were prepared within glass plates inside a gel-making apparatus (Bio-Rad). The resolving solution was produced by combining 2.1 ml solution A, 1.25 ml solution B and 1.5 ml distilled water. 50µl 10% APS and 5µl TEMED (Sigma-Aldrich) were then added to induce polymerization. Resolving solution was overlaid with 1ml isopropanol and allowed to set. The stacking solution was produced by combining 0.625 ml solution A , 1.25 ml solution C, 3ml distilled water, 25µl 10% APS and 5µl TEMED (Sigma-Aldrich). Isopropanol was washed away, and the gel completed by addition of stacking solution and plastic comb to allow the formation of loading wells. Stacking solution was allowed to set and gels were used or kept moist and stored at 4°C.

SDS-PAGE samples were prepared by addition of an appropriate volume of 5x Laemmli buffer (including β-M unless otherwise specified) and were boiled in a heating block for 5 min. Prior to loading, samples were briefly spun. Benchmark™ (Life Technologies) or Precision Plus Protein™ (Bio-Rad) protein ladders were used to assess band size. Electrophoresis was performed in a mini Protean® 3 apparatus (Bio-Rad) filled with SDS-PAGE running buffer at 200V (20mA) for 45-60 min, until the dye front ran off the gel.
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2.2.20.2. Coomassie Brilliant Blue Staining.

Completed gels were immersed in Coomassie Brilliant Blue R-250 and were microwaved for 30 seconds, then placed on a rocking table. Staining was allowed to continue for at least 4h. The stain was poured off and the stained gels washed briefly in deionised water, then immersed in Coomassie destain solution until satisfactorily destained. Completed gels were visualised using a scanner (Epsom), and wrapped in cellophane to dry.

2.2.20.3. Silver staining.

Protein gels were prepared and run as in SDS-PAGE protocol. All subsequent steps were carried out on a rocking table, using freshly prepared solutions. Completed gels were incubated in silver stain fix solution for 30 min, and then washed in 10% ethanol for 10 min and distilled water three times for 5 min. The gel was then incubated in Farmer’s reagent for 1 min, and washed in distilled water three times for 5 min. All subsequent steps were carried out without exposing the gel directly to light. The gel was incubated in 0.1% Silver Nitrate for 15 min, washed in distilled water for 20s, and then washed briefly with 20 ml freshly prepared silver stain developer. As soon as any cloudiness was evident in the solution, the developer was poured off and replaced with 30 ml fresh developer solution, until a desired level of staining was evident. The developer was then poured off and the gel washed once in 1% acetic acid for 5 min, and three times in distilled water for 5 min.

Any over-staining was alleviated by re-incubating the gel in Farmer’s reagent for 10-30 seconds, followed by thorough re-washing with distilled water. Completed gels were visualised using a scanner (Epsom), and wrapped in cellophane to dry.

2.2.21. Western Blotting.

Protein gels, samples and Benchmark™ prestained protein ladder (Life Technologies) were prepared and run as in SDS-PAGE protocol. Completed gels were loaded into a plastic frame and overlaid with a nitrocellulose membrane (Protran®, Whatman®, Schleischer & Scheull), such that the gel and membrane were sandwiched between pre-soaked fibre pads and blotting paper. Protein transfer was performed in a Criterion™ apparatus (Bio-Rad) filled with Western Blot transfer buffer for 45-60 min at 100V, or overnight at 30V. An ice pack (to maintain temperature) and a magnetic spinner bar (to allow buffer circulation) were also placed in the apparatus.

The membrane was then incubated in blocking buffer for 1h at room temperature, or overnight at 4°C, on a rocking table. The blocking buffer was poured off and the membrane washed once with PBS-T. Subsequent primary and secondary-HRP antibody incubations were performed in antibody dilution buffer for 2 h (primary) or 1h (secondary) at room temperature, or overnight at 4°C, on a rocking table. Between each incubation the membrane was washed 4 times for 5 min with PBS-T.
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Protein-antibody complexes were detected using the ECL™ western blotting analysis system (Amersham Bioscience, GE Healthcare) Chemiluminescence was visualised and images analysed using a LAS-3000 imaging system and Multigauge software (Fujifilm, Life Science)

2.2.22. Production of rAAV in HEK293T cells.

2.2.22.1. Transfection using polyethylenimine (PEI).

Linear 25kDa polyethylenimine (Polysciences Inc.) was provided by Dr James Ussher as a sterile 0.323g/L stock and stored at 4°C. The N content of the 0.323g/L stock was 7.5nmol/µl, and typically the 0.323g/L PEI stock was used such that the ration of nitrogen (N) in PEI to phosphorus (P) in the DNA construct to be transfected was 20:1. As such the volume of PEI stock used was determined following the formula below:

\[
\text{PEI (µl/plate) = (total µg plasmid DNA/plate) x (3nmol P/µg DNA) x 20 (N:P)}
\]

\[
7.5\text{nmol N/µl}
\]

2.2.22.2. Production of rAAV.

HEK293T transfection methods are based on personal communication from Dr James Ussher, Molecular virology group, School of Biological Sciences.

HEK293T cells were grown in batches of 5 x 15cm plates per transfection. The day prior to transfection, cells were seeded at 20 x 10^6 cells per plate in DF10 (without antibiotics) and cultured overnight at 37°C/5% CO₂. Cells were transfected with a total of 275µg plasmid DNA (per 5 plates - 55µg per plate), using PEI as described above. Three plasmids were used at an equimolar ratio in each transfection: 69.5µg plasmid encoding the vector backbone and gene of interest (Pam/CAG-NY-ESO-1-WPRE-bGHpA or pAM/CAG-eGFP-WPRE-bGHpA); 66.5µg of packaging plasmid (pNLRep or pAAV2/6) and 139µg of plasmid pFdelta6, which encodes adenoviral helper functions essential for rAAV production. Plasmids and PEI were separately diluted into 9.2ml (total volume) 150mM NaCl. The plasmid solution was filtered then both solutions combined, briefly vortexed, and incubated at room temperature for 15 min to allow formation of DNA/PEI complexes. 3.7 ml was then added drop-by-drop to each cell culture plate, and medium mixed by gentle swirling. Plates were incubated at 37°C/5% CO₂ for up to 16 h, then medium removed and replaced with 25ml fresh DF10 supplemented with penicillin and streptomycin. The transfected cells were returned to 37°C/5% CO₂ incubation, until being harvested 48 h after transfection as described below.
2.2.22.3. Harvesting of cells and lysate preparation.

Medium was removed and cells washed with PBS. 20 ml of PBS/5mM EDTA was added to each plate, and cells gently detached using a cell scraper and transferred to a 50ml Falcon tube. Plates were washed once more collecting residual cells, and these were added to the Falcon tube(s). Cells were centrifuged at 500 x g for 15 minutes, the supernatant discarded and cell pellets pooled and resuspended in 10ml of HEK293T lysis buffer. Pellets were stored at this point at -80°C if necessary.

Transfected cells were lysed by 3 freeze/thaw cycles alternating between liquid nitrogen and a 37°C water bath. Sodium deoxycholate was added to a final concentration of 0.5% to aid lysis, and clumps dispersed by pipetting. Benzonase® endonuclease (Sigma-Aldrich) was then added to a final concentration of 50U/ml, and lysates incubated at 37°C for 1h with periodic mixing. Lysates were clarified by centrifugation at 3300 x g for 30 min in a fixed-angle rotor (Sorvall RTH 750). Supernatant was collected, and stored at -20°C prior to virus purification via heparin affinity chromatography (below).

2.2.22.4. Purification of rAAV by heparin affinity chromatography (HAC).

To maintain sterility, all solutions used in HAC were filtered through a 0.2µm filter, and all steps were carried out in a class II biosafety cabinet. Sample and buffer loading was performed using a peristaltic pump, the lines for which had been stored in, and flushed with, 100% ethanol. The flow rates for column washing and sample loading was controlled at 1ml/min. HiTrap Heparin HP 1ml columns (GE Healthcare) were washed with 5CV 70% ethanol, then sequentially equilibrated with 5CV HAC buffer A, 15CV HAC buffer B and then 15CV HAC buffer A. The clarified virus-containing lysate was diluted with an equivalent volume of HAC buffer A and loaded onto the column. The flow-through was collected, and the column then washed with 30CV HAC buffer A. Appropriate volumes of HAC buffers A and B were combined to give NaCl concentrations of 200 and 600mM. The column was disconnected from the pump, washed by hand with 5CV of the 200mM NaCl-containing solution (this was discarded), and then virus eluted with 6CV of the 600mM NaCl-containing solution. This virus-containing fraction was collected and concentrated to 100µl at 3000rpm in a 100K MWCO Amicon Ultra centrifuge concentrator (Millipore). The concentrator was then washed with 4ml sterile cold PBS and the virus re-concentrated back to 100µl. The concentrated virus was collected, and the concentrator unit and filter gently washed with 400µl sterile cold PBS to maximise virus collection. The PBS fractions were combined and sterilised using an Acrodisc® 13mm 0.2µm filter (Pall Corporation) and stored in aliquots at -80°C.
2.2.22.5. Quantification of rAAV stocks.

rAAV-containing HAC samples were DNase I (Roche)-treated following manufacturers specifications in a total volume of 30µl at 37°C, 30 min, to digest any HEK293 DNA. DNase I was then inactivated by addition of EDTA to 25mM, and heating to 65°C for 10 min. Vector genomes were released from capsids by addition of Proteinase K to 0.5mg/ml and SDS to 0.5% in a total volume of 50µl followed by incubation at 37°C for 2h. Proteinase K was then inactivated by heating to 95°C for 20 min. Samples were diluted 1:200 in milliQ H₂O and 2µl used as PCR template.

Quantitative real-time PCR was performed on an Applied Biosystems 7900 HT Fast Real-Time PCR system (Applied Biosystems). Total PCR reaction volume was 10µl in 384wp wells. Amplification was performed using EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Invitrogen) and 300nM each of the forward and reverse bGHpA primer sets (Dr James Ussher). A ten-fold dilution series, spanning 7-log₁₀, of a bGHpA-encoding plasmid was used to create a standard curve. All samples were run in triplicate.

2.3. Cell culture methods.

2.3.1. Cell culture media.

All cell culture media were supplemented with Penicillin/Streptomycin (Gibco®) to a final concentration of 100U/ml and 100µg/ml respectively, and with GlutaMAX™-I (100x, Gibco®) to 1x. All cell culture media were sterilised by filtration with a 0.22µM filter (Millipore).

RPMI-1640 (Gibco®) was used as the base culture medium for all mixed peripheral blood mononuclear cells, T cells, transformed hematopoietic cell lines (T2, LCL, THP-1 and U937), and melanoma cell lines. RPMI-1640 supplemented with Penicillin, Streptomycin and GlutaMAX™-I was designated ‘R0’. For culture, R0 was supplemented with 5% v/v heat-inactivated AB Human Serum (Invitrogen), designated ‘Rs5’, or with 10% v/v heat-inactivated Fetal Bovine Serum (Invitrogen), designated ‘RF10’.

Dulbecco's Modified Eagle Medium (DMEM) was used as the base culture medium for fibroblast culture. DMEM supplemented with Penicillin, Streptomycin and GlutaMAX™-I was designated ‘D0’. For culture, D0 was supplemented with 10% v/v heat-inactivated Fetal Bovine Serum (Invitrogen; Carlsbad, CA, USA), designated ‘DF10’.

2.3.2. Cell culture equipment.

All primary hematopoietic cells were cultured in 96-, 48-, or 24-well plates (BD Biosciences), typically at a density of 1-5 x 10⁶/ml, and were maintained by having half of their medium discarded
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and replaced with fresh medium every 72-96h. Transformed hematopoietic cell lines (T2, LCL, THP-1 and U937) were cultured in T25-T75 flasks upright, typically at a density of $1 \times 10^6$/ml and in no more than 20ml RF10. These cultures were maintained passaged by discarding 25-50% of the cell suspension and replacing it with an equivalent volume of fresh RF10 every 72-96h. Adherent cells (primary fibroblasts and melanocytes, and transformed melanoma cell lines) were cultured in T25-T75 flasks in RF10 or DF10 as appropriate, and passaged using TrypLE™ Express (Life Technologies) when they reached ≤80% confluence.

2.3.3. Peripheral blood mononuclear cell (PBMC) isolation and ethical approvals.

Peripheral blood mononuclear cells (PBMC) were isolated from blood drawn from healthy donors and treated with 1000IU/ml heparin (Sigma-Aldrich) to prevent coagulation. 50ml Leucosep™ tubes (Greiner-One) were loaded with 15ml Lymphoprep™ (Axis-Shield) and centrifuged for 1 min at 300 xg so that the Lymphoprep™ sat below the tube membrane. Blood was diluted 1:1 with RPMI 1640 at RT and 35ml slowly applied to each Leucosep™ tube. Samples were then centrifuged for 15 min at 800 x g at low acceleration and no brake settings to allow buffy coat formation via density separation. Buffy coats were collected and pooled into a 50ml tube, then washed twice with RPMI 1640 to remove residual Lymphoprep™. PBMC were counted and resuspended in Rs5 for cryopreservation as described in section 2.3.4, or resuspended in MACS buffer for cell subset purification as described in section 2.3.8. Blood collection, PBMC isolation and all subsequent applications were carried out under approval from the Auckland Human Participants Ethics Committee (reference number 010558). All donors received project information packs and a consultation outlining potential PBMC uses, and submitted consent forms prior to sample collection.

2.3.4. Cryopreservation of mammalian cells.

Cells were typically cryopreserved in 1-20M aliquots. Cells were harvested and resuspended in 0.5ml of their standard culture medium per aliquot, to which was added 0.5ml freezing medium per aliquot. Freezing medium comprised 80% FBS and 20% DMSO (Sigma-Aldrich), giving a final concentration of ≥40% FBS and 10% DMSO. Cells were aliquoted into 1.8ml Nunc® Cryotube® vials (Thermo Fisher Scientific) and cooled to -80°C at 1°C/min in a cryopreservation container filled with isopropanol. Vials were subsequently stored in liquid nitrogen.

2.3.5. Thawing of cryopreserved mammalian cells.

Vials were removed from liquid nitrogen storage and warmed to 37°C in a water bath until defrosted. Cells were transferred to a 15ml Falcon tube and 2ml warmed R0 added drop by drop. Cells were incubated for 1 min and then a further 9-14ml warmed R0 added. Cells were mixed by pipette and then centrifuged at 1300rpm for 5 min. The supernatant was removed by pipette and cells resuspended
in appropriate culture medium for subsequent manipulation. In some cells were incubated for 45 min in culture medium supplemented with benzonase (Merck-Millipore; MA, USA).

2.3.6. Production of Lymphoblastoid B-cell lines (LCL).

B95-8 *Sanguinus oedipus* cells were provided by Dr See-Tarn Woo, Auckland District Health Board. These cells continuously shed infectious Epstein-Barr virus particles into their cell culture medium. As such, all work involving B95-8 culture, and subsequent EBV handling and LCL production, was carried out under PC2 conditions.

B95-8 cells were cultured and RF10 in T75 flasks (BD). Cells were split 1:4 before they reached confluence. To produce a stock of infectious EBV, cell culture medium was collected and transferred to a 50ml Falcon tube. This was centrifuged at 400 x g for 10 min to pellet any collected cells, and the supernatant then filtered through a 0.45µm filter (Millipore) and stored in aliquots at -80°C until required. B95-8 cells were detached using a minimal volume of TrypLE™ Express (Gibco®), combined with 5 volumes RF10 and pelleted by centrifugation at 400 x g for 10 min. The supernatant was discarded, and cells were cryopreserved as described above.

To produce LCL, whole PBMC isolated as described above were thawed, counted and resuspended at $\leq 2 \times 10^6$ cells/ml in 1.5 ml RF10. 2.5ml EBV-containing supernatant was thawed and combined with PBMC in a T25 flask (BD), and incubated at 37°C/5% CO₂ for 2 h. 1ml fresh RF10 containing PHA (Gibco®) and cyclosporin A (Sigma-Aldrich) to final concentrations of 0.5% v/v and 0.1µg/ml respectively, was added. Cells were fed with fresh RF10 as appropriate, and cells transferred to a T75 flask after 2 weeks. After 3-4 weeks culture LCL were typically visible growing in macroclusters. LCL lines were stained with anti-CD20, anti-CD3 and anti-CD14 to assess residual levels of T cells and monocytes, and were cryopreserved as described above.

2.3.7. Irradiation of mammalian cells.

LCL to be used as feeder cells were cultured to desired numbers in T75 flasks and RF10. Typically $5 \times 10^8$ LCL were harvested, resuspended in 100-120ml fresh RF10 in a 250ml Schott Bottle containing an autoclaved magnetic stirrer bar, and incubated on ice for 30 min. Cells remained on ice throughout irradiation, to minimise the activity of DNA repair enzymes, and were kept on a magnetic spinning plate, to ensure that cells remained in a homogenous suspension. Cells received 50Gy from a Cobalt-60 source. Following irradiation cells were cryopreserved and a sample cultured to ensure that no proliferation occurred. Typically irradiated LCL exhibited 0% viability after 5-7 days of culture.
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2.3.8. T cell subset isolation.

T cell subsets were purified using MACS® isolation kits, LS or LD columns and QuadroMACS or MidiMACS magnetic arrays (all Miltenyi Biotec Inc.). LS and LD columns were equilibrated with cold MACS buffer prior to use. All incubation and separation steps were carried out at 2-8°C.

2.3.8.1. CD4⁺ and CD8⁺ T cell purification.

CD4⁺ and CD8⁺ T cell enrichments were performed using the MACS human CD4⁺ T cell isolation kit and MACS human CD8⁺ T cell isolation kit, respectively. Freshly isolated or thawed PBMC were counted and resuspended in filtered MACS buffer (PBS supplemented with 0.5% FBS and 2mM EDTA). Non-target cells were labelled with biotin-conjugated monoclonal antibodies against CD14/15/16/19/36/56/123/235a and CD8 or CD4, respectively, for 5-10min, and then incubated with streptavidin-coated microbeads for a further 10min. Samples were made up to ≥0.5ml and applied to LS columns within a magnetic array. Unlabelled target cells (CD4⁺ or CD8⁺) passed through the column, while labelled non-target cells were retained within the magnetic field. The column was washed once with 3-5ml MACS buffer and total flowthrough containing target cells was collected. The purity of collected CD4⁺ or CD8⁺ cells was assessed by staining fractions before (A; Figure 2-1) or after separation (B-C; Figure 2-1) with anti-CD3, anti-CD4 and anti-CD8 antibodies and analysing samples via flow cytometry, as described in section 2.5.1. Following separation, samples were typically ≥95% CD3⁺CD4⁺ or CD3⁺ CD8⁺.
Untouched CD3+CD4+ and CD3+CD8+ T cells were sequentially isolated from whole PBMC, and subset purity assessed via flow cytometry for cell surface lineage markers. A: PBMC contain a mixed CD3+CD4+ and CD3+CD8+ pool. B: Untouched CD4+ T cells or C: Untouched CD8+ T cells were isolated from the total T cells via lineage-depletion to $\geq 95\%$ purity.

2.3.8.2. Naïve and memory CD8+ T cell subset isolation.

Untouched CD8+ T cells collected as above were incubated for 15 min with CD45RO MicroBeads. Samples were washed once with MACS buffer, resuspended in 0.5ml MACS buffer and passed over an equilibrated LD column. The column was then washed three times with 2ml MACS buffer and all flowthrough, containing CD45RO- T cells, was collected. All CD45RO+ memory T cells were retained on the column, and were collected by removing the column from the magnetic array and gently flushing with 5ml MACS buffer. The CD45RO+ T cell fraction typically comprised both a CD45RO+ CD45RA- and CD45RO+ CD45RAmid ‘transitional’ memory fraction – this heterogeneous mixture was used in subsequent assays. The untouched CD45RO- cell fraction typically comprised both a CD45RA+ CD45RO- CCR7+ naïve T cell fraction and a CD45RA+ CD45RO- CCR7- TEMRA fraction of varying magnitude. In order to remove TEMRA and isolate a pure naïve T cell population, the CD45RO- fraction was centrifuged, resuspended in MACS buffer and labelled with anti-CCR7-FITC for 15min. Cells were washed twice with MACS buffer to remove unbound antibody and then were incubated for 15 min with anti-FITC MicroBeads. Samples were washed again, resuspended in
0.5ml MACS buffer and passed over an equilibrated LD column. The column was then washed three times with 2ml MACS wash and all flowthrough, containing CCR7- T<sub>EMRA</sub> was collected. CCR7<sup>+</sup> naïve T cells were retained on the column, and were collected by removing the column from the magnetic array and gently flushing with 5ml MACS buffer.

The phenotype of pre- and post-separation samples was assessed staining fractions before or after separation with anti-CD8, anti-CD45RO, anti-CD45RA and anti-CCR7 antibodies and analysing samples via flow cytometry (A-B; Figure 2-2).

![Figure 2-2 CD8<sup>+</sup> naïve and memory subset isolation by MACS.](image)

| A | Total CD8<sup>+</sup> T cells were stained for CD45RA, CD45RO and CCR7 expression prior to MACS subset isolation. |
| B | Total CD8<sup>+</sup> CD45RO<sup>+</sup> memory cells (grey), CD8<sup>+</sup> CD45RA<sup>+</sup> CCR7- T<sub>EMRA</sub> (red) and CD8<sup>+</sup> CD45RA<sup>+</sup> CCR7<sup>+</sup> naïve (blue) cell subsets were purified. CD45RO<sup>+</sup> and CD45RA<sup>+</sup> CCR7<sup>+</sup> were used in subsequent culture and expansion experiments. One representative donor is shown. |

2.3.9. **Monocyte-derived dendritic cell (MoDC) production and culture.**

Monocytes were collected isolated from fresh or thawed PBMC using plate adherence. Briefly, PBMC were suspended in Rs5 at 1 x 10<sup>7</sup> cells/ml and 200ul seeded into 48 well plates wells. Plates were centrifuged briefly and incubated for 2h to allow monocyte adherence. Wells were gently washed and non-adherent cells removed, and replaced with fresh PBMC as above. This process was repeated until well floors were saturated with monocytes. Residual non-adherent PBMC were carefully removed by washing wells three times with 200ul Rs5 until only monocytes remained, and monocytes were then cultured in 500µl Rs1 supplemented with 20ng/ml IL-4 and 100ng/ml GM-CSF.
(Wolfl and Greenberg, 2014). Media and cytokines were refreshed every 48h to 3d and if desired MoDC were then stimulated with 10ng/ml LPS and 100ng/ml IFNγ overnight (Wolfl and Greenberg, 2014). Matured MoDC were loaded with peptide at time of maturation if used in T cell priming assays. Immature MoDC were transduced with rAAV vectors or used in other downstream assays as described.

2.3.10. T cell clone culture.

Single T cells were sorted based on pentamer or activation marker staining as described in section 2.5.3 and seeded into individual U-bottom 96-well-plate wells filled with 100µl Rs5 containing 5x10⁴ allogeneic LCL, previously irradiated with 50Gy as described in section 2.3.7. Additionally, in each plate some wells were seeded with no T cells in order to monitor feeder cell death, and some wells were seeded with 10-100 T cells to act as positive polyclonal outgrowth controls. Cells were allowed to recover for 1h at 37°C/5% CO₂, and then stimulated by addition of 100µl Rs5 containing 10ng/ml IL-12 (to mimic the presence of a matured APC and enable appropriate priming of effector function (Wolfl and Greenberg, 2014)); 10ng/ml IL-2 (to facilitate T cell growth and survival in the absence of other activated T cells); 5ng/ml IL-7; 10 ng/ml IL-21 (to promote retention of IL-7Rα expression, Figure 3-2) and 0.5% v/v Phytohaemagglutinin (Sigma-Aldrich) to activate the T cell through ligation and cross-linking of CD2, CD3 and other glycosylated surface proteins (O’Flynn et al., 1986).

Growing clones were fed by replacement of Rs5 supplemented with either IL-2 alone or IL-2, IL-7 and IL-21 every 72-96h; IL-12 was provided only during stimulation at day 1. Clones expanded using IL-7 and IL-21 were supplied with IL-2 only to day 5 of culture, as previous work within the host laboratory has shown that this level of supplementation facilitates naïve CD8⁺ T cell outgrowth without development of an exhausted phenotype (Ho, 2012), and were provided with IL-21 as long as continued expansion was evident (typically for 14 days following initial blast detection). As irradiated feeder cells died off, typically over the course of 5-7 days, outgrowth of T cell ‘blasts’ and expansion of clonal pellets was monitored visually. T cell blasts typically exhibited an irregular morphology and appeared ‘bright’ under light microscopy, in contrast to the darker and more granular apoptotic feeder cells. As blasting populations often did not survive through the course of a cloning experiment, wells were scored as being ‘positive’ for clone outgrowth as soon as blasts were evident. T cell blasts typically became evident between days 5 and 14. At D28 of culture surviving clones were assessed for antigen specificity via pentamer staining or IFNγ ELISPOT. At this timepoint, large pellets, entirely filling the well, typically contained ~5 x 10⁵ cells, representing < 20 population doublings, while smaller pellets typically contained ~ 1 x 10⁵ cells, representing < 17 population doublings. T cell outgrowth efficiency was defined as the % of seeded wells in which blasting T cells could be visually identified; cloning efficiency was defined as the % of surviving clones that were antigen-specific; while overall cloning efficiency was determined by the % of total wells that
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contained live antigen-specific clones available for secondary expansion. Clone pellets were designated as generation 1 (‘G1’).

Selected antigen-specific clones were transferred to 48- or 24-well plates and restimulated in the presence of $2 \times 10^6$ or $5 \times 10^6$ irradiated feeder LCL in 1 or 2ml Rs5 respectively. Clones were stimulated and expanded using PHA and cytokine conditions described above. Following secondary expansion, these generation 2 (G2) clones were used for phenotypic and functional assays as described in text, and aliquots of $1-10 \times 10^6$ cryopreserved. Clones are designated in-text by their donor source, epitope-specificity and seeded well number – for instance “U22 (donor) NLV (peptide epitope) 1G2 (well number).

2.3.11. Insect cell culture.

Low passage number Sf9 cells were provided by Dr James Dickson, School of Biological Sciences, University of Auckland. Cells were cultured at 28°C shaking at 130rpm in Sf900 III SFM medium without antibiotics in capped Erlenmeyer flasks.

2.4. Immunological assays.

2.4.1. Cell counting via Trypan Blue exclusion.

Cells to be counted were mixed by pipette and then diluted with an equal volume of 0.4% Trypan Blue Solution (Gibco®, Life Technologies). 10μl of this solution was loaded onto an Improved Neubauer bright-line double-ruling haemocytometer under a glass cover slip. Viable cells with intact cell membranes were able to exclude the dye and appeared clear, while cells with perturbed membranes were stained blue and excluded from counting.

2.4.2. Bright field microscopy and photography.

Cultured cells were viewed, and images were acquired using a Leica DMI3000 B inverted microscope fitted with a Leica DF290 camera and Leica Application Suite software (LAS) version 4.0 (all Leica Microsystems Ltd.)

2.4.3. Labelling of cells with amine-reactive fluorescent dyes.

CellTrace™ Violet; CellTracker Green CMFDA and CellTracker™ Orange CMRA (all Life Technologies) were reconstituted from powder in DMSO to 5μM, 10μM and 10μM respectively. Cells to be stained were washed with PBS to remove residual serum, and then resuspended in PBS containing dye at working concentration, at $1 \times 10^6$ cells/ml. Typically, for general cell labelling, cells were stained at 37°C for 20 min at 0.5μM CellTrace™ Violet, 0.5μM CellTracker Green CMFDA, or
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1µM CellTracker™ Orange CMRA. Concentrations were determined empirically to allow visualisation with optimised PMT voltages on the BD FACSAria™ II. For cell division experiments, T cells were stained at 37°C for 30 min using 5µM CellTrace™ Violet, to allow for maximum resolution in division peaks. Unlike CFSE, CellTrace™ Violet does not exhibit toxicity at concentrations above 1µM, nor does it need to be applied on ice to produce sharp peaks. Following staining, residual free dye was quenched by addition of 5 volumes of RF10. Cells were then pelleted, washed once with 1ml RF10, and resuspended in appropriate medium for downstream assays.

2.4.4. Enzyme-linked ImmunoSpot analysis (ELISPOT).

ELISPOT plate wells were pre-coated with ELISPOT capture antibody in ELISPOT dilution buffer overnight at 4°C. This solution was then removed and wells washed once with PBS, then blocked with RF10 for 2h at room temperature to prevent non-specific protein binding. Blocking solution was removed and wells washed twice with RPMI-1640.

Antigen-presenting cells or whole PBMC loaded with appropriate peptide or DMSO, and unpulsed T cell clones were plated in Rs5 in 96-well ELISPOT plates. Typically, if T2 or LCL cells were used as ‘APC’ (to screen T cell clone specificity), 50,000 were plated with 5-10,000 clonal T cells, whereas when PMBC were used to detect precursor frequencies 200,000 were plated. T cells stimulated with 0.5% v/v PHA or a known agonist peptide were plated as positive controls. Plates were then incubated for 48 h at 37°C/5% CO₂.

Cells were gently resuspended and removed by aspiration, and wells were washed twice with 200µl milliQ water. At these and all subsequent wash steps plates were incubated for 3 min per wash. Water was removed by aspiration. Wells were then washed 4 times with 200µl PBS-T. PBS-T, and all subsequent washes were removed by flicking and blotting plate. 100µl detection antibody solution was added to each well and the plate incubated for 2h at room temperature. Detection antibody solution was removed by aspiration and wells washed 4 times with 200µl PBS-T. 100µl 100x Streptavidin-HRP (BD) diluted to 1x in dilution buffer was then added to each well and incubated for 1h at room temperature. S-HRP was removed by aspiration and wells washed 4 times with PBS-T and then 4 times with PBS. Substrate solution was prepared fresh by combining 333µl AEC and 10ml 0.1M acetate solution. This was then filtered with a 0.45µm filter (Millipore) and 5µl H₂O₂ (30%) added. 100µl of substrate solution was added to each well and the plate monitored until satisfactory colour development was evident. The substrate solution was then discarded and the plates rinsed thoroughly with distilled water to stop the substrate reaction. The plate was allowed to dry in the dark at room temperature overnight and photographed. Antigen-specific T cell frequency was assessed by counting the spots in each well by eye using a dissection microscope, or clone specificity was assessed by comparing IFN-γ production in peptide-loaded vs control wells.
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2.4.5. Peptide preparation.

Peptides were provided as lyophilised stocks. Peptides were resuspended in DMSO to desired concentration as described in Table 2-10.

2.4.6. Polyclonal T cell stimulation and expansion.

Polyclonal T cell stimulation was performed using Dynabeads® Human T-Expander CD3/CD28 beads (Life Technologies, referred to as Dynabeads® in-text). These beads are magnetic, 4.5µm in diameter and are saturated with anti-human-CD3 and anti-human-CD28 antibodies (clones OKT3 and CD28.2, respectively) at a proprietary ratio optimised for T cell expansion.

PBMC were freshly isolated from blood as described in section 2.3.3, or thawed as described in section 2.3.5. PBMC were plated in 24 well plates in 2ml Rs5, at a density of up to 2 x 10⁶ cells/ml (i.e. 2 x 10⁶ cells/cm² plate floor area). Monocytes were allowed to adhere to the plate surface for 2h, and then non-adherent cells transferred to adjacent wells and incubated overnight to allow further monocyte depletion. Stringent depletion was necessary, as if beads were applied directly to PBMC the majority were internalised by phagocytic cells and were not accessed by T cells. As T cells typically comprise 75% of non-adherent lymphocytes, to activate T cells, Dynabeads® Human T-Expander CD3/CD28 beads were added at a ratio of 1.25 beads/lymphocyte, to give approximately 1 bead/T cell. Rs5 was supplemented with IL-12 (10ng/ml) and either 10ng/ml IL-2 or with 5ng/ml IL-7 and 10ng/ml IL-21, and cells coincubated with beads for 72h to ensure synchronous stimulation. At 72h, cells were collected and beads magnetically depleted. Beads were washed twice in Rs5 to ensure complete cell retention, and washes pooled with collected T cells. Separated bead and cell fractions were monitored visually to ensure that cell losses were minimal, and that no beads remained in the cell fraction. By 72h, T cells typically exhibited a blasting morphology. Stimulated T cells were replated at 1 x10⁶ cells/ml in Rs5 supplemented with cytokines as described above. Expansion wells were split as they reached confluence, and fed with Rs5 containing relevant cytokines as they were passaged. Expansions with 5ng/ml IL-7 were supported with 10ng/ml IL-21 until cells had passed their logarithmic division phase (typically day 14-21), after which cells were maintained in 5ng.ml IL-7 only. Typically, expansions lasted 21-30 days, during which time T cells expanded up to 1000-fold. Phenotyping and functional assays were carried out after expansions had finished dividing, as determined by no change in counted cell number within a 72h period.

2.4.7. Cytokine receptor detection and expression kinetics.

In the absence of cognate cytokine binding, γc cytokine receptor subunits are typically cycled to the cell membrane as rapidly as they are internalised to ensure constitutive availability. However, on cytokine binding these subunits are rapidly endocytosed and degraded (Hémar et al., 1995).
Additionally, many γc cytokines induce transcriptional down-regulation of their receptor (Alves et al., 2008; Vranjkovic et al., 2007). Because this modulation could impair flow cytometric detection of surface cytokine receptor subunits and lead to false-negative phenotypic characterisation of clonal T cell populations, we sought to determine the optimal timepoint for detection of surface IL-7Rα following IL-7 withdrawal. Several expanded T cell libraries were maintained in 10ng/ml IL-7 for 48h. At serial timepoints, samples were washed to remove exogenous IL-7, resuspended in cytokine-free Rs5 and incubated in the absence of cytokine until analysis, at which point T cells were stained with anti-CD8 and anti-IL-7Rα monoclonal antibodies. We found that on both CD8+ and CD8− T cell populations surface IL-7Rα expression was negligible in the presence of IL-7, became increasingly detectable with 6-24h of cytokine withdrawal, and reached maximum density in all donors within 36h of cytokine withdrawal, after which MFI remained stable (A, C; Figure 2-3). No change in T cell viability was observed at this timepoint. This pattern of detection was consistent for both of the available anti-IL-7Rα monoclonal antibodies: hIL-RRM21 and R34.34 at 36h (B; Figure 2-3). As such, 36h incubation in cytokine-free Rs5 was used prior to staining for any surface cytokine receptor.

**Figure 2-3 Kinetics of surface expression of IL-7Rα on T cells.**

Purified T cells from three donors were cultured for up to 48h as indicated, in the presence or absence of IL-7, and then stained for surface expression of CD8 and IL-7Rα. A, C: In both CD8+ and CD8− T cell subsets IL-7Rα is not detectable by flow cytometry in the presence of IL-7, and surface density increases after cytokine withdrawal to saturation at 24-36h. B: This expression pattern was consistent for both available monoclonal antibodies.
2.4.8. T cell clone-mediated cytotoxicity.

SK-Mel-23 (HLA-A2+ Melan-A+) melanoma cells were stained with CellTracker™ CMFDA as described in section 2.4.3 and plated into flat-bottom 96-well plate wells (BD Biosciences) in RF10, typically at 1.5 x 10^4 cells per well in 200µl total volume. Cells were centrifuged and allowed to settle and adhere for up to 6h until they displayed normal adherent morphology.

Clonal T cells were then added at titrated effector-to-target (E/T [X]) ratios, centrifuged to allow immediate access to the melanoma cells, and co-incubated for the duration of the assay (4-18h). Samples that contained no T cells, or T cell clones specific for an irrelevant HLA-A2-restricted epitope (typically CMV pp65_{495-503}) at E/T [4] were included as negative controls. Each condition was set up in triplicate. To assess melanoma cell morphology, cells were imaged via bright field microscopy as described in section 2.4.2.

To assess melanoma cell viability and specific lysis while controlling for sample collection and acquisition error, 80% of the cell-free supernatant was discarded and the remainder gently aspirated and transferred to a 1.2ml FACS tube. This fraction contained T cells, dead detached melanoma cells and weakly adherent live melanoma cells. 40µl of an enumeration master mix (PBS containing 1 x 10^4 CompBeads™ (BD) / 40µl) was then added to each well. Wells were again aspirated and solutions transferred to the relevant FACS tubes. To detach any remaining live melanoma cells, 40µl TrypLE™ Express was added to each well and plates were incubated for 5 min at 37°C. TrypLE™ Express was quenched by addition of 40µl RF10, and wells were again aspirated and detached cells transferred. Finally, wells were washed once more with 40µl PBS to collect any remaining detached cells and beads, and these were transferred to FACS tubes. Following all cell collection steps wells were assessed visually for efficiency of cell collection – typically no remaining melanoma cells or beads were observed. The collated samples were stored on ice.

Immediately prior to acquisition of each sample, DAPI was added to a final concentration of 0.5µg/ml to allow discrimination of live (CMFDA+ DAPI-) and dead (CMFDA+ DAPI+) melanoma cells.

Melanoma cells and CompBeaT™ gates were initially determined by light scatter (A; Figure 2-4). Live Melanoma cells in each sample were enumerated by gating the singlet CMFDA+ DAPI- population – example gates are shown in (B; Figure 2-4). Total event counts within the ‘CompBeaT™ gate’ and ‘live melanoma cell gate’ were tabulated for each sample. Bead counts in each sample were standardised to ‘100,000’, and live melanoma cells in each sample were normalised to ‘live cells per 100,000 beads’ based on the bead:cell ratio in said sample. Surviving melanoma cells in each sample were then expressed as % of the mean ‘no T cell clone’ live cell number, and specific lysis calculated by: 100% - surviving melanoma cell %.
Figure 2-4 Gating strategy for T cell clone mediated cytotoxicity assays.
Clonal T cells, living and dead melanoma cells and CompBeads were acquired and distinctly gated (left panels). Following doublet exclusion within the melanoma cell gate (middle panels), live (CMFDA+ DAPI-) melanoma cells were enumerated (right panels) and surviving cells/CompBead determined. A: Negligible cytotoxicity was observed in the absence of T cells; B-C: T cell mediated cytotoxicity was induced in a titratable manner as E/T was increased from 0.1 to 1.

2.5. Flow cytometry, cell sorting and immunocytochemistry methods.

2.5.1. Antibody staining.

2.5.1.1. Cell surface marker staining.
Cells were typically stained in either 15ml Falcon tubes, 5ml FACS tubes, or U-bottom 96 well plates (all BD Biosciences) in a final volume of 50µl (including antibodies) flow cytometry staining buffer (PBS/5% FBS v/v) supplemented with fluorophore-conjugated monoclonal antibodies at volumes listed in Table 2-5. To avoid sample-to-sample variation, wherever possible samples were stained using pre-prepared antibody master-mixes. Unless otherwise specified (as below), cell surface markers were stained on ice for 30 min, and unbound antibody removed by washing samples twice with 200µl flow cytometry wash buffer (PBS/1% FBS v/v). Individual antibodies were titrated by
staining appropriate protein-positive populations (typically PBMC, stimulated using PMA/I if required for protein expression). Typically PBMC comprised protein-positive and protein-negative populations, allowing assessment of background staining. Antibodies were titrated in serial two-fold dilutions, from double- to one-sixteenth the concentration recommended by manufacturer. Doses were chosen as the lowest concentration at which saturated staining was observed, in the absence of background fluorescence above that observed in unstained controls (Table 2-5).

2.5.1.2. General gating strategy using FlowJo software (Treestar; v10).

Events to be analysed were initially gated using an appropriate SSC area vs FSC area profile, avoiding debris and most cell doublets (A; E Figure 2-5). Within this gate, remaining doublet cells were excluded by sequentially gating only on events that displayed a linear FSC area vs height then SSC area vs height profile (B-C; F-G Figure 2-5). Dead cells were then excluded by DAPI or LIVE/DEAD® Fixable Blue fluorescence as described in section 2.5.2 (D; H Figure 2-5). Singlet live cells were then further assessed as per experimental parameters.

Figure 2-5 General flow cytometry gating strategy.

A-C: Singlet T cells or E-G: Singlet T2 cells were selected for analysis by sequential inclusion gating based on forward scatter (FSC) and side scatter (SSC) area (-A), height (-H) and width (-W) profiles. Within singlet gates, D; H: dead cells were excluded based on DAPI fluorescence. Only singlet live cells were subsequently analysed for cell surface marker expression.
2.5.1.3. Optimised CCR7 detection.

Two anti-CCR7-FITC conjugated clones were available: 150503 and G043H7. As chemokine receptors are cycled through early endosomes, to optimise surface capture and brightness we investigated the effect of altering staining temperature for each clone. Purified T cells from three donors were equilibrated on ice, at room temperature or at 37°C, and then stained with an equivalent concentration of one of the anti-CCR7 clones. Subsequently, samples were chilled and co-stained with anti-CD45RO to allow visualisation of distinct naïve (CCR7+ CD45RO−) central memory (CCR7+ CD45RO− and effector memory CCR7− CD45RO+) populations. No difference in CD45RO MFI was observed across the range of temperatures investigated (data not shown). We found that staining with clone 150503 was highly temperature dependent, as the CCR7 MFI of each of the CCR7+ populations progressively increased as the temperature increased, as did our ability to clearly distinguish the CD45RO− CCR7+/− fractions. Staining with clone G043H7 was also temperature dependent in that the MFI of CCR7+ populations was higher at RT and 37°C than on ice, but no difference was seen between RT and 37°C. Maximum MFI achieved with clone G043H7 was much greater than that with clone 150503, particularly within the naïve (CCR7+ CD45RO−) fraction, and this clone gave better visual distinction between the naïve and central memory populations (Figure 2-6). As such, all subsequent CCR7 staining was performed using clone G043H7 at or above RT for 15 min, after which cells were chilled and stained for remaining surface markers.
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Figure 2-6 Optimisation of surface CCR7 detection.
Total T cells were stained for surface CD45RO and CCR7 expression using fluorophore-matched antibody clones and temperatures indicated. One representative donor of three is shown. Subset gating was determined based on FMO controls. To quantify CCR7 surface density, median fluorescence intensity (MFI) was determined within each gated CD45RO/CCR7 subset. Graphs indicate mean MFI ± SEM for three donors.

2.5.1.4. Tetramer/Pentamer staining.
PBMC were typically stained in 5 ml capped FACS tubes (BD Falcon™) in 100µl total volume. To assess the optimal temperature for rapid pentamer binding, we compared incubation at 37°C and ice for 15 min using two CD8+ T cell clones specific for the Melan-A26-35 epitope (ELAGIGLTV A27L). Following ELA-pentamer-PE staining, cells were washed once with 1ml FACS wash at room temperature and then chilled on ice for 10 min and stained with anti-CD8a-Alexa Fluor700. We found that for both clones tested pentamer staining intensity was brighter at 37°C than on ice, while CD8a intensity was equivalent for both methods (Figure 2-7). As such, future pentamer staining was performed for 15 min at 37°C, after which unbound pentamer was removed by washing prior to standard cell surface marker staining.
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Figure 2-7 Optimisation of pentamer staining.
Two ELA26-35-restricted CD8+ T cell clones were sequentially stained with ELA-pentamer at temperatures indicated, then anti-CD8 on ice. Pentamer-PE and CD8-Alexa700 median fluorescence intensities (MFI) for both clones are shown.

2.5.1.5. Intracellular flow cytometry.
Where cells were to be stained for both surface markers and intracellular markers, staining was initially carried out as described in section 2.5.1.1. Following thorough removal of unbound antibodies, samples were fixed and permeabilised for 30 min on ice using 100µl Fix/Perm Buffer (BD Biosciences) per sample. Fix/Perm was removed by washing twice with 200µl Perm/Wash Buffer (BD Biosciences), and samples were then stained with fluorophore-conjugated monoclonal antibodies or fluorophore-matched isotype controls at volumes listed in Table 2-6, diluted in 50µl total volume Perm/Wash Buffer for 30 min on ice. Unbound antibody was removed by washing samples twice with 200µl Perm/Wash buffer, and samples were then resuspended in flow cytometry wash buffer and analysed within 24h. Individual antibodies were titrated by staining appropriate protein-positive populations (typically PBMC, stimulated using PMA/I if required for protein expression). Antibodies were titrated in serial two-fold dilutions, from double- to one-sixteenth the concentration recommended by manufacturer. Doses were chosen as the lowest concentration at which saturated staining was observed, in the absence of background fluorescence above that observed in unstained controls (Table 2-6).

2.5.1.6. Optimised detection of T cell clone effector cytokine production.
To stimulate the production of intracellular cytokines or effector granule proteins, T cells or PBMC were plated in 200µl Rs5 containing Phorbol-12-myristate-13-acetate (PMA), ionomycin and BD GolgiStop™ (Monensin) protein transport inhibitor. Where T cells were to be stimulated using peptide-pulsed APC, APC were pulsed overnight with peptide at relevant concentrations, washed 3
times to remove residual free peptide from solution, and plated in 96 well plate wells in 100µl. T cells were resuspended at desired concentration in a further 100µl Rs5 containing BD GolgiStop™ at a concentration of 1.34µl/ml (2x). T cells were then added to APC wells and samples mixed well by pipette. T cells and APC were allowed to equilibrate in BD GolgiStop™- containing medium for 30 min, and then pelleted to allow cell-cell contact and stimulation.

Intracellular accumulation of effector ‘Th1’ cytokines within T cells is typically assessed at 2-8h post stimulation. As the production of individual cytokines may follow discrete kinetics, to optimise the co-detection of IL-2, IFNγ and TNFα we investigated stimulation of purified T cells as described above for 4h and 6h, followed by staining with anti-CD8-Alexa Fluor®700; anti-IL-2-PE; anti-IFNγ-Alexa Fluor®647 and anti-TNFα-Alexa Fluor®488. Discrete CD4+ and CD8+ T cell gates were drawn, and cytokine accumulation assessed within each population by enumerating cytokine+ cells (population % +) and by determining the MFI within those cytokine+ events. Stimulated cells stained with anti-CD8 only, and unstimulated cells stained with the complete panel were used to define cytokine+ gates. Results from one representative donor are shown below. We found that for both CD4+ and CD8+ cells, the 6h timepoint was superior for detection of IFNγ, both in terms of the % expression and intracellular cytokine levels. CD4+ and CD8+ T cells displayed different kinetics of IL-2 and TNFα expression – IL-2 was best detected in CD4+ cells 6h after stimulation and TNFα expression remained consistently high at both timepoints. By contrast, in the CD8+ population both the % of cells and intensity of staining for both IL-2 and TNFα were greater at 4h then 6h. Interestingly, although the % and intensity of IFNγ increased from 4h to 6h in the CD8+ cells, a much greater % of the CD8+ than CD4+ population were IFNγ+ at 4h (75% vs <50%) and it was the most intensely staining of the cytokines (Figure 2-8). These data suggest that CD8+ T cells may have a more rapid onset of polyfunctional cytokine production, but a shorter window of production. As such, we determined that when assessing polyfunctional cytokine secretion in CD4+ cells a 6h timepoint was optimal, but when assessing CD8+ T cells (and CD8+ T cell clones in particular) a 4h timepoint was optimal.
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Figure 2-8 Relative kinetics of activated CD4* and CD8* T cell cytokine production.

Total T cells were stimulated with PMA and ionomycin in the presence of BD GolgiStop™ for times indicated. Surface CD4/CD8 and intracellular cytokines were detected by staining. Within CD4* and CD8* populations, cytokine-positive cells (%) were determined by gating based on stimulated cells stained with anti-CD4, anti-CD8 and fluorophore matched isotype control antibodies. Median fluorescence intensity (MFI) was determined within cytokine-positive cells. One representative donor of three is shown.

2.5.2. Doublet and dead cell discrimination.

When analysing unfixed samples, immediately prior to staining DAPI was added to each sample to a final concentration of 0.5µg/ml. Dead and dying cells with perturbed cell membranes were unable to exclude DAPI, and as such DAPI+ dead cells could be excluded from subsequent analysis. In some T cell experiments, DAPI was not used and dead cells were excluded based on an altered FSC/SSC light scatter profile, a procedure previously optimised within our laboratory (Ho, 2012). This method was only applied to pure populations of lymphocytes. In experiments that involved fixation and/or permabilisation of cells, prior to the start of the staining protocol samples were washed twice with PBS to remove serum and incubated for 30 min at RT in 100µl PBS containing LIVE/DEAD® Fixable Blue (Life Technologies) at 1:1000 v/v. Unbound dye was subsequently quenched with an equivalent volume of RF10, samples were pelleted and supernatant removed. As LIVE/DEAD® Fixable Blue dimly stains viable cells due to interaction with cell membrane proteins, heat-killed cells were also stained with LIVE/DEAD® Fixable Blue to act as a compensation control prior to data acquisition.

2.5.3. Live fluorescence-activated cell sorting (FACS) for T cell cloning.

2.5.3.1. Pentamer-guided cell sorting.

Candidate cells were sequentially stained for surface expression of CD8, CD20 with PE- or APC-conjugated pentamer (as described in section 2.5.1.4), and then for surface expression of CD3, CD8 and CD20 (as described in section 2.5.1.1) with the exception that all staining was carried out under
sterile conditions, using phenol-free Rs5 as final wash buffer. Cells were suspended at ≤5 million cells/ml for sorting. Pentamer⁺ cells were selected by gating on CD20⁻ CD3⁺CD8⁺ Pentamer⁺ single lymphocytes. Pentamer staining was validated by use of a no-pentamer FMO control.

### 2.5.3.2. Activation-marker-guided cell sorting.

Candidate cells were stained for surface expression of CD3, CD4, CD8, CD25, CD56, CD69, CD134 and CD137 following peptide-based expansion and recall as described in section 2.5.1.1 with the exception that all buffers were filter-sterilised and all staining was carried out under sterile conditions, using phenol-free Rs5 as final wash buffer. Cells were suspended at ≤5 million cells/ml for sorting.

Individual cell sorting was carried out using a BD FACSAria™ II cell sorter using a ‘single cell purity’ sort mask, an 85µm nozzle and 45psi system pressure. Stream amplitude and frequency settings were determined on a sort-by-sort basis following manufacturer’s specifications. To avoid post-sort toxicity, DAPI was not included as a viability marker during live cell sorting, and light scatter gating was accordingly conservatively stringent. All samples were initially gated using light scatter to select lymphocytes and exclude doublets, as described in 2.5.1.2. Following light scatter gating, selection gates were drawn as described below:

Peptide-activated CD4⁺ T cells were selected by gating on CD3⁺ CD4⁺ CD8⁻ CD25⁺/CD69⁺ CD134⁺ events. Peptide-activated CD8⁺ T cells were selected by gating on CD3⁺ CD4⁻ CD8⁺ CD25⁺/CD69⁺ CD137⁺ events. Activation marker expression gates were validated using: CD25/69/134/137 expression on peptide pulsed T cell clones; FMO controls for CD134 and CD137; and paired unstimulated, fully stained cultures.

Individual selected cells were sorted into wells of a 96-well U-bottom culture plate (BD Biosciences) containing 100µl Rs5 and 5 x 10⁴ LG2-LCL previously irradiated (50Gy). Individual cells were then stimulated and expanded into clonal populations as described in section 2.3.10.

### 2.5.4. Data acquisition and analysis.

Flow cytometry data was acquired using either a FACSCalibur™ or FACSAria™ II using CellQuest™ Pro or FACSDiva™ (v6) software, respectively (all BD Biosciences). When using the FACSCalibur™ fluorescence compensation was performed manually using individually stained cells or cytometry beads in channels FL-1 to FL-4. When using the FACSAria™ II compensation was performed by FACSDiva™ software. Acquisition thresholds were determined using unstained samples. 1 x 10⁴ to 1 x 10⁷ events were collected per experimental sample, depending on the nature of the experiment. Data were exported as .FCS and were analysed using FlowJo software v7.6 or vX.1 (Tree Star).
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2.5.5. Immunocytochemistry for detection of Melan-A.

2.5.5.1. Sample preparation and staining.

Primary human melanocytes and melanoma cell lines were seeded into chamber slides (BD) in duplicate at 10,000 cells/chamber in 500 µl RF10, and allowed to grow until 80-100% confluent. Media was removed by aspiration, and chambers washed 3 times with 200 µl TBS. Some melanoma cell lines, in particular SK-Mel-29, are poorly adherent, and so to minimise cell detachment all wash steps were performed by carefully running TBS down the sides of the chamber. Cells were fixed with acetone at -20°C for 5 min and then washed 3 times with TBS. Chambers were then incubated with 100 µl 0.25% casein for 10 min at room temperature to block non-specific binding sites, and washed 3 times for 5 min with TBS on a rocking table. One chamber for each cell line was then incubated with anti-Melan-A (clone M2-7C10; mouse IgG2b; Cell Marque) diluted to 1:100 in 100 µl TBS-1% FBS, while the other chamber was incubated in 100 µl TBS-1% FBS to act as a secondary-only control. Slides were incubated for 1h at RT on a rocking table, washed 3 times for 5 min with TBS and then incubated with goat-anti-mouse-IgG2b-Alexa Fluor®488 (Molecular Probes) and DAPI, diluted in 100 µl TBS-1% FBS at 1:200 and 1:2000 respectively, for 1h at room temperature on a rocking table protected from light. Chambers were washed 3 times for 5 min with TBS, then all TBS was aspirated. The chamber framework was removed, and cells coated with ProLong® Gold and a glass coverslip. Slides were stored at 4°C until visualised.

2.5.5.2. Visualisation and acquisition.

Images were collected using a Nikon Ni-U upright fluorescence microscope with a Spot Pursuit Slider camera and Spot software. Optimal exposure times for detection of Alexa Fluor®488 and DAPI fluorescence signals were empirically determined, but identical exposure times were used when acquiring images for matched experimental and secondary-only control chambers.

2.5.5.3. Image analysis.

Images were analysed and figures constructed using Cytosketch software (Build X, www.cytocode.com). Identical image fields for Alexa Fluor®488 and DAPI were superimposed, and Alexa Fluor®488 auto-fluorescence determined and thresholded to zero to determine genuine signal.

2.6. Data processing and statistical analyses.

Numerical data were processed, and graphs produced, using GraphPad Prism for Windows (version 4.03). All statistical analyses were carried out in GraphPad Prism. Specific statistical tests used, and p-values generated are indicated in relevant chapter sections and figures. Parametric analyses were utilised where data followed Gaussian distribution. Post-hoc corrections were used when dealing with
multiple comparisons, as detailed in specific figure legends. Differences were considered statistically significant at \( p = 0.05 \). Typically, significance levels were annotated as: * \( p = 0.05 \); ** \( p = 0.01 \); *** \( p = 0.001 \).
Chapter 3. T cell culture and cloning

3.1. Introduction and aims.

The application of homeostatic cytokines during in vitro expansion can provide insight into T cell survival requirements ex vivo, and T cell memory formation requirements in vivo. Previous Ph.D. thesis projects within our laboratory have demonstrated that purified naïve CD8\(^+\) T cells can be expanded using commercially available Dynabeads® Human T-Expander CD3/28 (Dynabeads®), supported by timed administration of the γc cytokines IL-2, IL-7 and IL-21, and retain desirable characteristics for adoptive cell therapy. The aims of this chapter were to: extend these observations by investigating both naïve and memory CD8\(^+\) T cell culture and maintenance; to investigate whether known antigen-specific memory populations could be retained through IL-7 + IL-21 mediated T cell expansion; and to develop and optimise a pentamer-guided CD8\(^+\) T cell cloning protocol based on IL-7 + IL-21 administration to expand large stocks of T cell clones directed against therapeutically relevant viral and cancer targets.
3.1.1. Methods.

3.1.2. Cell culture.

PBMC and T cells were cultured in sterile Rs5, as described in sections 2.3.1 and 2.3.2. Rs5 was supplemented with cytokines as described in text. During expansion and maintenance, Rs5 and supplementary cytokines were replenished as described in text.

3.1.3. Isolation of naïve and memory CD8+ T cells.

Pure CD8+ naïve (CD45RA+ CCR7+) and memory (CD45RA-) T cell populations were isolated by MACS (section 2.3.8.2). Cells were either maintained or expanded in Rs5 supplemented with IL-2, IL-7, IL-15 or IL-21 and combinations thereof as indicated in text for 21 days, and cell surface protein expression then assessed by flow cytometry as below.

3.1.4. Generation of polyclonal T cell libraries.

PBMC were collected from blood as described in section 2.3.3 and monocytes stringently depleted via sequential plate adherence (section 2.3.9). Non-adherent lymphocytes were stimulated with Dynabeads® Human T-Expander CD3/CD28 beads (Life Technologies, referred to as Dynabeads® in-text) for 72h in the presence of either IL-2 and -12, or IL-2; -12; -7 and -21 (10ng/ml) and then allowed to expand as described in section 2.4.6. T cell phenotype, CD4:CD8 ratio and Tetramer+ cell content were assessed at D0, 7, 14 and 21.

3.1.5. Tracking of Tetramer/Pentamer-specific T cell populations.

At days 0, 7, 14 and 21 CD8+ T cells specific for HLA-A2-restricted peptides ELA26-35 (A27L) (Melan-A/MART-1), GIL58-66 (Influenza matrix) GLC280-288 (BMLF1), NLV495-503 (pp65) were detected within polyclonal T cell expansions by staining for CD8 and pentamer-specific cells as described in section 2.5.1.4. Antigen-specific cells were expressed as pentamer+ % within the total CD8+ T cell fraction.

3.1.6. Cytokine-mediated T cell survival.

Pure CD4+ and CD8+ T cells were isolated via MACS (section 2.3.8.1) and plated in Rs5 alone or Rs5 supplemented with IL-2, -4, -7 or -15 for 12d. Surviving T cells were counted via Trypan Blue exclusion and expressed as % of initial T cell population.

3.1.7. Cell surface protein expression.

Surface expression of CD3, CD4, CD8, CD27, CD28, CD45RA, CD45RO and CD62L was analysed via flow cytometry as described in section 2.5.1.1. Surface expression of CCR7 and CD127 was also
determined by flow cytometry, with modifications as described in sections 2.5.1.3 and 2.4.7 respectively. Antibodies and doses used are listed in Table 2-5. Live/Dead discrimination was carried out using DAPI (section 2.5.2).

3.1.8. Pentamer-guided live-cell sorting for CD8+ T cell cloning.

Pentamer staining was carried out on fresh or frozen PBMC as described in section 2.5.1.4. Single CD3+CD8+pentamer+ cells were seeded via FACS and stimulated as described in sections 2.5.3.1 and 2.3.10 in the presence of either IL-2 and -12, or IL-2; -12; -7 and -21. Successfully expanded T cell clones were validated for specificity by pentamer staining. T cell outgrowth and cloning efficiency were assessed as described in section 2.3.10.
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3.2. Results.

3.2.1. Common γc cytokines modulate CD8+ T cell phenotype during in vitro expansion and maintenance.

Previous work within our laboratory has indicated that administration of the common γc cytokines IL-7 and IL-21 to naïve CD8+ T cells during stimulation and expansion using Dynabeads® results in the retention of naïve T cell-defining surface markers, specifically CD45RA and CCR7, and near-universal expression of the co-stimulatory molecules CD27 and CD28, which are constitutively expressed by naïve cells, but heterogeneous across in vivo memory T cell populations (Appay et al., 2008), in the resting Dynabead®-expanded population. This work, and other reports (Wolfli and Greenberg, 2014), also demonstrated that administration of cytokines at a concentration of ≥5ng/ml in medium supplemented with 5% v/v human serum is sufficient and necessary to support T cell expansion and maintenance. As such, these conditions were used during polyclonal T cell expansion in this chapter.

Pure naïve and mixed CD45RO+ TCM/EM cells were isolated as described in sections 2.3.8.1 - 2.3.8.2. TEMRA (CCR7– CD45RO CD45RA+) were removed from the CD45RA+ fraction (section 2.3.8.2) because their frequency is highly variable on a donor-to-donor basis, and because TEMRA have been showed to proliferate poorly in response to TCR-mediated stimulation but strongly in response to IL-15 (Geginat et al., 2003a; Willinger et al., 2005) potentially confounding analysis of the naïve samples. Naïve and memory cells were either: stimulated with Dynabeads® for 72h, after which time beads were removed to ensure a synchronous stimulation across all cells, and expanded to d22; or left unstimulated in culture for the same period. Stimulated and cultured cells were treated with cytokines IL-2, IL-7, IL-15 (10ng/ml) alone, or combinations of IL-2 + IL-7 and IL-15 + IL-7. Stimulated samples were also treated with IL-7 + IL-21, to assess the effect of IL-21 inclusion during T cell expansion. Expression of secondary lymphoid organ (SLO) homing markers (CCR7 and CD62L) and co-stimulatory molecules (CD27 and CD28) was assessed via flow cytometry at d22 of expansion or culture.

Due to low donor number and donor variability phenotypic changes were not assessed statistically, but several trends were evident when surface marker expression was assessed at a population level. During culture of naïve T cells (left panel, A, Figure 3-1) Losses of CCR7, CD62L, CD28, and CD27/CD28/CD62 triple-expression were observed on populations cultured in IL-2 and IL-15 alone (decreases of 15-40% on naive populations and 10-50% on memory populations), but not IL-7 alone. Interestingly, co-administration of IL-7 with IL-2 or IL-15 increased retention of these markers and marker combinations, probably because of a lack of CD25 and CD122 expression on unstimulated naïve cells. These data are in accordance with previous observations made within our laboratory.
(Brooks, 2007; Ho, 2012). During culture of memory cells (right panel, A, Figure 3-1) a similar pattern emerged, but with the distinction between cytokine conditions being more marked. Again, co-administration of IL-7 with IL-2 or IL-15 increased retention of CCR7 and CD62L, but not CD28, relative to IL-2 or IL-15 alone. In both naïve and memory culture experiments, negligible loss of CD27 was observed in any cytokine condition. As true naïve and T_{CM/EM} cells express IL-7Rα \textit{in vivo} (Bachmann et al., 2005) differential death rates due to a lack of IL-7 responsiveness are unlikely to be the cause of the differential population phenotype observed on memory culture.

We found that following naïve cell expansion (left panel, B, Figure 3-1), no loss of CCR7 or CD27 was observed at a population level under any cytokine condition tested. By contrast, CD62L was retained on all cells expanded in IL-7 or IL-7 + IL-21, but lost on ~30% of cells expanded in IL-2 or IL-15, with no apparent rescue on inclusion of IL-7. Similarly, CD28 expression was retained on all cells expanded in IL-7 + IL-21, with minor population loss on expansion in IL-7 alone, but CD28 expression was lost in ~50% of cells expanded in IL-2 and ~40% of cells expanded in IL-15, again with no apparent rescue on IL-7 inclusion. Similar results were apparent on stimulation of memory cells (right panel, B, Figure 3-1), with negligible CD27 expression loss observed under any condition. Although the maximal expression at a population level was lower than that seen in naïve expansions, IL-7 + IL-21 and IL-7 alone allowed the greatest retention of CD28 and CD62L. Interestingly, the expression levels of CCR7 were typically higher post-memory expansion for IL-2 and IL-2 + IL-7 than those seen in culture alone, which may reflect biased outgrowth of T_{CM} precursors over T_{EM}, as T_{CM}, although typically forming a smaller fraction of PBMC, exhibit a much greater proliferative potential (Geginat et al., 2003a)

IL-7Rα has been reported to be lost from the T cell surface during activation, and to be re-expressed on functional memory cells (Alves, 2008 ). To assess whether common γc cytokine exposure played a role in this modulation, surface density of IL-7Rα was assessed post-cession of naïve and memory expansions, following 48h cytokine withdrawal to allow maximal IL-7Rα surface accumulation (section 2.4.7). Although all cells appeared to express detectable IL-7Rα, MFI was significantly higher in naïve and memory cells treated with IL-7 + IL-21 when compared to all other conditions \((p = <0.001, \text{Figure 3-2}).\)

Taken together, these data suggest that in both naïve and pooled memory populations, application of IL-7 + IL-21 during expansion is important for the retention of co-stimulatory markers, SLO-homing markers, and an IL-7Rα_{bright} phenotype, although IL-7 alone may be sufficient to retain this phenotype during post-expansion \textit{in vitro} maintenance.
Figure 3-1 Common γε cytokines modulate naïve and memory CD8* T cell phenotype during expansion and maintenance.

Pure CD8* naïve and memory T cells were isolated as described in section 2.3.8.2. Cells were either B: stimulated with Dynabeads® Human T-expander CD3/28 as described in section 2.4.6 and expanded for 22 days in Rs5 supplemented with cytokine as shown (5ng/ml, refreshed within ≤96h) or A: left unstimulated and maintained under the same conditions. Only expanding cells were treated with IL-21. At D22 surface marker expression was detected by flow cytometry (sections 2.5.1.1 and 2.5.1.3). Data shown represents two donors, represented by individual colour-matched spots.
Figure 3-2 Common γc cytokine exposure during T cell expansion modulates IL-7Rα surface density.

Pure naïve and memory CD8+ T cells isolated and expanded as described in section 3.1.3 were washed and maintained for 48h in cytokine-free Rs5. Surface CD8 and IL-7Rα were detected by flow cytometry (sections 2.5.1.1 and 2.4.7). IL-7Rα-PE MFI was determined by subtraction of PE signal of paired CD8-only samples. Data shown as mean ± SEM MFI of triplicate samples from one representative donor (of two). ***p = <0.001; *p = <0.05 two-way ANOVA and Bonferroni post-test.

3.2.2. Polyclonal T cell library expansion in the presence of IL-7 and IL-21 allows retention of diverse antigen-specific memory populations.

Having established that IL-7 and IL-21 were optimal cytokines for retention of CD28, CD62L, CCR7 and IL-7Rα expression in both naïve and memory CD8+ T cells, we next sought to confirm that application of these cytokines to mixed CD4+ and CD8+ T cell expansion from PBMC allowed retention of antigen-specific T cells of various reported phenotypes, as this study aimed to utilise pentamer-guided T cell sorting to expand CD8+ T cell clones specific for a variety of tumour-associated and virus-derived proteins. Antigen-specific populations for which peptide:MHC pentamers are available have been described as residing in vivo in a spectrum of phenotypic states, from naïve to late effector memory (Appay et al., 2002; Appay et al., 2008; Dunbar et al., 2000). Specifically, we investigated whether T cells specific for ELA26-35 A27L (Melan-A protein, naïve phenotype), GILM59-66 (Influenza matrix protein, TCM-early T EM), GLC280-288 (Epstein-Barr virus BMLF1 protein, early-late T EM) and NLV429-503 (Cytomegalovirus pp65 protein, late T EM to T EMRA) were retained or lost during polyclonal T cell expansion. The development of these expanded T cell libraries (termed ‘PBMCexp’ in-text) also provided a useful resource for future assays, as described in
relevant sections. PBMC from several donors were depleted of monocytes by plate adherence, and non-adherent PBMC expanded using Dynabeads® as described in detail in section 2.4.6. T cells typically expanded up to 1000-fold numerically over 28 days, and a significant skewing of the CD4:CD8 T cell ratio was observed, such that CD8\(^+\) T cells typically constituted \(\geq 60\%\) of the final library. This skewing is a recognised consequence of Dynabead®-mediated expansion (Li and Kurlander, 2010; Rasmussen et al., 2010), and does not appear to be a consequence of relative CD4\(^+\) and CD8\(^+\) T cell survival in IL-7, as this cytokine maintained both cell types equivalently with \(>90\%\) survival over 10 days in culture (B; Figure 3-3). Pre- and post-expansion CD4\(^+\)/CD8\(^+\) frequency and pentamer-specific cell detection in one representative donor, positive for all four antigen-specific populations, are shown in A; Figure 3-3. Within the total CD8\(^+\) T cell population, ELA\(_{26-35}\) and GIL\(_{58-66}\)–specific cells appeared to undergo a 10-fold increase in frequency, GLC\(_{280-288}\)–specific cells underwent a ~threefold increase in frequency, while NLV\(_{495-503}\)–specific cells were retained but underwent a ~50\% reduction in frequency. Given that proliferative potential is inversely correlated with differentiation status (Geginat et al., 2003a), these relative fold-increases or decreases were anticipated, but in all cases d22 pentamer\(^+\) populations represented a notable absolute expansion of precursor cell numbers. These data suggested that expansion in IL-7 + IL-21 was appropriate for cloning naïve and memory T cells of any starting phenotype.
Figure 3-3 Expansion of PBMC in the presence of IL-7 + IL-21 allows retention of antigen-specific populations of diverse memory phenotypes.

Non-adherent PBMC were stimulated with Dynabeads® as described in section 2.4.6. A: Prior to expansion, PBMC were stained with antibodies against CD3, CD4, CD8 and with pentamers as shown. Pentamer-specific cells are shown as % of all CD3⁺CD8⁺ events. At d22 of expansion, following cessation of T cell proliferation, stains were repeated and pentamer-specific T cells detected again as described. Pentamer gates were drawn based on matched FMO (no pentamer) control samples. One representative donor of eight is shown. B: Pure CD4⁺ and CD8⁺ T cell populations were isolated as described in section 2.3.8.1 and cultured for 10d in Rs5 + 10ng/ml cytokine as indicated. Media and cytokines were refreshed every 72h. At d10 viable cells were counted via trypan blue exclusion and expressed as % of initially seeded cells. Data shown represents mean + SEM of three donors, dotted line represents initial seeding number.
3.2.3. Tetramer/Pentamer-guided CD8+ T cell cloning.

Having established that IL-7 and IL-21 were appropriate support cytokines for the expansion of diverse naïve and memory T cell populations, this study sought to optimise pentamer-guided single cell cloning using the BD FACSARia™ II. Pentameric peptide:MHC constructs are an extremely powerful tools for antigen-specific T cell visualization and isolation (Dunbar et al., 1999). An overview of all pentamer-guided T cell cloning performed in this chapter is shown below Figure 3-4, and T cell cloning methodology is described in detail in sections 2.3.10 and 2.5.3. In brief, individually collected CD8+/pentamer+ T cells were stimulated using PHA in the presence of irradiated LCL feeder cells. As only one T cell was present per well, IL-12 was administered during the first 24h of activation, to mimic the presence of a mature APC (Lee et al., 2007), and IL-2 was administered during the first 72h of activation, to standardise the IL-2 signal received in the absence of other activated T cells. This brief application of IL-2 has been shown by our laboratory not to alter the phenotypic effects of IL-7 + IL-21 administration (Ho thesis, 2012).

3.2.3.1. Pentamer-mediated CD8+ T cell cloning overview.

Figure 3-4 Overview of Pentamer-mediated CD8+ T cell cloning.

Schematic detailing T cell cloning strategy employed in chapters 3 and 4. One representative CD8/Pentamer co-stain and gate is shown (left panel), as is one image of a large, rapidly metabolising ‘G1’ clone in round-bottom 96wp (central panels). Serial T cell clone stimulation and outgrowth was carried out and assessed as described in section 2.3.10. Phenotypic and functional characterisation of G3 ELA28-35-specific clones expanded from a common precursor pool in IL-2 or IL-7+21 was carried out in chapter 4.
3.2.3.2. Tracking T cell clone outgrowth kinetics.

Initial experimentation aimed to define the outgrowth kinetics of PHA-stimulated T cell clones by tracking the appearance of T cell blasts in U-bottom wells. NLV-pentamer-specific cells were collected from PBMC as this antigen-specific population was typically the largest and easiest to define. Following stimulation, actively growing T cells could be visually distinguished from dying LCL by their ‘blasting’ morphology and light refraction characteristics (D; Figure 3-5). Newly emergent T cells became visible at d3 at the earliest, but typically from d7, with appearance of new clones continuing until d16 (A; Figure 3-5). Many clones became visible but did not form large pellets or survive beyond 14d post-appearance. Clones that did emerge were stained again for CD8 expression and NLV-pentamer specificity. In this large pilot study >95% of screened clones from two donors (n=59) were CD8+, and that >80% of screened clones were CD8+ and NLV-specific (B-C; Figure 3-5). In total, 198/301 seeded wells produced clones, and 48/59 clones screened were confirmed as NLV-specific, for an overall cloning efficiency of 53.5%.
Figure 3-5 T cell clone outgrowth kinetics and visualisation.

CD8⁺ NLV-pentamer⁺ single T cells were isolated as described in section 2.5.3.1, and stimulated and expanded as described in section 2.3.10 supported by IL-2, -12, -7, -18, and -21. A: The emergence of visually detectable T cell blasts was tracked across four plates from two donors. Data represents the number of wells containing previously unseen T cell blasts at each day of analysis. B: Clone pellets were stained for surface CD8 and NLV-pentamer (sections 2.5.1.1 and 2.5.1.4) at d22. Data represents clones exhibiting CD8⁻; CD8⁺ pentamer⁻ and CD8⁺ pentamer⁺ staining patterns as demonstrated in C. D: Emerging T cell blasts could be visually detected as highly refractive ‘blasting’ cells, typically from D3 onwards. Typically, by D14 large clonal pellets could be detected, while irradiated LCL had been cleared from the wells. T cells indicated in each panel by *. All images acquired as described in section 2.4.2. D0-D10 200x magnification; scale bars represent 50µm. D14 50x magnification, scale bar represents 500µm.
3.2.3.3. Comparative efficiency of IL-2 and IL-2; -7; -21-mediated T cell cloning.

Antigen-specific T cell cloning has traditionally relied on continuous administration of IL-2 to promote maximal T cell expansion (Yee et al., 1999; Yee et al., 2002b). Several studies have indicated that long-term IL-2 is deleterious for T cell survival on restimulation, and drives T cells towards an effector phenotype (Hinrichs et al., 2008; Ring et al., 2012). Having defined IL-7 + IL-21 mediated pentamer–guided clonal expansion as being highly efficient (section 3.2.2), subsequent experimentation aimed to compare clonal expansion using IL-2 alone to this optimised regimen, also comparing the inclusion and exclusion of IL-21 to further define its contribution to T cell outgrowth. CD8+ T cells specific for pentamer-ELA26-35 (Melan-A protein), -SLL157.165 (NY-ESO-1) -GLC280-288 (Epstein-Barr virus BMLF1) and -NLV495-503 (Cytomegalovirus pp65) were isolated from three donors and expanded as described in sections 2.3.10 and 2.5.3.1. For each pentamer-specific population assessed we found that administration of IL-7 or IL-7 + IL-21 greatly improved both total T cell clone outgrowth and the appearance of large clonal pellets available for subsequent restimulation (A; Figure 3-6). For epitopes where >3 plates of clones were assessed (NLV and ELA), these results were highly significant (p= <0.01; two-way ANOVA and Bonferroni posttest). The inclusion of IL-21 increased total T cell clone and large T cell clone yields compared to IL-7 alone, but these results did not reach statistical significance. When pentamer-specificity was assessed in surviving clones at d28, it was found that inclusion of IL-7 or IL-7 + IL-21 enhanced overall cloning efficiency for all epitopes (B; Figure 3-6). In total, only one SLL157.165 -specific clone and five NLV495-503 -specific clones grown in IL-2 alone were available for secondary restimulation to create ‘G2’ stocks, compared to ten ELA26-35 -specific; seven GLC280-288 -specific; nine SLL157.165 -specific and twenty NLV495-503 -specific clones generated in IL-7 + IL-21. Each of these clones was restimulated as described in section 2.3.10 and total expansion number and population doubling from single cell precursor determined. In determining population doubling number no T cell death was assumed to have occurred, as this could not be detected or quantified. G2 clone expansions are summarised in Figure 3-7. All clones exhibited between 20 and ≤27 population doublings from single-cell precursor, with ELA26-35 -specific clones generated in IL-7 + IL-21 exhibiting the highest mean population doubling number (Figure 3-7).
Figure 3-6 Administration of IL-7 and IL-21 improves CD8+ T cell cloning efficiency and cell proliferation.

A: Expansion of pentamer-stained and sorted T cell clones specific for nonamer peptides (as indicated in graph titles) was visually assessed over 22d. Data represents pooled results from all plates and donors for each peptide epitope in each cytokine expansion condition. ‘T cell growth’ was defined as any well in which T cell blasts could be observed over 22d, irrespective of survival. ‘Large clones’ were defined as macroscopically visible T cell pellets that survived to d22. N= total number of 96-well plates per condition per epitope. Data is expressed as mean ± SEM % of T cell+ or large-clone+ wells within each plate; as such, statistical analyses were only conducted for NLV95-503 and ELA26.35–specific T cell clones. **p= <0.01; two-way ANOVA and Bonferroni post-test comparing each cytokine condition.

B: All T cell clones surviving to d22 were stained for surface CD8 and pentamer (sections 2.5.1.1 and 2.5.1.4). Data represents mean ± SEM pentamer-specific clones expressed as % of total clones screened for each epitope and each cytokine condition.
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Figure 3-7 Summary of T cell clone G2 proliferation.

All T cell clones generated in section 3.2.3.3 were enumerated post-secondary restimulation (G2). Viable cells were counted by trypan blue exclusion. Proliferation is expressed per clone as total cell number (left axis) and as ‘population doublings’ from single cell level (right axis), assuming no cell death during expansion.

3.2.3.4. Phenotypic analysis of antigen-specific T cells before pentamer-guided cloning.

Having produced a suite of G2 clones expanded in IL-7 + IL-21, it was important to investigate the memory subset and co-stimulatory marker profile of each clone, to assess whether the retention of co-stimulatory and homing markers, and IL-7Rα expression observed in sections 3.2.1- 3.2.2 could also be detected in these clones. In order to contextualise the G2 phenotypes observed, we also sought to define the likely starting phenotype of each pentamer-specific population prior to pentamer-guided cell sorting. To do this CD45RA, CD45RO, CCR7, CD27 and CD28 expression within each pentamer-specific population from relevant donor PBMC was analysed via flow cytometry (Figure 3-8). These analyses found that pentamer-SLL-specific precursor cells from donor M56 exhibited an early T\textsubscript{EM} phenotype (CD45RA\textsuperscript{−}, CD45RO\textsuperscript{+}, CCR7\textsuperscript{−}, CD27\textsuperscript{+}, CD28\textsuperscript{+}, A; Figure 3-8). Pentamer-ELA-specific precursor cells from donor M56 exhibited an mixed naïve and T\textsubscript{CM} phenotype (CD45RA\textsuperscript{+}, CD45RO\textsuperscript{−}, CCR7\textsuperscript{−}, CD27\textsuperscript{−}, CD28\textsuperscript{+}, B; Figure 3-8) and that pentamer-ELA-specific precursor cells from donor M56 exhibited a naïve phenotype (CD45RA\textsuperscript{−}, CD45RO\textsuperscript{−}, CCR7\textsuperscript{−}, CD27\textsuperscript{−}, CD28\textsuperscript{+}, B; Figure 3-8). Pentamer-NLV-specific precursor cells from both donors M56 and U22 primarily
exhibited mixed TEM and TEMRA phenotypes (CD45RA^+/-, CD45RO^+/-, CCR7-, CD27^+/-, CD28^-, C; Figure 3-8) although in both donors some CD27^+ CD28^+ CD45RO^+ CCR7^+ TCM could be observed.

Figure 3-8 Pentamer-specific precursor phenotype pre-cloning.

PBMC were stained with antibodies against CD3, CD8, CD27, CD28, CD45RA, CD45RO and CCR7, and with pentamers prior to pentamer-assisted single-cell sorting. Events within each CD8^+ pentamer^+ gate (red) are back-gated onto total CD8^+ T cells for equivalent marker combinations (grey). Pentamer gates were drawn based on matched FMO (no pentamer) control samples, and marker gates drawn based on samples stained for CD3 and CD8 only. Plots were generated from several experiments – specific antibody-fluorophore combinations are annotated. A: SLL-specific cells in donor M56 PBMC. No SLL-precursor population was detected in donor U22. B: ELA-specific cells in donors M56 and U22 PBMC. C: NLV-specific cells in donors M56 and U22. Donors M56 and U22 were the source of nearly all G2 clones generated. Data representative of two precursor analyses per donor.
3.2.3.5. Memory phenotype analysis of IL-2 and IL-7; -21-generated T cell clones.

This study next assessed the phenotype of each IL-2-only or IL-7 + IL-21-generated clone following cessation of G2 proliferation. Each clone was stained for CD8, CD45RA, CD45RO, CCR7, CD27 and CD28 and CD62L, and assessed via flow cytometry (A; Figure 3-9). All GLC-specific clones generated in IL-7 + IL-21 exhibited a T_Em phenotype, with partial retention of CD62L and CD27, and complete expression of CD28. When comparing IL-2 generated and IL7 + IL-21 generated NLV-specific clones it was found that IL-2 clones were all late T_Em phenotype (as T_EMRA precursors are typically poorly- or non-proliferative it is likely that none were expanded to G2) with no retention of CD27, CCR7 or CD62L. By contrast, some NLV-specific clones generated in IL-7 + IL-21 exhibited retention of CD62L, CCR7 and CD45RA expression, and at least one clone exhibited an early T_CM phenotype (C; Figure 3-9). These CD45RA+ cells were not T_EMRA, as all clones expressed CD45ROhi. IL-7 + IL-21 generated NLV clones also exhibited mixed levels of CD27 and CD28 expression. SLL-specific clones generated in IL-7 + IL-21 typically exhibited a CD27+ CD28+ CCR7+ CD62L+ CD45RA+ CD45ROmid phenotype, while ELA-specific clones generated in IL-7 + IL-21 all exhibited a CD27+ CD28+ CCR7+ CD62L+ CD45RA+ CD45ROdim phenotype. This unique phenotype, typically retained through >24 population doublings marks these ELA and SLL clones as being early T_CM or T_SCM (Gattinoni et al., 2011; Gattinoni et al., 2009a). Interestingly, these clones exhibited a rounded morphology in culture, reminiscent of naïve T cells (B; Figure 3-9). One representative clone from each pentamer-specificity and cytokine condition is shown in (C; Figure 3-9), highlighting the differences in phenotype. As no ELA clones were generated in IL-2 alone in these experiments, a pre-existing IL-2 generated clone designated ELA 2D10 generated by Professor Rod Dunbar was used as a comparator. This clone was also used in several subsequent experiments, as indicated in text.
Figure 3-9 CD8+ T cell clones expanded in IL-7 and IL-21 exhibit surface expression of co-stimulatory and secondary lymphoid organ-homing proteins, and CD45RA isoform.

All G2 clones were stained with antibodies against CD8, CD27, CD28, CD62L, CD45RA, CD45RO and CCR7 as described in sections 2.5.1.1 and 2.5.1.3, and phenotype was assessed via flow cytometry. A: Gates for each marker were drawn based on CD8-only stained controls, and data is presented as the % of all CD8+ events that were positive for each surface marker within each clonal population. B: Bright-field microscopy of ELA26-35-specific clone U22 1C10 indicating spherical morphology. Image acquired at 400x; scale bar represents 50µm. C: Representative flow cytometry analyses of IL-7 + IL-21 generated T cell clones specific for ELA26-35 (top), SLL157-165 (middle) and NLV495-503 (bottom) are shown (blue), overlaid with representative epitope-matched, IL-2 generated, “effector” phenotype clones (red). Analyses of epitope-specific clones were conducted in different experiments and with different fluorophore combinations (indicated on each panel), but within each epitope-pairing IL-2 and IL-7/21 clones were stained with matched antibody fluorophore combinations.
3.2.3.6. Retention of IL-7Rα and homeostatic cytokine responsiveness by IL-2; -7; -21-generated T cell clones of various specificities.

Having demonstrated that T cell clones generated in IL7 + IL-21 do retain co-stimulatory and SLO-homing marker expression, and in some cases exhibit a unique TCM/SCM phenotype, we next asked whether expansion in IL-7 + IL-21 also allowed retention of IL-7Rα expression and homeostatic cytokine responsiveness. IL-7Rα expression is a hallmark of healthy memory T cells, and is a differentiating factor between true ‘memory’ and ‘effector’ populations (Hand et al., 2007). To establish a method for assessing cytokine responsiveness, we first assessed which common γc cytokines were able to maintain viable PBMCexp in culture (A; Figure 3-10). Within 8 days PBMCexp unsupported by cytokine showed negligible viability. Although IL-21 did promote some cell survival above the ‘no cytokine’ condition, PBMCexp could not be maintained in it. By contrast, PBMCexp could be maintained without homeostatic division in IL-4 and IL-7, while IL-2 and IL-15 promoted TCR-independent T cell proliferation (A; Figure 3-10). To track the rate of cytokine-responsive or non-responsive G2 T cell clone proliferation or death, we washed and cultured two ELA-specific IL-7 + IL-21 generated TCM/SCM clones and effector clone ELA 2D10 in Rs5 alone or supplemented with IL-2 or IL-7 (B; Figure 3-10). We found that all T cell clones rapidly died in the absence of cytokine support – this was an important finding as it indicated that our G2 TCM/SCM clones had not undergone any transformative event and were still cytokine-dependent. ELA 2D10 and both G2 TCM/SCM clones were able to proliferate in response to IL-2, but interestingly only TCM/SCM clones were able to survive in the presence of IL-7 alone, suggesting that clones grown in IL-7 + IL-21 may retain IL-7Rα expression (B; Figure 3-10). To confirm this, IL-2 and IL-7 + IL-21-generated G2 clones of each pentamer specificity (where available) were analysed for surface IL-7Rα expression via flow cytometry (section 2.4.7). We found that all IL-7 + IL-21-generated clones did retain IL-7Rα expression, while this was not seen in any IL-2-generated G2 stocks. Importantly, retention of IL-7Rα expression was phenotype-independent, as TEM NLV- and GLC-specific clones grown in IL-7 + IL-21 were also IL-7Rα+ (C; Figure 3-10) On incubation of several IL-2- and IL-7 + IL-21-generated G2 clone stocks in IL-2, IL-7 or IL-15, we found that not only were IL-7 + IL-21 clones solely responsive to IL-7, they also exhibited a stronger proliferative response to IL-2 and IL-15 than did IL-2-generated G2 clones, potentially suggesting a higher level of common IL-2/15R beta chain (CD122) expression. Differential CD122 expression on paired IL-2 and IL-7 + IL-21 polarised clones was further assessed in section 4.3.8.
Figure 3-10 retention of IL-7Rα expression and homeostatic cytokine responsiveness by IL-7 + 21 generated clones.

A-B; D: T cell viability was assessed by trypan blue exclusion, and viable T cells expressed as % of initially seeded cell number. In all assays Rs5 + cytokine (10ng/ml) was refreshed within <96h  A: PBMCexp were washed and incubated in Rs5 + cytokine as indicated for 8d. Data representative of three donors (black, grey, white circles).  B: One IL-2-generated ELA26-35-specific clone (2D10) and two IL-7/21-generated ELA26-35-specific clones (U22 1D9; 1B11) were washed and plated in Rs5 alone (no cytokine); Rs5 + IL2 or Rs5 + IL-7. Wells were sampled at timepoints indicated. Data shown as mean ± SEM of duplicate samples per clone per timepoint.  C: Epitope-specific T cell clones were washed and incubated in Rs5 alone for 48h (section 2.4.7) and surface IL-7Rα detected via flow cytometry.  D: Epitope-specific T cell clones generated in IL-2 alone (black) or IL-7 + IL-21 (grey) were washed and incubated in Rs5 alone or with cytokines as indicated for 10d.
3.3. Chapter summary.

In this chapter we investigated the application of different common γc cytokines to pure naïve and memory CD8+ T cells during in vitro maintenance or expansion following anti-CD3/CD28 stimulation. We found that IL-2 and IL-15 signalling during T cell maintenance caused the loss of CCR7, CD62L and CD28 expression on both naïve and memory T cells, but that these markers were retained on culture in IL-7 and co-administration of IL-7 could arrest IL-2- and IL-15-induced phenotypic alteration at a population level. Further, we found that following common γc cytokine-supported naïve or memory T cell expansion CD62L and CD28 loss was even more marked in the presence of IL-2 or IL-15, with no apparent rescue on IL-7 co-administration. By contrast, expansion in IL-7 or IL-7 + IL-21 allowed retention of CD62L and CD28 at pre-expansion levels. Fold-expansion in each cytokine was not tracked in this experiment, and as such we cannot definitively rule out differential cell proliferation as a causative factor in phenotypic change, although samples receiving or lacking IL-7 co-administration expanded approximately equivalently. CD27 expression showed no modulation under any condition, and appeared to be common γc cytokine insensitive in vitro. Importantly, only expansion in the presence of both IL-7 + IL-21 allowed retention of an IL-7RαIII phenotype and this could be detected in both expanded naïve and memory T cells. Based on these findings we applied the use of IL-7 + IL-21 to total CD4+ and CD8+ T cell expansion to produce large polyclonal T cell libraries. Antigen-specific T cell populations of diverse reported memory phenotypes could all be retained within these libraries, demonstrating that IL-7 + IL-21 do not preclude even highly differentiated memory T cell expansion and maintenance in vitro.

Based on these data we investigated the application of IL-7 + IL-21 in supporting the outgrowth of memory T cells specific for the peptide NLV_{495-503} (CMV, pp65) following pentamer-guided FACS and found this protocol to efficiently facilitate pentamer-specific clonal outgrowth. Further, on extending this T cell cloning strategy across T cells specific for several virus or tumour-derived proteins, we found that a regimen involving continuous administration of IL-7 + IL-21, following a brief period of IL-2 + IL-12 exposure, was superior to administration of brief IL-12/continuous IL-2 in terms of overall T cell outgrowth, T cell clone health and persistence, and overall pentamer-specific cloning efficiency.

On analysing T cell clones expanded to G2 we found that application of IL-7 + IL-21 during T cell clone outgrowth allowed the retention of CD62L, CD27 and CD28 through serial restimulations, even when expanding antigen-specific cells exhibiting a highly differentiated precursor phenotype. Most importantly, we found that when expanding T cells specific for the tumour-associated antigen Melan-A (peptide ELA_{26-35}A27L) or the cancer-testis antigen NY-ESO-1 (peptide SLL_{157-165}), co-expression of CD27, CD28, CD62L and CCR7 could be retained, often universally at a population level, and clones exhibited co-expression of the CD45RA and RO isoforms. This phenotype was retained through 22-
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27 population doublings, and marks these cells as exhibiting an early T\textsubscript{CM} or potentially T\textsubscript{SCM} phenotype. To our knowledge this phenotype has not previously been reported for \textit{in vitro} generated antigen-specific CD8\textsuperscript{+} T cell clones derived from single-cell precursors. G2 clones of all specificities expanded in the presence of IL-7 + IL-21, but not continuous IL-2, retained IL-7R\textalpha expression and responsiveness to the homeostatic cytokines IL-7, -4 and -15, demonstrating a transition to true memory status. The wider significance of these results is discussed in detail in chapter 7.
Chapter 4. Functional and phenotypic characterisation of CD8+ Melan-A/MART-1 specific T cell clones

4.1. Introduction and aims.

In chapter three, we demonstrated that application of IL-7 and IL-21 during polyclonal or clonal T cell culture and expansion allowed retention of co-stimulatory and SLO-homing markers, IL-7Rα expression and homeostatic cytokine responsiveness. In particular, we developed a set of ELA26-35 specific T cell clones that appeared to exhibit a TCM/SCM phenotype. In this chapter we aimed to more fully investigate the functional and phenotypic characteristics of this clone set, particularly focusing on their potential application in adoptive immunotherapy. As such, the surface phenotype of these cells was further characterised and the ability of these cells to recognise and kill melanoma cell lines was investigated, as was their cytokine production and effector capacity.

The availability of genetically identical clonal cells allowed us to more fully characterise the process by which T cells stimulated under different conditions undergo protein expression and metabolic changes during effector transition, expansion, and restoration of memory status. To investigate the dynamic alteration of phenotypic markers, and the specific cytokine-signalling-mediated plasticity of each relevant surface marker G2 clones were again stimulated in IL-2 alone or an optimised cytokine regimen and expanded to create paired G3 stocks.

4.2.1. Cell culture.

PBMC and T cells were cultured in sterile Rs5, and T2 and LCL were cultured in sterile RF10 as described in sections 2.3.1 and 2.3.2. Rs5 was supplemented with cytokines as described in text. During expansion and maintenance, Rs5 and supplementary cytokines were replenished as described in text.

4.2.2. Parallel expansion of IL-7/21-derived G2 ELA_{26-35}-specific clones.

G2 ELA_{26-35} clones produced in chapter 3 were further expanded into paired “IL-2” and “IL-7/21” G3 stocks as described below. Functional analyses in this chapter were carried out using both G2 and G3 stocks, as noted in text.

4.2.3. Labelling of T cells with CellTrace™ Violet.

Labelling was carried out as described in section 2.4.3. For detection of pre-labelled T cells within a heterogeneous cellular mixture, labelling was carried out at 0.05-0.1µM. For labelling of T cells to assess proliferation T cells were labelled at 5µM.

Figure 4-1 Generation of paired G3 ELA_{26-35}-specific clone stocks for phenotypic and functional analyses.
4.2.4. Intracellular staining of cytokines.

Cytokine production by T cells was detected as described in section 2.5.1.5 and 2.5.1.6. Prior to fixation, cells were stained with Live/Dead™ fixable dyes as described in section 2.5.2.

4.2.5. Cell surface protein expression.

Surface expression of CD3, CD4, CD8, CD27, CD28, CD45RA, CD45RO, CD62L, CD95, KLRG1 and PD-1 was analysed via flow cytometry as described in section 2.5.1.1. Surface expression of CCR7 and CD127 was also determined by flow cytometry, with modifications as described in sections 2.5.1.3 and 2.4.7 respectively. Cytokine receptor subunits were detected as described in section 2.4.7. Antibodies and doses used are listed in Table 2-5. Live/Dead discrimination was carried out using DAPI (section 2.5.2).

4.2.6. T cell mediated cytotoxicity.

Cytotoxicity assays were carried out as described in section 2.4.8

4.2.7. Peptide loading.

T2 and LCL were incubated in RF10 supplemented with peptide at concentrations indicated for 2h and then washed three times to remove any free peptide prior to co-incubation with CellTrace™ Violet-labelled T cell clones.
4.3. Results.

4.3.1. Development of an ‘on-cell’ pMHC:TCR affinity assay.

The affinity of the TCR for cognate peptide has typically been assessed through binding in solution or in peptide elution studies and found to be low – with $K_D$ in the range of 1-50µM, and characterised by fast off-rates (Cole et al., 2007). However as TCR engagement is assisted by accessory CD8 molecules, by TCR:pMHC microcluster formation and by cell:cell adhesion contacts that prolong TCR:pMHC interaction time, the true ability of a T cell to recognise its cognate epitope presented by an APC or on a target cell may be distinct from TCR affinity for soluble peptide in isolation. To assess the limit at which each ELA-specific G2 clone, and ELA clone 2D10, were able to recognise ELA26-35 in an HLA-A2-bound context on the surface of APC, we developed an ‘on-cell’ assay peptide presentation assay. Due to the large number of samples required in each experiment, in some assays T cell clones were labelled with CellTrace™ Violet only to mediate detection rather than being co-stained with anti-CD3 or anti-CD8 antibodies (noted in-text). As some proliferation-measuring dyes, notable CFSE, can have cytotoxic effects at high dose, we first investigated whether CellTrace™ Violet staining interfered with detection of T cell activation. We found that when left unstained, or when labelled with maximum and minimum detectable titrated doses of CellTrace™ Violet, neither an NLV-specific clone stimulated by peptide (A; Figure 4-2) or PBMCexp stimulated by Dynabeads® (B; Figure 4-2) showed any deficit in surface expression of CD137, a well-characterised co-stimulatory activation marker known to be expressed on T cells 24-48h post TCR-stimulus (Wolfl et al., 2007), indicating that CellTrace™ Violet staining was appropriate for our investigations.
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4.3.1.1. CellTrace Violet staining does not impair T cell activation or CD137 detection

Figure 4-2 CellTrace™ Violet staining does not impair T cell activation or CD137 detection.
A: NLV 4D9 clonal cells or B: PBMCexp were left unstained or stained with CellTrace™ Violet at low (0.01µM) or high (5µM) concentrations. Cells were then washed and incubated in the presence of A: T2 cells previously loaded with 1µM NLV495-503 peptide or no peptide; or B: Dynabeads® at a 2:1 bead:cell ratio or media alone. Samples were washed and surface CD137 expression determined by flow cytometry (section 2.5.1.1). Data is shown as representative overlay plots of unstimulated (grey) and stimulated (red) cells at each CellTrace™ Violet concentration.

4.3.1.2. Saturating T cell activation is E:T ratio-dependent.

In developing our ‘on-cell’ affinity assay we first wanted to determine an effector:target ratio that would reliably saturate T cell:APC contact and T cell activation, in order to minimise assay variability and ensure that affinity was not under-reported. We utilised the CD8⁺ T cell clone ELA 2D10 (isolated and expanded within the host laboratory) for these experiments, as this clone was capable of recognising the HLA-A2⁺ Melan-A⁺ melanoma cell line SK-Mel-23 (Figure 4-6; Figure 4-7) on coculture and also showed complete activation when exposed to HLA-A2⁺ T2 cells loaded with ELA26-35 peptide at 1µM (A; Figure 4-3). T2 cells are transporter-associated with antigen processing (TAP)-deficient and do not load peptide onto MHC I through the normal endogenous pathway, meaning that they express empty surface HLA-A2 molecules that can be readily peptide-loaded (Luft et al., 2001). Given these results, we incubated ELA 2D10 with T2 and HLA-A2⁺ lymphoblastoid cell line cells (LCL, section 2.3.6) loaded with 1µM ELA26-35 at titrated APC:T cell ratios (C; Figure 4-3). We found that for both APC types, APC:T of ≥2:1 was required to induce saturated T cell clone activation (>95%). As such, this ratio was used in all ‘on-cell’ affinity assays.
Figure 4-3 Optimisation of APC: T cell clone ratio for reliable saturation of T cell activation.

A: T cell clone ELA 2D10 was shown to exhibit saturated CD137 expression at a population level when incubated for 24h with Dynabeads® or with T2 cells loaded with 1µM ELA26-35 peptide. 2D10 TCR is of sufficient pMHC affinity to recognise Melan-A+ SK-Mel-23 cells. Surface CD8 and CD137 were detected by flow cytometry (section 2.5.1.1). Data shown as % cells within the CD8+ gate that express CD137. B: PBMCexp, T2 and LCL were stained for surface HLA-A2 expression (section 2.5.1.1). *p=<0.05, Student’s T-test. C: T2 or LCL were incubated for 2h in Rs5 + 1µM ELA26-35, then washed three times and incubated with CellTrace™ Violet-stained ELA 2D10 at APC:T cell ratios indicated for 24h. CD8+ T cell clone activation was determined as in A.

4.3.1.3. T2 exhibit greater SSP presentation capacity then LCL; CD137 is a more sensitive reporter of T cell activation than IFNγ production.

Having defined an optimal APC:T cell ratio, we next wanted to assess which APC type, and which T cell activation reporter measure would be optimal. T cell activation is commonly quantified by upregulation of surface activation markers such as CD69 or CD137 at defined timepoints, by measurement of degranulation and surface CD107a detection, or by production of effector cytokines such as IFNγ. Each of these has advantages and limitations: not all T cells are constitutively effector-granule+ (Takata and Takiguchi, 2006), detection of intracellular cytokine accumulation is relatively rapid but labour intensive, while detection of CD137 requires 18-24h of T cell:APC interaction (Wehler et al., 2008). Further, some studies have suggested that different effector functions exhibit different TCR-signalling intensity thresholds (Hemmer et al., 1998). To determine the capacity of T2 and LCL to present SSP ELA26-35 to ELA 2D10, and to compare the sensitivity and utility of surface marker upregulation vs intracellular cytokine accumulation we loaded both APC with ELA26-35 over a 7-log10 range, and assessed CD137 up-regulation at 24h and IFNγ production at 4h. Interestingly, we found that although T2 cells express significantly lower levels of surface HLA-A2 than do LCL (*p=<0.05, Student’s T-test, Figure 4-3), T2 were significantly more potent at inducing IFNγ production (A; Figure 4-4) or CD137 upregulation (B; Figure 4-4) than LCL at matched peptide
concentrations (two-way ANOVA and Bonferroni posttest, \(p\)-values noted on figure), presumably because their empty surface HLA-A2 molecules are immediately available for peptide binding, whereas LCL must ingest and cross-present short peptides. We also compared IFN\(\gamma\) induction and CD137 upregulation at matched peptide concentrations within each APC type – for both T2 (C; Figure 4-4) and LCL (D; Figure 4-4) CD137 was found to be significantly more sensitive, especially within the range of 1-100nM, where the largest biological differences were observed (two-way ANOVA and Bonferroni posttest, \(p\)-values noted on figure). Based on these data we selected CD137 expression at 24h as our optimal T cell activation readout, but selected HLA-A2\(^*\) LCL as our APC, in order to more realistically gauge the likely T cell responsiveness to endogenously presented, rather than exogenously saturated, epitopes.

**Figure 4-4 optimisation of APC and reporter for ‘on-cell’ T cell p:MHC affinity assessment.**

T2 or LCL were incubated for 2h in Rs5 + ELA\(_{26,35}\) at concentrations indicated, then washed three times and incubated with CellTrace™ Violet-stained ELA 2D10 at 3:1 for either 6h in the presence of GolgiStop™ (IFN\(\gamma\) readout) or 24h in Rs5 alone (CD137 readout). Samples were stained for surface CD137 (section 2.5.1.1) or intracellular IFN\(\gamma\) (section 2.5.1.5). A: Comparison of IFN\(\gamma\) induction by T2 cells vs LCL at matched [ELA\(_{26,35}\)]. B: Comparison of CD137 induction by T2 cells vs LCL at matched [ELA\(_{26,35}\)]. C: Comparison of CD137 vs IFN\(\gamma\) induction by T2 at matched [ELA\(_{26,35}\)]. D: Comparison of CD137 vs IFN\(\gamma\) induction by LCL at matched [ELA\(_{26,35}\)]. CD137 gating was based on clone 2D10 incubated with APC not loaded with peptide; IFN\(\gamma\) gating was based on the above as well as stimulated cells stained with a fluorophore-matched isotype control. A-D: Data shown as mean +/- SEM of triplicate samples per condition. ***\(p = <0.001\); **\(p = <0.01\); *\(p = <0.05\) two-way ANOVA and Bonferroni post-test.
4.3.2. G2 ELA\textsubscript{26-35}-specific clones exhibit a range of ‘on-cell’ TCR:pMHC affinities.

Having designed and optimised an ‘on-cell’ affinity measurement assay, we investigated the ability of all G2 ELA\textsubscript{26-35}-specific clone stocks (derived from donors U22 and M56) and effector clone ELA\textsubscript{2D10} to recognise LCL loaded with ELA\textsubscript{26-35} over a 7-log\textsubscript{10} range (Figure 4-5). We found that clones exhibited diverse ‘on-cell’ peptide affinities but could be broadly divided into four groups: clones U22\textsubscript{1C10} and 1C8 failed to upregulate CD137 above 10\% of cells even at 10\mu M loading; clones U22\textsubscript{1E10A}, 1G11, 1D9, and 1B11 exhibited saturated activation only at 10\mu M loading, and showed <50\% activation at 10nM; clone U22 1E10A showed saturating activation to 1\mu M loading but showed <50\% activation at 10nM; and clones M56 1C11, U22 1C5, and effector clone ELA\textsubscript{2D10} showed saturated activation at 100nM and >50\% activation at 10nM loading. This allowed us to establish an affinity hierarchy for future experiments.

**Figure 4-5 ELA\textsubscript{26-35}-specific clones exhibit a range of ‘on-cell’ TCR:pMHC affinities.**

HLA-A2\textsuperscript{+} LCL were incubated for 2h in Rs5 + ELA\textsubscript{26-35}at concentrations indicated, then washed three times and incubated with CellTrace\textsuperscript{TM} Violet-stained ELA\textsubscript{26-35}-specific clones as noted in figure. Samples were stained for surface CD8 and CD137 (section 2.5.1.1) and CD137\textsuperscript{+} events expressed as \% of total CD8\textsuperscript{+} CellTrace\textsuperscript{TM} Violet\textsuperscript{+} events. Data shown as mean +/- SEM of duplicate samples per condition per clone.

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4.3.3. Available melanoma lines exhibit varying patterns and intensities of Melan-A protein expression.

Having determined the relative ‘on-cell’ peptide affinities of each G2 clone and clone ELA 2D10, we sought to determine whether a functional TCR threshold existed below which clones would be unable to recognise Melan-A+ melanoma cells, and thus be unable to exert therapeutic function. Interestingly, Schmid et al (2010) have recently proposed that a TCR $K_D$ threshold exists above which T cell effector function cannot be enhanced – similarly, we wanted to investigate whether surface density of ELA26-35 presentation by Melan-A+ cell lines would render T cell clones displaying up to 2-log$_{10}$ differences in ‘on-cell’ affinity functionally equivalent in terms of target cell recognition. In order to carry out this experiment we needed to first characterise HLA-A2+ melanoma cell lines available within the laboratory as being Melan-A+/. We investigated Melan-A expression in melanocytes and all available melanoma cell lines by immunocytochemistry (section 2.5.5 and Figure 4-6) and by intracellular flow cytometry (as Melan-A is primarily contained within intracellular melanosomes) (section 2.5.1.5 and Figure 4-7). In doing so we were able to establish a hierarchy of Melan-A expression: lines Me275 and SK-Mel-23 were Melan-A$^{bright}$ with a homogeneous expression pattern; SK-Mel-29 was Melan-A$^{bright}$ with a heterogeneous expression pattern; DAGI was Melan-A$^{dim}$ with a heterogeneous expression pattern; and lines Na8, Trombelli and MZ2 (HLA-A2+) did not express Melan-A.
Melanoma cell lines exhibit various patterns of Melan-A expression via ICC.

Melanocytes and melanoma lines were cultured in 8-well chamber slides (section 2.5.5) and Melan-A expression assessed in fixed cell by staining with clone M2-C710 + secondary detection antibody, or with secondary alone (SOC). All samples were counterstained with DAPI to allow visualisation of nuclei. Images acquired and processed as described in section 2.5.5. 

- **A-B:** melanocyte control; 
- **C-D:** Me275; 
- **E-F:** Sk-Mel-23; 
- **G-H:** SK-Mel-29; 
- **I-L:** DAGI; 
- **M-N:** Na8; 
- **O-P:** MZ2; 
- **Q-R:** Trombelli.

Figure 4-6 Melanoma cell lines exhibit various patterns of Melan-A expression via ICC.
Figure 4-7 Melanoma cell lines exhibit various patterns of Melan-A expression via ICFC.

Melanocytes and melanoma lines were fixed and permeabilised as described in section 2.5.1.5 and Melan-A expression assessed by staining with clone M2-C710, or with isotype control, after which samples were washed and stained with Alexa488 - secondary detection antibody. Data shown as overlaid histograms demonstrating SOC fluorescence (grey filled) and M2-C710 + Alexa488-secondary fluorescence (black lines).
4.3.4. G2 ELA\textsubscript{26-35 (A27L)}-specific clones are able to recognise naturally presented EAAGIGLTV on Melan-A\textsuperscript{*} melanoma cells lines.

Having established a hierarchy of melanoma cell line Melan-A expression, we assessed the ability of each G2 clone and ELA 2D10 to recognise HLA-A\textsuperscript{2} Melan-A\textsuperscript{hi} (SK-Mel-23), Melan-A\textsuperscript{lo} (DAGI) and Melan-A\textsuperscript{*} (Trombelli) cell lines. Importantly, the pentamer-bound epitope against which these clones were selected is heteroclitic with an A27L substitution designed to maximise TCR affinity, while the originally identified epitope presented by melanoma cells is EAAGIGLTV (Dunbar et al., 1999). Clones were cultured with confluent melanoma cells for 24h and then T cell activation assessed by surface CD137 detection (section 2.5.1.1) as described below (Figure 4-8). In order to contextualise T cell response levels, these data were combined with previously presented affinity data at 10nM and 100nM LCL peptide loading, the range at which affinity differences were most apparent.

Interestingly, all responsive clones (excluding U22 1C10 and 1C8) showed equivalent or greater activation by SK-Mel-23 than by 100nM-loaded LCL, suggesting that SK-Mel-23 presents a high surface density of HLA-A2:EAA\textsubscript{26-35} complexes. Indeed, clones 1G11, 1B11 and 1D9 actually showed greater responses to SK-Mel-23 than to 1µM-loaded LCL (Figure 4-5). Further, while clones typically exhibited equivalent or lower activation by DAGI than by 10nM-loaded LCL, two clones (M56 1C11 and U22 1D9) exhibited unusually strong responses to DAGI, equivalent to or greater than those seen vs 100nM-loaded LCL. This suggests that these two clones may in fact exhibit a higher affinity for the wild-type sequence (EAA) than for the heteroclitic peptide (ELA), although this was not investigated through peptide challenge in this thesis. Finally, as clones with extremely diverse ‘on-cell’ affinities exhibited saturating activation by SK-Mel-23 (U22 1C5 - U22 1D9, exhibiting a 2-log\textsubscript{10} difference in saturated responses to LCL) it is possible that TCR affinity and target recognition may be decoupled in anti-Melan-A cytotoxicity. Further functional analyses, including analysis of melanoma cell killing were carried out in sections 4.3.6 - 4.3.12 following production of G3 clone stocks.
Figure 4-8 ELA\textsubscript{26,35}-specific T cell clones are able to recognise Melan-A\textsuperscript{+} melanoma cell lines.

Melan-A\textsuperscript{+HI} (Sk-Mel-23), Melan-A\textsuperscript{+LO} (DAGI) and Melan-A\textsuperscript{-} (Trombelli) melanoma cells were cultured to confluency in flat-bottom 96wps. 5 x 10\textsuperscript{4} CellTrace\textsuperscript{TM} Violet ELA\textsubscript{26,35}-specific clonal cells were added to wells and incubated for 24h. All cells were then collected and stained for surface CD8 and CD137 (section 2.5.1.1) and CD137\textsuperscript{+} events expressed as % of total CD8\textsuperscript{+} CellTrace\textsuperscript{TM} Violet\textsuperscript{+} events. Data shown as mean +SEM CD137\textsuperscript{+} clonal cells from duplicate samples per condition. 10nM and 100nM T cell activation data was generated as shown in Figure 4-5.
4.3.5. Parallel G3 expansions exhibit differential proliferation and metabolism.

Clonal T cell populations represent a relatively unique opportunity within human immunological research to conduct parallel restimulation and expansion experiments starting with genetically identical precursor cells, in order to investigate the role of particular interventions (in this case common γc cytokine administration) in inducing phenotypic changes. As such we generated, starting from G2 clone stocks, parallel G3 clone stocks expanded in continuous IL-2 alone (2), or IL-7 + IL-21 with withdrawal of IL-2 at d5 (7/21) as described in section 2.3.10 and shown in Figure 4-1. Fold-expansion of these restimulations was tracked at d14 and d22, by which time proliferation had plateaued. We found that IL-2 and IL-7/21 G3 expansions reached equivalent cell numbers at d14 (A; Figure 4-9) but that IL-7/21 expansions did not significantly increase in cell number between D14 and D22 (A; Figure 4-9) while IL-2 expansions did (p=<0.05; paired T-test A; Figure 4-9). We also attempted paired expansions with clone ELA 2D10, but this clone was unable to expand in the absence of IL-2 and showed complete contraction at D22 in the IL-7/21 condition, reinforcing its IL-7Rα-effector status (B; Figure 4-9). Interestingly, we found that although IL-2 and IL7/21 expansions exhibited equivalent cell numbers and densities in culture at D14, IL-7/21 expansions appeared to be metabolically quiescent, showing minimal media acidification, while IL-2 expansions showed extensive media yellowing, typically within a 24-48h timeperiod from media replenishment. (C; Figure 4-9). This was not solely due to continued proliferation, as yellowing continued beyond d22 and cessation of proliferation (based on counting via Trypan Blue exclusion at d28; data not shown), indicating that chronic IL-2 exposure had either induced or maintained a metabolic switch to aerobic glycolysis as typically exhibited by rapidly dividing effector cells (Oestreich et al., 2014). G3 stocks were maintained in culture until d28, at which point phenotypic and functional analyses were carried out.
Figure 4-9 Expansion of G3 ELA26-35-specific clone stocks.

G2 ELA26-35-specific clones generated in chapter 3 were expanded in parallel as described in Figure 4-1, starting with 1x10^6 T cells/expansion. **A**: Fold-expansion was assessed by counting viable cells at D14 and D22. *p* < 0.05, paired T-test comparing mean fold-expansion within each condition at D14 and D22. **B**: IL-7Rα effector clone ELA 2D10 was unable to expand following IL-2 withdrawal. Data shows mean +SEM fold-expansion for duplicate samples per condition. **C**: Parallel IL-2 and IL-7/21 expansions showed differential metabolic activity at D14, despite equivalent T cell number and density.
4.3.6. Parallel G3 expansions exhibit differential retention or loss of G2 early T<sub>CM/SCM</sub> phenotype.

In chapter 3, we demonstrated that polyclonal T cell expansion in IL-7/21 resulted in the retention of co-stimulatory markers, SLO-homing markers, and IL-7Rα, while expansion in IL-2 alone promoted the loss of these surface molecules (Figure 3-1; Figure 3-2). Further, we demonstrated that ELA<sub>26-35</sub>-specific G2 clones expanded in IL-7/21 exhibited a unique T<sub>CM/SCM</sub> phenotype based on expression of CD45RA, CD27, CD28, CD62L and CCR7, despite having undergone up to 26 population doublings.

To expand on these findings, we undertook a more thorough phenotypic and functional characterisation of matched G2, G3 (IL-2) and G3 (IL-7/21) clone sets, to assess which aspects of this unique phenotype were fixed, and which could be modulated by renewed chronic IL-2 exposure.

We first assessed cell surface phenotype by flow cytometric analysis of CD45RA, CD45RO CD27, CD28, CD62L, CD95 and CCR7 on all G2, G3 (IL-2) and G3 (IL-7/21). We saw no significant changes in T<sub>CM/SCM</sub> phenotype between G2 and G3 (IL-7/21) stocks, but found three major patterns of cytokine-mediated differentiation when comparing G3 (IL-2) and G3 (IL-7/21) stocks: firstly no change in CD27 expression was observed; secondly, significant down-regulation (on a population basis) of CCR7, CD28 and CD62L was observed; and finally, a significant increase in CD45RO expression (on a population basis) and a significant upregulation of CD95 (Fas) surface density was observed (B; Figure 4-10). All G2 and G3 stocks were CD95<sup>+</sup>, a hallmark of all antigen-experienced cells. These results are in direct accordance with those seen in section 3.2.1. Although it is possible that continued proliferation played a role in these surface marker alterations, we deemed this unlikely as the G3 (IL-2) stocks typically only underwent one additional population doubling compared to G3 (IL-7/21) stocks. One representative phenotype staining pattern for matched G2, G3 (IL-2) and G3 (IL-7/21) expansions is shown in (A; Figure 4-10). These data indicate that G3 (IL-7/21) clone stocks retain their unique T<sub>CM/SCM</sub> phenotype through >30 population doublings.
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Figure 4-10 G3 clone stocks expanded in IL-2 and IL-7/21 exhibit differential retention of G2 surface marker phenotype.

G2 and d28 G3 stock phenotype was assessed via flow cytometry for surface markers as described in sections 2.5.1.1 - 2.5.1.3. A: one representative G2/G3 (IL-7/21)/G2 (IL-2) clone set of 9 is shown. Marker gates are based on CD8-only stained controls. B: Data represents % of CD8+ events positive for each surface marker within each G2 and G3 population. Data represents 9 individual clone sets. ***p < 0.001 paired T-test.
4.3.7. All G3 clones exhibit polyfunctional cytokine production.

Polyfunctional cytokine secretion, notably the retention of IL-2 production concomitant with IFNγ-production capacity is an important effector function of anti-tumour CTL. To investigate the capacity of G2 and G3 clone stocks to produce IL-2, TNFα and IFNγ, cells were non-specifically stimulated with PMA/I as described in section 2.5.1.6 and cytokines detected via flow cytometry (section 2.5.1.5). PMA/I was chosen as the non-specific stimulus to normalise activation in a set of clones exhibiting diverse TCR:pMHC affinities, and because PMA/I proved superior to Dynabeads® in eliciting triple-cytokine production in PBMCexp (A; Figure 4-11). Interestingly we saw that all clone sets were capable of producing IL-2, TNFα and IFNγ, with notable increase in TNFα and IFNγ production in both IL-2 and IL-7/21 G3 clone sets. This is consistent with progressive acquisition of effector function on repeated stimulation, and also suggests that cytokine production potential and co-stimulatory/SLO-homing marker expression may be decoupled (B; Figure 4-11). Gating strategies for two representative clone sets are shown in (C; Figure 4-11).
Figure 4-11 Polyfunctional cytokine secretion.

A-C: All samples were stained for surface CD8, and then fixed and permeabilised and co-stained for intracellular cytokines as described in section 2.5.1.6 and 2.5.1.5. All data represent cytokine* events within all live CD8* events, gated based on isotype control fluorescence. A: PBMC from two donors were stimulated as indicated and cytokine production assessed. Data represents mean + SEM % cytokine* events. B: ELA26-35-specific G2 (grey triangles) and G3 (white/grey circles) clone pairs, and SLL9 G2 (grey triangles) and G3 stocks (IL-7/21 only, white circles) were stimulated with PMA/I for 4h and intracellular cytokines detected as described. Triple-cytokine* events were calculated by gating on IL-2/TNFα* events within all CD8*/IFNγ* events. C: Gating strategy is shown for two representative ELA26-35-specific G2 and G3 clone sets.
4.3.8. Parallel G3 expansions exhibit differential retention or loss of cytokine receptor subunit expression and homeostatic cytokine responsiveness.

We next assessed the retention of common γc cytokine receptor subunits on G2 and G3 clones via flow cytometry following 48h cytokine withdrawal as described in section 2.4.7. We found that both G2 and G3 (IL-7/21) stocks constitutively expressed CD25 (IL-2Rα), but that CD25 expression was significantly down-regulated on G3 (IL-2) stocks, potentially due to receptor attenuation from chronic IL-2 signalling. We found that G3 (IL-7/21) clones exhibited significantly higher surface density of CD122 (IL-2Rβ), although staining for this marker was extremely dim in all samples. Finally, IL-7Rα expression was retained in all G2 and G3 (IL-7/21) clones, but completely lost in all G3 (IL-2) stocks, suggesting either that chronic IL-2 signalling silences the transcription of IL-7Rα, or that G3 (IL-2) clones fail to transition from an IL-7Rα effector phase to an IL-7Rα+ memory population post-stimulation (**p<0.01, paired T-test; A; Figure 4-12). These patterns of cytokine receptor subunit expression correlated with responsiveness to γc cytokine exposure in vitro. Both G3 (IL-2) and (IL-7/21) clones died in the absence of cytokine, but G3 (IL-7/21) clones exhibited significantly greater survival in IL-4 and IL-7, and while G3 (IL-2) clones could be maintained in IL-2 and IL-15, only G3 (IL-7/21) clones demonstrated proliferation in response to these cytokines. (**p<0.01, paired T-test; B; Figure 4-12)
Figure 4-12 ELA_{26-35}-specific G3 clone stocks expanded in IL-2 and IL-7/21 exhibit differential retention of cytokine receptor subunits and *in vitro* cytokine responsiveness.

**A**: All G2 and G3 clones were washed and incubated in cytokine-free Rs5 for 48h (section 2.4.7). Surface cytokine receptor subunit expression was detected by flow cytometry (section 2.5.1.1). Data shown as MFI for each of 9 matched G2 (grey triangle), G3 IL-7/21 (white circle) and G3 IL-2 (grey circle) clones. MFI determined by subtraction of matched unstained MFI for each sample. **p =< 0.01, paired T-test.**

**B**: All G2 and G3 clones were washed and incubated in Rs5 alone or supplemented with cytokine as indicated (10ng/ml, Rs5 and media exchange within 96h) for 12d. At d12 viable cells were enumerated by trypan blue exclusion and expressed as % of initially seeded cell number.
4.3.9. G3 clones do not exhibit exhaustion marker expression.

Chronically stimulated, or highly-differentiated CD8+ T cells exposed to high antigenic burden or repeated strong TCR signalling may become ‘exhausted’ and lose effector and proliferative function, a common fate of T cells during chronic viral infection, or within an immunosuppressive tumour microenvironment. This state is typically characterised by the expression of inhibitory surface markers such as PD-1, KLRG1 and CD57, which act to antagonise TCR-mediated signalling and potentially to induce cell death. We assessed all G2 and G3 clones for expression of PD-1 (A-B; Figure 4-13) and KLRG1 (D; Figure 4-13) but found no evidence of expression. PD-1 is known to be induced following TCR-stimulation in an NFAT-1 dependent manner (Lu et al., 2014b; Oestreich et al., 2008). Interestingly, when we stimulated G3 (IL-7/21) clonal cells with Dynabeads® for 24h we saw only minor, dim up-regulation of PD-1 (C; Figure 4-13) potentially suggesting that continued IL-7/21 signalling represses PD-1 expression.

Figure 4-13 ELA26-35-specific clones do not express the exhaustion markers KLRG1 or PD-1.

A; B; D: Pan T cells, ELA clone 2D10 and six ELA26-35-specific G2/G3 clone sets were stained for surface expression of CD3 alone or CD3, PD-1 and KLRG-1 and analysed via flow cytometry (section 2.5.1.1). PD-1 and KLRG-1 gates were drawn based on positive cells observed within the Pan-T and ELA-2D10 samples. A: Data shown as % PD-1+ cells within all CD3+ events for each G2 and G3 clone sample. B: gating strategy for PD-1 detection – top panel; CD3-only (grey filled) and CD3/PD-1 (black line) samples are shown for T cells, ELA 2D10 and one G2 clone (U22 1C5). Bottom panel – CD3/PD-1 co-expression and gating. C: Six G2 clones were left unstimulated or stimulated with Dynabeads® for 24h and then stained for surface CD8 and PD-1 expression. Data shows one representative clone unstimulated (grey) or stimulated (black) compared to a constitutively PD-1+ T cell clone (red). D: Gating strategy for KLRG1-detection. Data shown as histogram overlays for CD3 only (grey) and CD3/KLRG1 (black) samples showing KLRG1 fluorescence within all CD3+ events. Total T cells and three representative G3 IL-7/21 clones from two donors are shown.
4.3.10. G3 expansion potential and phenotype are modulated by stimulus and by common γc cytokine exposure

Data collected in sections 4.3.6 - 4.3.9 indicated that post-expansion, G2 clones re-stimulated in the presence of IL-2 or IL-7/21 exhibited differential surface marker phenotypes, apparent memory/effector states, and differential responsiveness to homeostatic cytokines. However, these data give only before and after snapshots of clone phenotype and function. As such, we asked whether the modulation of markers that appear to be cytokine-responsive (CCR7, CD62L and CD28) was dynamic over the course of an expansion, and whether the nature of the stimulus or the cytokine support provided played a greater role. One representative G2 clone (U22 1C5) was split into equivalent samples and stimulated with either Dynabeads® or PHA/LCL as described previously in text. Within each stimulation condition, one sample was expanded using IL-2 only conditions, and the other using IL-7/21 conditions as previously described. Expansions were sampled at d10, d15, d20, d27. At each timepoint fold-expansion was determined by trypan blue exclusion, while surface expression of CD8, CD28, CD62L and CCR7 was assessed via flow cytometry. We found that within each stimulation condition, clones expanded in IL-2-only exhibited greater fold-expansion than those treated with IL-7/21, although within each stimulation condition cell expansion at d10 was equivalent and divergence in cell number typically emerged from d15 onwards. Interestingly, PHA/LCL stimulation elicited greater expansion than Dynabeads® - this was cytokine-independent, as PHA + IL-7/21 elicited greater clone expansion than Dynabeads® + IL-2, even at d27. When assessing surface marker expression we found that in all samples CCR7 expression was lost at d10, and progressively re-appeared at a population level over the course of the expansion (top left; Figure 4-14). In both IL-7/21 samples CCR7 return occurred at a greater rate than in IL-2 samples. By contrast, all samples were >80% CD28+ at d10, and CD28 was retained at this level in both IL-7/21 expansions over 27d, while being progressively lost in both IL-2 expansions. CD28 loss was linked to chronic IL-2 exposure rather than continued cell division, as rates of loss were equivalent in both PHA and Dynabead® expansions, which proliferated at vastly different rates, and CD28 was retained in the PHA IL+7/21 expansion, which exhibited greater proliferation than the Dynabead/IL-2 sample (top right; Figure 4-14). CD62L exhibited a similar pattern to CD28, with constant expression in both IL-7/21 conditions, and progressive loss in both IL-2 conditions (bottom left; Figure 4-14).
ELA26-35-specific G2 clone U22 1C5 was stimulated with either Dynabeads® or PHA/LCL as described in sections 2.4.6 and 2.3.10, respectively, and expanded in IL-2 alone or IL-7 and IL-21 within each stimulation condition, as shown in Figure 4-1. At D10, D15, D20 and D27 samples of each expansion were collected and counted by trypan blue exclusion to allow tracking of fold-expansion (bottom right). Samples were also stained at these days for surface CD8 alone, or CD8, CD28, CCR7 and CD62L, and surface expression assessed by flow cytometry. Data shown as % of CD8+ events that express surface marker as indicated. Gating was based on fluorescence in paired CD8-only stained samples. Data representative of two independent experiments.

One particularly important question we sought to answer was whether G2 stocks expanded in IL-7/21 ever truly entered an effector phase. T cell activation and effector transition is accompanied by transient loss of surface CD3 and upregulation of co-stimulatory molecules such as CD137. T cell activation and effector transition is also characterised by ADAM17 (MMP)-mediated shedding of CD62L (Yang et al., 2011; Zhao et al., 2001). In order to assess whether IL-7/21 expanded cells ever entered this phase, this experiment was repeated with analysis of surface markers at d1, d3, d7 and d10, to inform the early events not addressed in Figure 4-14.

In all samples, cytokine treatment played no differentiating role between d0 and d10, probably because IL-2 was added in both samples, and produced by the activated clonal cells. As such, only stimulation conditions are discussed. Both PHA and Dynabeads® induce loss of surface CD3 and expression of surface CD137, indicating that T cells have been appropriately stimulated (A; Figure 4-15). At 24h, Dynabeads® mediated only a small decrease in CD27+ %, while PHA induced loss of CD27 on the majority of cells. Interestingly, by 72h this relationship was reversed, with CD27...
returning to near-unstimulated levels, while in Dynabeads® samples CD27 expression and surface density (MFI) continued to decrease (B; Figure 4-15). This may be one factor in PHA promoting greater proliferation, as the high levels of surface CD27 will be available for CD70-mediated costimulatory signalling from both the LCL and activated T cells. The reverse was observed for CD28 - expression was almost entirely lost in Dynabeads® samples (potentially due to direct ligation by anti-CD28) while minimal modulation was observed on PHA samples (C; Figure 4-15) By d3 CD28 returned to normal levels on all samples at a population level, but surface density remained lower on Dynabeads® samples (C; Figure 4-15). CCR7 was rapidly lost from both bead samples at 24h, but retained on PHA samples. In all samples, CCR7 expression at a population level progressively declined until dropping to zero at d10 (as seen in Figure 4-14) (D; Figure 4-15). Finally, all samples did enter a CD62L effector phase, with no surface CD62L apparent at d1 and d3 (F; Figure 4-15). Interestingly, by d7 CD62L returned to normal levels, concurrent with surface CCR7 loss and co-incident with rapid cell division.

Taken together these data suggest that all samples do enter an early effector phase between d0 and d7, but that subsequent cytokine exposure, rather than proliferation level or method of stimulation, is the determining factor in surface marker expression.
Figure 4-15 G3 clones show rapid modulation of surface markers on stimulation.

ELA26-35 specific G2 clone U22 1C5 was stimulated with either Dynabeads® or PHA/LCL as describe in sections 2.4.6 and 2.3.10, respectively, and treated in IL-2 alone or IL-7 and IL-21 within each stimulation condition, as shown in Figure 4-1.

A-F: Samples were stained at these day indicated for surface markers as described in sections 2.5.1.1 (CD3, CD8, CD27, CD28, CD62L and CD137) and as described in 2.5.1.3 for CCR7. In all samples MFI was determined by subtraction of paired CD8-only MFI in relevant channels, and individual marker gating was based on fluorescence in paired CD8-only stained samples. All plots show mean + SEM MFI and/or % marker+ events within all live CD8+ events for triplicate samples.

A: CD3 and CD137 MFI were determined in unstimulated G2 clone samples and in samples stimulated for 24h as indicated.

B: CD27 %+ and MFI were determined at 24 and 72h, and 72h only (MFI) respectively for each stimulation condition.

C: CD28 %+ and MFI were determined at 24 and 72h, and 72h only (MFI) respectively for each stimulation condition.

D-F: Samples were analysed at d0, d1, d3, d7 and d10 and %+ CCR7 (D), CD28 (E) and CD62L (F) determined as described.

4.3.11. Parallel G3 expansions exhibit differential proliferative capacity on exposure to Melan-A+ melanoma cells.

Differential proliferative capacity on antigen re-encounter is a defining characteristic of T cells at different stages of differentiation, and intratumoural proliferation and effector formation by memory cells is emerging as an important effector function and prognostic indicator (Nakano et al., 2001). To assess if G3 (IL-7/21) and (IL-2) clones exhibited different proliferative potential we cultured them for 48h in the presence of HLA-A2+ Melan-A+ (SK-Mel-23) and Melan-A+ (Trombelli) tumour lines, or in the presence of Dynabeads®. Proliferation was assessed through CellTrace™ Violet dilution (A; Figure 4-16). We found that no antigen-independent proliferation occurred, and G3 (IL-7/21)
clones showed significantly greater division than matched G3 (IL-2) clones in response to SK-Mel-23 (\(**p<0.01\), paired T-test) (B; Figure 4-16). G3 (IL-7/21) clones also showed higher proliferation in response to Dynabeads\textregistered, although this may reflect relative CD28 expression (Figure 4-10) and co-stimulatory signalling supply rather than a general proliferation defect (B; Figure 4-16).

Figure 4-16 G3 clone stocks expanded in IL-2 and IL-7/21 exhibit differential proliferation on co-culture with Melan-A\textsuperscript{+} melanoma cells.

G3 T cell clone pairs were stained with CellTrace\textsuperscript{TM} Violet for proliferation as described in 2.4.3. Clone samples were plated in flat-bottom 96wp-wells alone or in the presence of Dynabeads\textregistered human T-expander CD3/28 at 1:1, or plated into wells containing 1 x 10\(^4\) Tubellini (Melan-A\textsuperscript{-}) or SK-Mel-23 (Melan-A\textsuperscript{+}) cells. All samples were stained for surface CD8, and CellTrace Violet fluorescence within all CD8\(^+\) events determined. A: Data shown as histograms demonstrating G3 IL-2 (grey) or G3 IL-7/21 (black) division peaks in each condition. One representative clone pair of three is shown. B: Divided T cell gating was determined based on CellTrace\textsuperscript{TM} Violet fluorescence in non-divided ‘clone only’ samples. Data shown as mean +/- SEM % divided T cells in each condition for duplicate samples of three clone pairs. \(**p<0.01\), paired T-test for each stimulation condition.

Data collected in sections 4.3.2 - 4.3.4 demonstrated that despite having relatively low TCR:pMHC affinity, G2 ELA<sub>26-35</sub>-specific cells were able to recognise both Melan-A<sup>bright</sup> and Melan-A<sup[dim] dim</sup> melanoma cells. We sought to extend these observations by assessing the cytotoxic capacity of G3 clones in comparison to an effector clone (2D10) using HLA-A2<sup*</sup>Melan-A<sup+</sup> SK-Mel-23 as a target. Initial flow-cytometric cytotoxicity measurements (section 2.4.8) indicated that in comparison to 2D10, all G3 clones exhibited little cytotoxicity within 4-6h, but when incubated overnight were equivalently capable of clearing killing a confluent well of SK-Mel-23, especially at E:T of 1:1 and above (Figure 4-17). Visual analysis indicated that G3 clones were able to rapidly perturb SK-Mel-23 cell morphology, inducing rounding and detachment at 4h in the same way as 2D10 (middle panels; Figure 4-18) followed by clearance of all adherent cells by 18h (right panels; Figure 4-18), but that this did not translate into measurable cell death at 4-6h.

Figure 4-17 G3-clone mediated cytotoxicity is time-dependent.

Cytotoxicity assays were carried out as described in section 2.4.8 at 4h, 6h and 18h timepoints. At each timepoint, ELA<sub>26-35</sub> effector (2D10), G3 (IL-2) and G3 (IL-7/21) clonal cells were incubated with Melan-A<sup+</sup> SK-Mel-23 cells in flat-bottom 96wp at titrated E:T ratios from 0.5:1 to 4:1. SK-Mel-23 cells in the absence of clone, and a non-specific clone (M56 NLV 1G6A) were included as negative controls in each assay. Data represents duplicate samples per condition per clone. One representative G3 clone pair (U22 1E10A) of three tested is shown.
Figure 4-18 G3 clones induce altered SK-Mel-23 morphology within 4h.

SK-Mel-23 cell morphology was imaged as described in section 2.4.2 at 4h and 18h, prior to well harvesting and cytotoxicity quantification. Cells incubated with ELA 2D10 and U22 1D9 (G3) exhibit similar rounded morphology at 4h, distinct from their normal elongated/spindly appearance in the absence of T cells ("no clone"). Cells incubated with ELA 2D10 and U22 1D9 exhibit T cell clusters and SK-Mel-23 plate clearance at 18h, consistent with high levels of melanoma cell death. Images acquired at 100x magnification (section 2.4.2). Scale bars represent 100µm.
As the G3 clones used in these assays (1E10A, 1D9, 1G11) exhibited lower TCR affinity than 2D10 (A; Figure 4-19), we asked whether this delayed cytotoxicity was an affinity-mediated defect. To answer this question we repeated killing assays using two further ELA_{26-35}-specific clones grown in IL-7 but derived from a different donor (U05), that both exhibited 10-fold higher ‘on-cell’ TCR:pMHC affinity than 2D10 (B; Figure 4-19). These clones were produced and provided by Dr Anna Brooks. Interestingly, these high-affinity clones exhibited the same pattern of poor comparative early killing, but equivalent comparative late killing to clone 2D10 (C; Figure 4-19), indicating that delayed cytotoxicity was not TCR:pMHC affinity-related, but rather reflected cell-intrinsic regulation of cytolytic capacity.

**Figure 4-19** Delayed G3 clone cytotoxicity is TCR:pMHC-affinity-independent.

A: G3 ELA_{26-35} clones 1D9, 1E10A and 1G11 exhibit lower TCR:pMHC affinity than clone 2D10. B: IL-7-generated ELA_{26-35}-specific clones derived from donor U05 exhibit >10-fold higher TCR:pMHC affinity than clone 2D10. A; B: T cell clones incubated with M56-LCL loaded with titrated [ELA_{26-35}] cell clone activation data generated as described in Figure 4-5 and section 2.4.8. Data shown as mean +/- SEM of duplicate samples. C: Cytotoxicity assays were carried out at 4h and 18h as described in Figure 4-17 and section 2.4.8, comparing U05 C5 and U05 C8 to ELA 2D10.
CD8+ T cell mediated toxicity is typically mediated by formation of an ‘immune synapse’ between T cell and target, polarised release within this synapse of effector granules containing perforin, a pore-forming protein that permeabilises the target cell membrane and facilitates entry of granzyme B, a serine protease that induces caspase-mediated apoptosis. To assess whether G3 clones contained effector granules needed to induce apoptosis, all G3 clone pairs, U05 C5 and U05 C8 and ELA 2D10 were incubated in the presence of GolgiStop™ and left unstimulated, or stimulated with PMA/I for 4h, after which intracellular perforin and granzyme content was assessed by flow cytometry (section 2.5.1.5). As GolgiStop™ only prevents the trafficking and release of de novo synthesised proteins, any pre-formed effector granules will still be discharged on T cell stimulation in this system. We found that all G3 clones, and IL-7-generated clones derived from donor U05, contained equivalent pre-formed perforin (A, unstimulated; Figure 4-20) to effector clone 2D10, and were capable of releasing this on stimulation (A, PMA/I; Figure 4-20). By contrast we found that G3 clones and clones derived from donor U05 contained negligible pre-formed granzyme B (B, unstimulated; Figure 4-20), but accumulated high levels of de novo-synthesised granzyme B 4h after stimulation (B, PMA/I; Figure 4-20).

**Figure 4-20** ELA26.35-specific G3 clones contain pre-formed perforin, but negligible pre-formed granzyme B.

ELA26.35-specific G3 clones (IL-2 and IL-7/21), U05 clones and 2D10 (‘effector’) were incubated in Rs5 supplemented with GolgiStop™ for 1h, and then left unstimulated, or were stimulated with PMA/Ionomycin for 4h as indicated. All samples were stained for surface CD8, and then fixed and permeabilised using BD Fix/perm, and stained for A: intracellular perforin and B: intracellular granzyme B as described in section 2.5.1.5. Data are shown as MFI for 9 paired G3 clones, two U05 clones and 2D10. MFI was determined by subtraction of paired isotype control-stained samples for each clone.
Chapter 4 – Results II

To better define the kinetics of granzyme B production, we again stimulated the three clone pairs used in initial killing assays and clone 2D10 in the presence of GolgiStop™, and assessed intracellular granzyme B pre-stimulation, and 30, 60, 120 and 180 minutes post-stimulation (Figure 4-21). We confirmed that G3 clones did not contain pre-formed granzyme B, and found that granzyme B became detectable only 2h after stimulation, with further accumulation observed 3h post-stimulation (Figure 4-21). These data suggest that on initial SK-Mel-23 encounter and degranulation G3 clones were only capable of releasing perforin, while granzyme B-mediated killing only occurred on subsequent encounters beyond 4h, after de novo synthesis of granzyme B (and additional perforin). This confirms visual observations whereby G3 clones were able to rapidly alter the visible morphology of SK-Mel-23 cells, as perforin release damages target cell membranes, and melanoma cells have been shown to rapidly undergo membrane internalisation and repair on perforin-mediated injury to prevent necrosis (Keefe et al.). Typically this membrane internalisation also facilitates granzyme B uptake, resulting in apoptosis, but as G3 clones have no initial granzyme B to deliver, SK-Mel-23 cells are able to repair their membrane and exclude DAPI at early timepoints. Following granzyme B upregulation after initial SK-Mel-23 encounter, G3 clones become equivalently cytotoxic to effector clone 2D10.

![Granzyme B upregulation](Figure 4-21)

Figure 4-21 ELA\textsubscript{26.35}-specific G3 clones upregulate granzyme B following stimulation.

G3 clones (IL-2 and IL-7/21) and ELA 2D10 (‘effector’) were incubated in Rs5 supplemented with GolgiStop™ for 1h, and then left unstimulated, or were stimulated with PMA/Ionomycin for up to 3h. All samples were stained for surface CD8, and then fixed and permeabilised using BD Fix/perm, and stained for intracellular granzyme B as described in section 2.5.1.5 at timepoints indicated. Data are shown as MFI for 3 paired G3 clones and ELA 2D10. Granzyme B MFI was determined by subtraction of paired isotype control-stained samples for each clone.
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4.4. Chapter summary

In this chapter we developed and validated an assay for assessing the ‘on-cell’ TCR:pMHC affinity of CD8+ T cell clones based on titrated short peptide loading of profession APC. We found that the suite of ELA_{26-35}-specific clones generated in chapter 3 exhibited a range of TCR affinities in this system, with saturating responses ranging over \(2\log_{10}\), and typically being in the high nM range. Nonetheless, we subsequently demonstrated that all CD8+ clones able to recognise \(\leq 10\mu\text{M} \) ELA peptide presented by APC were also able to recognise both Melan-A^+ (HI) and Melan-A^+ (LO) melanoma cells in an HLA-A2-restricted context.

To further investigate the apparent T_CM/T_SCM phenotype exhibited by G2 ELA_{26-35}-specific clones and to characterise the contribution of IL-7 + IL-21 exposure to this phenotype we re-expanded G2 stocks under IL-7 + IL-21 or IL-2 only conditions, to create paired G3 stocks for each clone. A more comprehensive phenotype analysis showed that G3 (7/21) retained the same CD45RA+CD45RO+CCR7+CD62L+CD27+CD28+ phenotype as their G2 precursors, but CCR7, CD62L, CD28 and CD45RA expression was lost on G3 (IL-2) clones, while CD45RO expression was increased. Further, we found that all clones expressed CD95, marking them as ‘antigen-experienced’ but that CD95 surface expression was increased on G3 (IL-2) clones. G2 and G3 (IL-7/21) expressed surface CD25, CD122 and IL-7Rα at significantly higher levels than G3 (IL-2) clones, and correspondingly only G2 and G3 (IL-2) clones could be maintained in IL-7 or proliferate in response to IL-15. Demonstration of a CD45RA+CD45RO+CCR7+CD62L+CD27+CD28+CD95+CD122+IL-7Rα+ phenotype, coupled with their ability to maintain this phenotype through serial expansions and \(\geq 30\) population doublings demonstrated that these G3 clones were indeed T_SCM, and also showed that retention of these markers was due to experiencing only a short IL-2 exposure with maintenance in IL-7 + IL-21. We investigated the modulation of G2-G3 transitional phenotype over time and found that all cells entered a true effector phase and exhibited a similar magnitude of cell division, but only cells in the presence of IL-7 + IL-21 and the absence of chronic IL-2 were able to ‘rest’ as a memory population. This suggested that phenotypic differentiation and proliferation were decoupled in these clones and that cytokine exposure and signalling was the key determinant of phenotype.

Further functional characterisation revealed that all G2 and G3 clones tested (ELA_{26-35} and SLL_{157-165} specific) were capable of polyfunctional cytokine production and did not express the exhaustion marker KLRG1 or the checkpoint inhibitor PD-1. We found that G3 (IL-7/21) clones displayed superior ability to proliferate on co-culture with Melan-A+ melanoma cells when compared with paired G3 (IL-2) clones. Finally, we found that all G3 clones exhibited delayed, but effective cytotoxic capacity. This was mediated by a lack of pre-formed granzyme B, but all clones were able to upregulate and discharge granzyme B following TCR:pMHC activation. The wider significance of these results is discussed in detail in chapter 7.
Chapter 5.  

T cell activation marker expression

5.1. Introduction.

T cell activation following TCR ligation is associated with temporal up- and down-regulation of surface markers important in T cell co-stimulation for survival and proliferation, in nutrient acquisition, and cell adhesion and trafficking. The availability of these markers on the cell surface makes them amenable to detection by flow cytometry if their temporal regulation can be predicted.

Work in this chapter aimed to characterise the CD3-dependent induction of candidate surface proteins (Table 5-1) on activated T cells in vitro. This was done to define reliable CD4+ and CD8+ activation markers that could be used to isolate antigen-specific T cells in a pentamer-independent system.

In order to define the conditions needed for in vitro presentation of synthetic peptides to T cells, a method for expression of recombinant NY-ESO-1, a well-characterised and immunogenic cancer-testis antigen, in Sf9 cells was developed. This model antigen was used to order to compare the kinetics of processing and presentation of synthetic long and short peptides to full length protein.

The final aim of this chapter was to develop a protocol for the MoDC-independent expansion of memory T cells from PBMC to establish a population from which antigen-specific cells could be isolated. As such, the overall goal of this chapter was to establish the technical components of a system whereby antigen-specific T cells could be expanded using SLP and then isolated via activation-marker guided FACS on SLP recall. The application of this protocol was also further explored in chapter 6.
5.2. Methods.

5.2.1. Cells.

PBMC and PBMCexp were cultured in sterile Rs5, as described in sections 2.3.1 and 2.3.2. Rs5 was supplemented with cytokines as described in text. During expansion and maintenance, Rs5 and supplementary cytokines were replenished as described in text. LCL were generated as described in section 2.3.6 and maintained in RF10. T2 cells were maintained in RF10. Sf9 cells were maintained in SF900-III supplemented with 1% v/v FBS.

5.2.2. Activation of T cells by CD3/CD28 cross-linking.

PBMCexp were used to investigate T cell activation. Cells were plated in 96wp – U-bottom wells and coated with LEAF-OKT3 and/or LEAF-CD28.2 at concentrations indicted in text for 10 min at 37°C. Unbound antibody was removed by washing, and goat-anti-mouse-IgG (Biolegend) applied at 40µg/ml in 100µl Rs5 to cross-link bound antibodies. Prior to staining, cells were washed to remove any unbound cross-linker, and briefly incubated with mouse IgG to saturate prevent any non-specific binding. In all experiments, expression of CD137 within the CD8+ T cell fraction was assessed via flow cytometry to determine T cell activation.

5.2.3. Activation of T cells by plate-bound antibodies.

PBMCexp were used to investigate T cell activation. 96wp-flat bottom wells were coated at concentrations indicated with LEAF-OKT3 and/or LEAF-CD28.2 diluted in PBS overnight at 4°C. Wells were then washed and blocked with Rs5 prior to T cell addition. In certain experiments, CD28.2 was included in T cell culture medium to provide in trans signalling. In all experiments, expression of CD137 within the CD8+ T cell fraction was assessed via flow cytometry to determine T cell activation.

5.2.4. Flow cytometry for surface markers and intracellular cytokines.

Surface expression of CD3, CD4, CD8, CD25, CD38, CD56, CD69, CD71, CD134, CD137, CD154, ICOS and HLA-DR was analysed via flow cytometry as described in section 2.5.1.1 and intracellular flow cytometry for IFNγ was carried out as described in section 2.5.1.5 using antibodies and doses listed in Table 2-5. Live/Dead discrimination was carried out using DAPI or Live/Dead™ fixable dyes (section 2.5.2).
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5.2.5. Peptide and protein processing and presentation assays.

In all assays CD8+ T cell clone activation was assessed by detecting surface CD137 expression on clonal T cell labelled with 0.1µM CellTrace™ Violet. T cell activation was expressed as % CD137+ events within all CellTrace™ Violet+ events. All peptide loading of APC or PBMCexp was carried out in Rs5 for periods indicated in-text, and prior to T cell:APC coincubation, all peptide-loaded cells were washed three times to remove any remaining free peptide.

5.2.6. Production of rNY-ESO-1-His6.

NY-ESO-1 cloning and pFastBacHTB vector construction were carried out as described in sections 2.2.7 - 2.2.17. Recombinant baculovirus was produced as described in sections 2.2.18 - 2.2.19.1 with the assistance of Dr James Dickson. rNY-ESO-1-His6 was purified as described in section 2.2.19.2. Optimisation steps are described in-text.
5.3. Results.

5.3.1. T cell activation is reliably induced by Dynabeads® Human T-Expander CD3/CD28 beads.

As we aimed to profile the timecourse of TCR-dependent induction of cell surface activation markers, we first sought to determine the optimal means of stimulating T cells in vitro to reliably induce saturating TCR/CD3-mediated activation. Antigen-independent in vitro T cell activation has been assessed through the use of many stimuli, including lectins such as PHA and concanavalin A (Medina et al., 1994; O'Flynn et al., 1985; O'Flynn et al., 1986), superantigens (SAg) such as Staphylococcal Enterotoxins A and B (SEA/B) (Herrmann et al., 1989; McLeod et al., 1998), and agonistic antibodies directed against CD3 and co-stimulatory CD2, CD27 and CD28 molecules (Kalamasz et al., 2004; Li and Kurlander, 2010). As we aimed to specifically investigate TCR/CD3-mediated T cell activation at a global level in pure T cell cultures, we excluded lectins (which act through cross-linking and capping of multiple glycosylated surface moieties, potentially introducing confounding TCR-independent signalling) and SEA/B (which act through cross-linking TCR and MHCII on only a subset of T cells) from these analyses (Kappler et al., 1989; Leca et al., 1986). Instead, we compared the delivery of agonistic CD3 and CD28 signals through soluble antibody cross-linking, through plate-bound antibodies, and through T cell: Dynabead® interaction. In all experiments described through sections 5.3.1.1 - 5.3.1.2 PBMCexp libraries (pure T cells) generated as described in sections 2.4.6 and 3.2.2 were used, and the well-established T cell activation marker CD137, known to be expressed on T cells 24-48h post TCR-stimulus (Wolfl et al., 2007) was used as a reporter for T cell activation. In all experiments, antibody clone OKT3 was used to bind CD3, and antibody clone CD28.2 was used to bind CD28, as these are the antibody clones conjugated to Dynabeads®.

5.3.1.1. Dynabeads® Human T-Expander CD3/28 beads elicit superior T cell activation than CD3–CD28 cross-linking.

We first compared T cell activation by incubation with Dynabeads® at a 1:1 bead/cell ratio, with activation by cross-linking of soluble anti-CD3 and anti-CD28 (section 5.2.2). Soluble anti-CD3 and anti-CD28 were initially used at a 2:1 ratio, at titrated concentrations from 0.625 - 20µg/ml OKT3 (A; Figure 5-1). We found that although T cell activation and surface density of CD137 was increased in a titratable manner on cross-linking, only a subset of PBMCexp ever became active (~15-40%), and at all concentrations tested Dynabeads® induced significantly greater PBMCexp activation at a population level and significantly greater CD137 surface density (*p<0.05; A; Figure 5-1). To assess whether surface CD3 or CD28 binding was insufficient at concentrations tested, we incubated
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PBMCexp with titrated doses of Alexa Fluor®-488-labelled OKT3 and CD28.2. We found that surface binding of CD3 was saturated at only 0.625µg/ml OKT3 (C; Figure 5-1) but that ≥ 10µg/ml CD28.2 was required to saturate surface CD28 (D; Figure 5-1). As such we repeated cross-linking using OKT3 and CD28.2 at 1:1 and 2:1 concentration ratios, ranging from 10 - 40µg/ml CD28.2, and compared T cell activation and surface CD137 density to 1:1 PBMCexp: Dynabeads®. We found that PBMCexp activation again increased in a titratable manner based on OKT3 concentration, and that within each OKT3 concentration condition, significantly higher T cell activation and CD137 surface density was induced at a 1:1 OKT3:CD28.2 ratio when compared to 2:1 (*p= <0.05; B; Figure 5-1), consistent with the idea that CD28 co-stimulation lowers T cell activation threshold in the presence of sub-optimal TCR/CD3 stimulation (Hemmer et al., 1998; Zhang et al., 2002). Nonetheless we again observed that only a subset of PBMCexp became activated through cross-linking (~15-70%) and that PBMCexp activation by Dynabeads® was significantly greater at a population level than by any cross-linking condition (*p= <0.05; **p= <0.01; B; Figure 5-1). Based on these data, and on the extremely high concentration of soluble OKT3 and CD28.2 needed to induce notable PBMCexp activation, we excluded cross-linking as a viable means of T cell activation in future assays.
Figure 5-1 Dynabeads® Human T-Expander CD3/28 beads elicit superior T cell activation than CD3–CD28 cross-linking.

A; B: Purified T cells were activated by cross-linking of soluble OKT3 and CD28.2 as described in section 5.2.2, following incubation at concentrations indicated, or were incubated with Dynabeads® at 1:1. All samples incubated for 24h and surface CD3 and CD137 detected via flow cytometry (section 2.5.1.1). Data shown as mean + SEM of mean CD137+% within all CD3+ events; and as mean + SEM CD137 MFI within the CD137+ population, normalised to % of maximum MFI within each donor. Data is representative of two donors with all samples assessed in triplicate. **p =< 0.01; *p =< 0.05 two-way ANOVA and Bonferroni post-test. T cells were incubated with C: OKT3 – Alexa488 or D: CD28.2 – Alexa488 at concentrations indicated. Data shown as MFI of singlicate samples, representative of two independent experiments.
5.3.1.2. Dynabeads® Human T-Expander CD3/28 elicit superior T cell activation than plate-bound OKT3 and CD28.2 antibodies.

We next compared T cell activation by plate-bound OKT3 alone, OKT3 and CD28.2, and plate-bound OKT3 supported by soluble CD28.2 against titrated PBMCexp:Dynabead® exposure (Figure 5-2). Plate bound-antibodies effectively ‘cross-link’ CD3 without application of a cross-linking antibody, and allow the formation of a polarised pseudo-synapse between cell and plate floor. We found that induction of CD137 in this system was CD28-independent, as plate-bound CD28.2 alone did not induce any T cell activation, while no difference was observed in concentration-matched OKT3 alone or OKT3 plus plate bound or soluble CD28.2 samples, in terms of either PBMCexp activation at a population level, or CD137 surface density on activated cells (Figure 5-2). These findings differ from the enhancement in CD137 expression observed through CD28 co-stimulation when using a soluble cross-linker (B; Figure 5-1), and are consistent with plate-bound OKT3 being a more potent CD3 stimulus than soluble OKT3. We found that PBMCexp activation was OKT3 concentration dependent (**p<0.01; Figure 5-2), but that only at the highest concentration assessed (10µg/ml) was complete PBMCexp activation reliably induced. By contrast, Incubation with Dynabeads® led to saturated PBMCexp activation at 0.5:1 and 1:1, and the surface density of CD137 induced by Dynabeads® at 1:1 was significantly greater than that observed with in any plate-bound condition (*p<0.05; Figure 5-2). Based on these data, we used Dynabeads® at a 1:1 bead:cell ratio to assess T cell activation marker expression in all future experiments.
Figure 5-2 Dynabeads® Human T-Expander CD3/28 elicit superior T cell activation than plate-bound OKT3 and CD28.2 antibodies.

T cells were incubated for 24h in Rs5 only or in Rs5 containing soluble CD28.2 (annotated as ‘/CD28.2’) in plate wells coated with antibodies OKT3 and/or CD28.2 (annotated as ‘+CD28.2’) at concentrations indicated, or in wells containing Dynabeads® at bead:cell ratios indicated. Plates were coated as described in section 5.2.3. All samples were incubated for 24h and then surface CD3 and CD137 detected via flow cytometry (section 2.5.1.1). Data shown as mean + SEM CD137+ T cells in triplicate samples from one representative experiment. ***p = <0.001; **p = <0.01; *p = <0.05 two-way ANOVA and Bonferroni post-test.

5.3.1.3. Dynabeads® Human T-Expander CD3/28 beads do not interfere with flow cytometric assessment of T cell surface markers.

As Dynabeads® are saturated by OKT3 and CD28.2 at a proprietary ratio, they are unable to bind further antibody (confirmed in Figure 5-3), and it is not necessary to remove these from samples prior to Fluorophore-conjugated antibody staining and flow cytometric analysis. Dynabeads® also exhibit distinct SSC and FSC characteristics from T cells, and unbound beads can easily be excluded from flow cytometric analysis based on their SSC/FSC profile. However, Activated T cells and Dynabeads® do become tightly bound on activation, particularly 24-48h post-incubation, and these associations cannot always be easily broken by manual dispersement, or easily detected by light scatter based on the minor relative SSC/FSC contribution of the bead. Dynabeads® exhibit unique
auto-fluorescence across several detection channels on the BD FACS Aria™ II, which may interfere with analysis of T cell surface proteins labelled by Fluorophore-conjugated antibodies. In order to exclude T cell:bead doubles from flow cytometric analysis of T cell activation marker expression, we investigated the relative auto-fluorescence properties of unlabelled PBMCexp and Dynabeads® across all detection channels and filter sets on the BD FACS Aria II. We found that while PBMCexp auto-fluorescence was low across most channels, Dynabeads® exhibited particularly high relative auto-fluorescence at emission windows of 610-695 nm, and at 785 nm, when excited by a 488 nm laser (channels ‘PE-CF594’; PE-TR; ‘PerCP-Cy5.5’ and ‘PE-Cy7’), and that in each of these channels the fold-difference in bead/cell auto-fluorescent signal ratio was greater than 10, allowing easy discrimination (Figure 5-4).

To investigate whether fluorescence in these channels would allow us to distinguish cells and bead:cell doubles, and whether doing so was necessary, we activated PBMC with Dynabeads® at 1:1 and analysed samples at 6h, 24h, 48h and 72h (Figure 5-5). We found that the singlet cell and bead:cell doublet populations increasingly merged from 6–72h as PBMC took on a blasts morphology. When analysing events within a combined singlet cell/bead:cell doublet SSC/FSC gate (left panels; Figure 5-5), we found that only a minority of events were bead:cell doublets (~22% at 6h – ~6% at 72h), and that these two populations could be discretely gated based on dual PE-Cy7 and PerCP-Cy5.5 fluorescence (gates 1 and 2, middle panels; Figure 5-5). Exclusion of bead:cell doublets was necessary, as single cell and bead:cell doubles exhibited overlapping SSC/FSC profiles at 24h, 48h and 72h when back-imposed (right panels; Figure 5-5).

![Figure 5-3 Dynabeads® do not bind flow cytometry antibodies.](image)

Dynabeads® were incubated with mouse IgG1-, IgG2a- and IgG2b-fluorophore-conjugated antibodies as indicated. Fluorescence was determined in the FITC, PE and APC channels for beads alone (grey, filled) or beads incubated with Fluorophore-conjugated antibodies (black line).
Figure 5-4 Dynabeads® can be distinguished by their auto-fluorescence profile on the BD FACS Aria™ II.

Dynabeads® human T-Expander CD3/28 beads and T cells were acquired on the BD FACS Aria™ II and MFI determined in each available channel. Data shown as singlicate MFI values for T cells (Cell) and Dynabeads® (Bead) and as Fold-difference determined by bead MFI / T cell MFI in each channel. Dotted line indicates a Bead/cell fold-difference cutoff of ‘10’.
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Figure 5-5 Dynabead® – T cell doublets can be distinguished based on Dynabead® auto-fluorescence.

PBMCexp were stimulated with Dynabeads®human T-Expander CD3/28 beads for 72h. Bead:cell samples were collected at 6h, 24h, 48h and 72h and acquired on the BD FACS Aria™ II. Left panels: T cells and bead:cell doublets were collectively gated based on SSC/FSC profile at each timepoint. Middle panels: within these gates, T cells (gate 1) and bead:cell doublets (gate 2) could be distinguished based on bead dual-fluorescence in the PerCyCy5.5 and PE-Cy7 channels. Right panels: Gates 1 (red) and 2 (blue) show overlapping SSC/FSC profiles when back-gated onto original SSC/FSC gate.
5.3.2. CD4⁺ and CD8⁺ T cells exhibit unique activation marker expression kinetics.

Having optimised a system for inducing reliable CD3-mediated T cell activation, and for exclusion bead:cell doublet-free analysis of activated T cells by flow cytometry, we proceeded to investigate the kinetics of expression of a panel of putative or well-described T cell activation markers, with the aim of identifying a suite of surface markers that would reliably indicate activated CD4⁺ and CD8⁺ T cells during an easily assessable in vitro ‘activation window’ 18-36h post-stimulation. T cell activation is typically accompanied by modulation of cell surface proteins involved in co-stimulation, typically of the CD28 or TNFR superfamilies (Watts, 2010); proteins involved in cell:cell or cell:extracellular matrix adhesion; proteins involved in nutrient or amino acid uptake or processing; and proteins involved in cytokine reception and signal transduction (further discussed in chapters 1 and 7). As such we assessed, via flow cytometry, the expression (at a population-level) of the candidate activation markers detailed in Table 5-1 on total T cells (section 2.3.8.1) prior to stimulation and at timepoints from 8h-72h (Figure 5-6).

Table 5-1 Candidate activation markers

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25</td>
<td>IL-2 receptor subunit alpha</td>
<td>(Fazekas De St. Groth et al., 2004)</td>
</tr>
<tr>
<td>CD38</td>
<td>Cyclic adenosine diphosphate ribose synthesis</td>
<td>(Sandoval-Montes and Santos-Argumedo, 2005)</td>
</tr>
<tr>
<td>CD56</td>
<td>Cell:cell adhesion via Fibroblast growth factor receptor 1</td>
<td>(Kelly-Rogers et al., 2006)</td>
</tr>
<tr>
<td>CD69</td>
<td>Cell:cell adhesion via Galectin-1</td>
<td>(Mardiney et al., 1996)</td>
</tr>
<tr>
<td>CD71</td>
<td>Transferrin receptor (iron uptake)</td>
<td>(Pattanapanyasat and Hoy, 1991)</td>
</tr>
<tr>
<td>CD134</td>
<td>Tumour necrosis factor receptor superfamily member; co-stimulation for proliferation and survival</td>
<td>(Godfrey et al., 1994)</td>
</tr>
<tr>
<td>CD137</td>
<td>Tumour necrosis factor receptor superfamily member; co-stimulation for proliferation and survival</td>
<td>(Wolfl et al., 2007)</td>
</tr>
<tr>
<td>CD154</td>
<td>Tumour necrosis factor superfamily member; interaction with APC through CD40</td>
<td>(Frentsch et al., 2005)</td>
</tr>
<tr>
<td>ICOS</td>
<td>CD28-superfamily member; co-stimulation for proliferation and survival</td>
<td>(Hutloff et al., 1999)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Exogenous peptide epitope presentation</td>
<td>(Salgado et al., 2002)</td>
</tr>
</tbody>
</table>
5.3.2.1. Activation marker induction by Dynabeads®.

We found that CD4⁺ and CD8⁺ cells shared expression and kinetics of some activation markers, but exhibited strikingly different patterns for others. All activation markers showed low baseline levels of expression in unstimulated CD4⁺ and CD8⁺ T cells with the exception of CD38, which was seen on ~20% of CD4⁺ cells across three donors. Both CD4⁺ and CD8⁺ T cells showed rapid expression of CD69 on all cells at 8h and this level of expression was sustained to 48h before starting to diminish. Both CD4⁺ and CD8⁺ T cells also showed rapid induction of CD71, to >60% and >80% of all cells respectively at 8h, with expression on ~90-100% of cells seen from 16-72h. Interestingly, neither subset expressed HLA-DR or CD56 in response to stimulation. CD8⁺ T cells also failed to express CD154, while CD4⁺ cells showed rapid CD154 induction on only ~50% of all cells. This expression level was maintained to 48h and then returned to near baseline. On both CD4⁺ and CD8⁺ T cells CD38 was slowly and progressively induced, with expression peaking at 72h on ~90% and ~75% of all cells respectively. CD134 showed a very similar pattern on CD8⁺ T cells, progressively increasing to a peak of ~80% at 48h. By contrast CD134 was rapidly induced on CD4⁺ T cells, reaching levels of ~80-90% expression at 16-32h and >95% expression from 48h. Conversely, CD137 was rapidly induced on all CD8⁺ T cells by 16h and expression was evident at this level through the course of the assay, while on CD4⁺ T cells CD137 expression peaked at ~75% of cells at 16h and then progressively declined. ICOS expression was slowly induced on CD8⁺ T cells, reaching ~80% at 32h and peaking at 48h. ICOS induction was more rapid on CD4⁺ T cells, reaching >80% at 24h and progressively increasing beyond this timepoint. Finally, CD25 showed rapid and equivalent expression kinetics on both CD4⁺ and CD8⁺ T cells, reaching ~80% by 16h and being expressed on >90% of cells assessed from 24-72h. Based on these data we defined CD69, CD71, CD25, CD134 and ICOS as appropriate markers for detecting activated CD4⁺ T cells within a convenient ‘activation window’ 16-32h and CD25, CD69, CD71 and CD137 as appropriate for detecting activated CD8⁺ T cells within this same window (Figure 5-6).
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Figure 5-6 CD4+ and CD8+ T cells exhibit unique activation marker expression profiles and kinetics.

T cells were isolated as described in section 2.3.8 and seeded into flat-bottom 96-wp at 2 x 10^5 per well. Individual samples were set up for each timepoint and T cells were left unstimulated or were stimulated by addition of Dynabeads® human T-expander beads at a bead:cell ratio of 2:1. Surface CD4, CD8 and activation marker expression was assessed via flow cytometry (section 2.5.1) at each timepoint. Activation marker gates were defined based on matched CD4/CD8-only stained controls. Beads were excluded from analysis based on autofluorescence (Figure 5.4–Figure 5.5). Data shown as % of events within all CD4+ (left) or CD8+ (right) gates that exhibited expression of each activation marker. Data represents mean ± SEM %+ events from three donors. Dotted lines represent ≥80% T cell expression between 18h and 36h. Two independent repeats of this experiment were conducted.
5.3.2.2. Activation marker induction by TCR:pMHC.

W next sought to confirm that the expression kinetics observed on stimulation with a potent anti-CD3/28 agonist were consistent on genuine TCR:pMHC interaction. As such, we investigated CD137, CD25, CD69 and CD38 expression on ELA\textsubscript{26-35}-specific T cell clones (generated and characterised in chapters 3 and 4) after ELA\textsubscript{26-35} stimulation in two settings. Firstly, we incubated CellTrace™ Violet-stained clonal cells with HLA-A2+ LCL loaded with 1\,µM ELA\textsubscript{26-35} or loaded with no peptide as described in section 5.2.5, and assessed surface expression of CD25, CD69, CD137 and CD38 on clonal cells via flow cytometry at 24h (section 2.5.1.1). Consistent with our findings in section 5.3.2.1, we saw that three clones tested became entirely CD25\textsuperscript{+}, CD69\textsuperscript{+} and CD137\textsuperscript{+} at 24h, but showed heterogeneous and variable CD38 expression (~20-60\% of cells) (A; Figure 5-7).

Subsequently we asked whether timed detection of these activation markers would allow isolation of peptide-activated cells from a heterogeneous cell pool. To investigate this we ‘spiked’ ELA\textsubscript{26-35}-specific clonal cells into autologous PBMC such that clonal cells made up ~5\% of total cells, and incubated this cellular pool in Rs5 supplemented with 10\,µM ELA\textsubscript{26-35} for 24h. All cells were then stained for surface CD25, CD69, CD137 and CD38 at 24h. We found that negligible activation marker expression could be observed on any cells in the absence of peptide, but on ELA\textsubscript{26-35} challenge a distinct population of CD25\textsuperscript{+} CD137\textsuperscript{+} cells could be detected within a discrete ‘collection gate’ (left panel; C, Figure 5-7). These cells were also entirely CD69\textsuperscript{+} (right panel; C; Figure 5-7). Cells within this collection gate were sorted as described in section 2.5.3.2 and cultured for 24h to allow surface re-expression of TCR. When subsequently stained with ELA-pentamer, these cells were found to constitute an entirely pentamer-specific population (D; Figure 5-7). These data confirmed that temporal detection of activation marker expression allowed collection of peptide-activated CD8\textsuperscript{+} T cells with high specificity. This approach was further characterised and explored in Chapter 6.
Figure 5-7 CD8+ ELA26-35 T cell clones exhibit peptide-dependent activation marker upregulation.

A: CellTrace™ Violet-stained ELA26-35-specific clones were incubated for 24h with autologous PBMC loaded with 1µM ELA26-35 (black contours) or with no peptide (light grey contours). Data shown represents activation marker expression within the CellTrace™ Violet+ gate. B: Three clones treated as in (A) showed consistent upregulation of CD25, CD69 and CD137, but only partial induction of CD38. C: ELA26-35-specific clonal cells were spiked into ELA26-35-loaded autologous PBMCexp at ~5%. At 24h all cells were stained for activation marker expression, and CD25+CD137+ cells collected. All cells within this gate expressed CD69. D: CD25+CD137+ cells collected in (C) were analysed for ELA-pentamer binding and proved positive.

5.3.3. Synthetic long (SLP) and short peptide (SSP) presentation to CD8+ T cell clones

Having validated a flow-cytometric means of detecting TCR:pMHC-activated T cells, we next aimed to investigate a system that would allow the expansion of detectable numbers of T cells directed against therapeutically relevant viral and cancer associated proteins. As even memory T cell populations are often present at ≤0.1% of total T cells, peptide- or protein-mediated expansion in vitro is necessary to establish a sufficient pool of antigen-specific T cells to allow single-cell isolation and cloning. Several groups have described peptide-mediated expansion utilising in vitro-generated and matured MoDC as antigen-presenting cells (APC) (Chen et al., 2009; Chiriva-Internati et al., 2002; Wolfl and Greenberg, 2014). MoDC are believed to be indispensable for in vitro naïve T cell priming and expansion, because of their ability to deliver appropriate co-stimulatory signals to naïve T cells. This system, although potent, does have drawbacks – MoDC are relatively laborious to
produce, and clinical PBMC samples from which T cells must be expanded are often too low in number to allow MoDC production. Furthermore, the outgrowth of memory cells in the absence of MoDC has been described (Ho et al., 2006). In sections 5.3.3 - 5.3.5 we investigated the ability of professional and non-professional APC to process and present short synthetic peptides (SSP), long synthetic peptides (SLP) and full length recombinant protein, in order to develop a MoDC-independent method of rapidly expanding memory T cells to allow single-cell cloning and epitope discovery.

5.3.3.1. SLP processing and cross-presentation by professional APC lines is not affected by serum, but is time-dependent.

We first investigated requirements for media serum content and peptide uptake and processing time using T2 cells. T2 are TAP-deficient, and as such are unable to traffic endogenously-derived peptides to the cell surface in MHCI, or to ‘cross-present’ exogenously-derived peptide antigens through the classical TAP-mediated cross-presentation pathway (Andersen et al., 1999; Fehres et al., 2014). Nonetheless T2 cells have been shown to be proficient at presentation of exogenous short peptides, presumably through direct loading of empty MHC-I complexes (Zandvliet et al., 2012), and are able to present peptide epitopes to CD8+ T cells when infected by viruses (Zhou et al., 1993).

We utilised the well-characterised pp65-derived synthetic short peptide (SSP) NLV<sub>495-503</sub> (NLV<sub>9</sub>), for which several peptide-specific clones had been isolated in chapter 3, and two long synthetic peptides (SLP): ILA<sub>32</sub> and CAG<sub>49</sub> (see Table 2-10 for full sequences). T2 were loaded with each peptide for 1h or 16h at 10µM in the serum-free (R0; AIM-V) or serum-containing (Rs1; Rs5) medium and then co-incubated with CellTrace™ Violet labelled clonal cells. T cell activation was determined by surface CD137 upregulation as described in sections 5.2.4 - 5.2.5. We found that media serum content had only a minor effect on peptide uptake and presentation – this was primarily mediated by poorer T2 survival in R0 (data not shown), while presentation in AIM-V, Rs1 and Rs5 was roughly equivalent within each peptide and timepoint condition (C; Figure 5-8). Importantly, this indicated that the presence of serum proteins did not negatively affect peptide loading into empty T2 cell surface MHC-I molecules. As such, all subsequent work was carried out in Rs5.

We found that peptide loading and processing time played a major role in subsequent presentation of SLP-derived epitopes. Activation of NLV<sub>9</sub>-specific clone 4D9 by T2 loaded with NLV<sub>9</sub> for 1h or 16h was equivalent (A, C; Figure 5-8), whereas activation by T2 loaded with ILA<sub>32</sub> or CAG<sub>49</sub> was noticeably higher after 16h loading then 1h loading – in fact 1h ILA<sub>32</sub> loading gave no T cell activation above background (B, C; Figure 5-8). Interestingly, T cell clone activation was not dependent on peptide size, as CAG<sub>49</sub> was better processed and presented than ILA<sub>32</sub> at both
timepoints, suggesting that peptide sequence or conformation may play a role in facilitating either peptide uptake or peptide processing by T2.

Figure 5-8 SLP processing by professional APC lines is not affected by serum, but is time-dependent.

HLA-A2+ T2 cells were incubated in serum-free (AIM-V/R0) or serum-containing medium (Rs1/Rs5) alone or supplemented with 10μM SSP NLV9, SLP ILA32 or SLP CAG49 for 1h or 16h. Samples were then washed three times and incubated with CellTrace™ Violet-stained NLV9-specific clone (NLV 4D9) at 2.5:1 for 24h. T cell clone activation was determined by flow cytometric detection of surface CD137 (section 2.5.1.1). C: Data shown is the mean + SEM CD137+ population (%) of duplicate samples for each condition. Data is representative of two independent experiments. CD137 gating was defined based on T cells incubated with non-loaded T2.

A; B: Representative samples of CD137 expression within the CellTrace™ Violet+ gate showing clone incubated with non-loaded (grey), 1h-loaded (solid black) and 16h-loaded (dashed black) T2 for NLV9 and CAG49, respectively.

5.3.3.2. Professional and non-professional APC are able to process and cross-present SLP to CD8+ T cell clones.

Having demonstrated that TAP-deficient APC were able to efficiently present SLP-derived peptide epitopes after a sufficient uptake and processing window, we next extended our analysis to compare TAP-deficient (T2) and TAP-competent (LCL) professional APC; and non-professional APC (PBMCexp). We found LCL were able to process and present SLP in a time-dependent in the same way as T2 cells (A; Figure 5-9), although LCL processing and presentation kinetics appeared to be slower than T2, potentially because LCL are able to divert internalised SLP to a proteasome/TAP-dependent classical cross-presentation pathway, and because a smaller pool of empty HLA-A2 is available for NLV9 loading. As such, even though LCL typically express significantly higher surface HLA-A2 than T2 (B; Figure 4-3), they induce less T cell activation after a 1h loading time (A; Figure
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5-9). PBMCexp were able to present SSP, although presentation was less efficient than T2 or LCL after a 1h pulse – again this may relate to the significantly lower surface density of HLA-A2 we characterised in (B; Figure 4-3). Interestingly, although no T cell clone activation was seen when PBMCexp were loaded with ILA\textsubscript{32}, a small but detectable activated population was induced by PBMCexp loaded with CAG\textsubscript{49} for 16h (A; Figure 5-9). To confirm these results, we loaded LCL and PBMCexp for 16h with titrated doses of NLV\textsubscript{9} or CAG\textsubscript{49} across a 5-log\textsubscript{10} series (B; Figure 5-9). Incubation of these samples with clone NLV\textsubscript{9} 4D9 revealed an approximate 2-log\textsubscript{10} difference in saturating presentation of NLV\textsubscript{9}; suggested that LCL could present SLP-derived epitopes as efficiently as PBMCexp could present SSP; and, importantly, confirmed that PBMCexp loaded with 10\mu M CAG\textsubscript{49} were able to induce a level of T cell clone activation roughly equivalent to PBMCexp loaded with \leq 10nM short peptide (B; Figure 5-9). These data indicate that non-professional APC are able to process and present SLP, but do so inefficiently and require extended uptake and processing time.

![Figure 5-9 Professional and non-professional APC are able to process and present SLP to T cell clones.](image)

**A**: HLA-A2\textsuperscript{+} T2, LCL and PBMCexp were incubated in Rs5 alone or Rs5 + 10\mu M NLV\textsubscript{9}, ILA\textsubscript{32} or CAG\textsubscript{49} for 1h or 16h. Samples were then washed three times and incubated with a Cell Trace\textsuperscript{™} Violet-stained NLV\textsubscript{9}-specific clone (NLV 4D9) at 2.5:1 for 24h. T cell activation was determined by flow cytometric detection of surface CD137. Data shown is the mean + SEM CD137\textsuperscript{+} population (%) of duplicate samples for each condition. Data representative of two independent experiments.

**B**: LCL or PBMCexp were incubated for 16h in Rs5 + titrated doses of NLV\textsubscript{9} or CAG\textsubscript{49} as indicated. Samples were washed and incubated with clone as in A. T cell clone activation was determined as in A. Data shown as individual samples representative of two independent experiments.

As previous experiments (section 4.3.1.2) had indicated that even in a saturating system involving presentation of SSP (albeit to relatively low affinity clones) a clone:APC ratio of \geq 1:1 was required for complete induction of CD137 on all responder cells, we investigated whether altering the NLV\textsubscript{9}-
specific clone:PBMCexp ratio would enable superior SLP-mediated activation. We incubated PBMCexp or T2 with 10µM NLV₉ or CAG₄₉ for 16h as previously described, and then assessed activation of NLV clone M56 1G6A. Interestingly, although responses to all loaded T2 cells and to PBMCexp loaded with NLV₉ were strong (B; Figure 5-10), as observed previously, we now also saw a strong, titratable, response to PBMCexp loaded with CAG₄₉, consistent across three donor PBMCexp and two independent experiments, with ~40-75% of clonal cells being activated at 2:1 and ~80-100% of clonal cells being activated at 20:1 (A, B; Figure 5-10). To investigate whether this increased apparent presentation capacity was clone-dependent, we compared the ‘on-cell’ TCR:pMHC affinities of clones M56 1G6A and 4D9 (used in prior experiments) and found that 1G6A exhibited an approximately 2-log₁₀-greater affinity for peptide NLV₉ when presented by T2 cells (Figure 5-11). We concluded that this increased TCR:pMHC affinity was responsible for the greater sensitivity of detection of SLP processing and presentation, and highlights the importance of reporter cell sensitivity in these assays. Taken together, these data indicate that PBMCexp are able to process SLP and ‘cross-present’ epitopes to high-affinity CD8⁺ T cells, suggesting that T cells present in a heterogeneous PBMC mixture may be capable of contributing to memory T cell activation and expansion.

Figure 5-10 Non-professional APC are able to process and present SLP to T cell clones.

A: PBMCexp were loaded with no peptide, 10µM NLV₉ or CAG₄₉ for 16h, then washed three times and incubated with CellTrace™ Violet-stained clone M56 1G6A at E/T 1:2 or 1:20 as indicated. Samples were then stained for surface CD3 and CD137 (section 2.5.1.1) and CD137 expression within the CD3⁺ CellTrace™ Violet population assessed. B: Data shown as T cell clone activation (% CD137⁺) induced by three donor PBMCexp per peptide and E:T condition, and by T2 controls as annotated.
Figure 5-11 **NLV<sub>495-503</sub>-specific clones 4D9 and M56 1G6A exhibit differential TCR:pMHC affinity.**

T2 cells were loaded with NLV<sub>9</sub> for 2h at concentrations indicated. T2 cells were washed three times and then incubated with CellTrace™ Violet-stained NLV 4D9 or M56 1G6A at 2.5:1 for 24h. Samples were stained for surface CD8 and CD137 expression. T cell clone activation was determined by gating on the CD137<sup>+</sup> fraction of all CD8<sup>+</sup> CellTrace™ Violet<sup>+</sup> events. CD137 gating was based on CD8<sup>+</sup> CellTrace™ Violet-only FMO samples. Data shown as mean of duplicate samples.

5.3.3.3. **Processed SLP are not shed from the surface of LCL as minimal peptide epitopes.**

Despite our previous data demonstrating apparent cross-presentation by LCL and PBMCexp it was possible that peptide CAG<sub>49</sub> was being naturally hydrolysed during long incubations at 37°C, to yield NLV<sub>9</sub>, or that serum or surface peptidyl dipeptidases and dipetidyl peptidases were binding and processing CAG<sub>49</sub> to yield free NLV<sub>9</sub> independent of any intracellular processing. To investigate these possibilities, and with the knowledge that PBMCexp could present SSP but not SLP to NLV<sub>9</sub>-specific clones following a brief (2h) loading period only (A; Figure 5-9), we incubated CAG<sub>49</sub> at 10µM for 16h in either Rs5 or RF10 alone, or in the presence of 5x10<sup>4</sup> LCL. Following incubation, samples were centrifuged and acellular supernatant carefully transferred and applied to PBMCexp for 2h, to allow any NLV<sub>9</sub> generated *de novo* by heat or peptidase activity to be loaded onto PBMCexp HLA-A2. At the same time, PBMCexp were also treated with 10µM fresh CAG<sub>49</sub> for 2h to act as a presentation-negative control, and with 10nM NLV<sub>9</sub> that had been incubated in Rs5 overnight. We found that negligible T cell activation was induced by fresh CAG<sub>49</sub> loaded PBMCexp, consistent with previous experiments (A; Figure 5-9), while NLV<sub>9</sub> incubated overnight remained efficiently presented, indicating that the minimal epitope alone was resistant to hydrolysis and heat-mediated degradation (Figure 5-12). We found that loading of PBMCexp with any 16h supernatant yielded negligible T cell activation – although a significant (*p*<0.05, 2-way ANOVA) difference was observed between both LCL-absent and LCL-present media conditions, in all cases T cell activation
was at the lower limit of detection and significantly ($p<0.001$, 2-way ANOVA) lower than that observed when pulsing with NLV₉ alone at 10nM (3-log₁₀ lower than initial CAG₄₉ concentration). These data do not completely exclude the possibility that surface peptidases contribute to NLV₉ release from SLP with subsequent immediate internalisation by LCL, but they do indicate that neither heat, nor serum peptidase activity nor surface peptidase activity results in processing and release of significant levels of free soluble NLV₉.

**Figure 5-12 Processed SLP are not shed from the surface of LCL as minimal peptide epitopes.**

CAG₉ was incubated for 16h in: Rs5 alone; Rs5 + LCL (5 x 10⁴/well); RF10 alone; or RF10 + LCL (5 x 10⁴/well). Samples were then centrifuged to pellet any cells and supernatant applied to PBMCexp for 2h. PBMCexp were also incubated with 10nM pre-incubated NLV₉ and 10μM fresh CAG₉ for 2h. PBMCexp were then washed three times and incubated with CellTrace™ Violet-stained NLV 4D9 at 2.5:1. Samples were stained for surface CD8 and CD137 expression (section 2.5.1.1) and T cell clone activation was determined by flow cytometric detection of surface CD137 within the CD8⁺ CellTrace™ Violet⁺ gate. Data shown as mean + SEM CD137⁺ % of triplicate samples from one representative donor (of two). ***$p < 0.001$; *$p < 0.05$ two-way ANOVA and Bonferroni post-test.
5.3.4. Production of full-length rNY-ESO-1-His<sub>6</sub>.

The host laboratory had access to several SLP derived from the cancer-testis antigen NY-ESO-1, a well-characterised immunogenic and therapeutically relevant target in cancer immunotherapy (Gnjatic et al., 2006). We sought to express and purify a full-length recombinant from of NY-ESO-1, in order to compare the relative efficiency of processing and presentation of this protein compared to available SLP, extending our observations in section 5.3.3.

5.3.4.1. Amplification of NY-ESO-1 cDNA and expression vector production.

Full-length and truncated NY-ESO-1 have been produced in E.coli and isolated under cGMP conditions with sufficient purity to be administered in clinical vaccination trials (Lowe et al., 2011; Murphy et al., 2005). These products have typically been collected under denaturing conditions as NY-ESO-1 forms aggregates and inclusion bodies in E.coli, putatively due to its high level of hydrophobicity and disordered C-terminus (Gnjatic et al., 2006), or requirement for specific chaperones or chaperonins during folding.

Production of recombinant proteins using a Baculovirus vector offers several advantages over E.coli – the host Sf9 cell line (Table 2-3) is eukaryotic, allowing for post-translational modification and chaperoning; this system avoids the need to remove endotoxin from protein preparations, which may impact immunological assays, and the Baculovirus/Sf9 system results in high per-cell protein production (Kost et al., 2005).

In order to produce rNY-ESO-1 we designed expression primers to amplify the full-length NY-ESO-1 sequence, incorporating BamHI/HindIII restriction sites to allow subsequent cloning into the pfastBacHTB vector (Life Technologies). This vector allows incorporation of an in-frame N-terminal His<sub>6</sub>-tag to facilitate eventual purification by nickel- or cobalt-affinity chromatography, and expression of the transgene is under the control of a strong polyhedrin promoter. The transgene cassette is bordered by transposon Tn7 sites, allowing transfer of the entire cassette into a bacmid encoding a min-attnTn7 integration sites within LacZ (section 2.2.16).

Expression primers NYESO1_HTB_F and NYESO1_HTB_R were designed using Geneious software (V.7.1) and screened for cross-reactivity by NCBI BLAST. The primer pair was found only to amplify NY-ESO-1 (CTAG1B) and the closely related gene LAGE-1 (CTAG2) (A; Figure 5-13). NY-ESO-1 and LAGE-1 share a common initiation of transcription sequence, and exhibit 90% protein homology across their shared sequence, but the LAGE-1 gene encodes a longer transcript of approximately 750bp (Mandic et al., 2003), allowing differentiation based on size.

Full length NY-ESO-1 transcripts were amplified using cDNA derived from testes RNA (Ambion; 2.2.7) as a template. AGE analysis confirmed that PCR products at both ~550bp and ~750bp were
amplified, and that amplification could be improved by inclusion of 1-2x PCR enhancer solution (B; Figure 5-13). The smaller of these was excised and recovered from the gel, co-digested with BamHI/HindIII, and ligated into the plasmid pFactBacHTB (Invitrogen; sections 2.2.8 - 2.2.11). Competent E.coli DH5α™ were transformed with pFastBacHTB-rNYESO1 (sections 2.2.12 - 2.2.13) and ampicillin-resistant colonies selected and propagated in small broth cultures. Minipreps from several cultures were isolated and sequenced using primers NYESO1_Seq_1F and NYESO1_Seq_RC, and plasmids showing correct sequence and insert orientation were selected and used to transform competent E.coli DH10α™ containing a helper plasmid encoding a Tn7 transposase and tetracycline resistance (sections 2.2.15 - 2.2.17).
Figure 5-13 Amplification of NY-ESO-1 (CTAGIA/B) DNA.

A: NCBI BLAST of primers NY-ESO-1_F and _R revealing specificity for only CTAGIA/B (NY-ESO-1) and CTAG2 (LAGE-1/CAMEL). B: Optimised amplification of NY-ESO-1 from testes RNA template via RT-PCR with Platinum Pfx DNA polymerase. PCR products were analysed via AGE. NY-ESO-1 major band (~550bp) and LAGE-1 minor band (~750bp) are indicated. E0: no PCR enhancer; E1: 1x PCR enhancer; E2: 2x PCR enhancer; F: fibroblast RNA template; NT: no template control.
Recombinant bacmid DNA was isolated from DH10α™ and transgene insertion and orientation confirmed by PCR. Recombinant bacmids were transfected into Sf9 cells and resulting recombinant baculoviral P1, P2 and P3 stocks were collected as described in sections 2.2.17 - 2.2.18. Titrated doses of high-titre P3 stocks were used to optimise small-scale protein expression in 10ml Sf9 cultures, comparing Sf9 cell survival and protein expression levels. We found that Sf9 viability decreased in a titratable fashion to only 25% of uninfected controls at the highest dilution tested (1:2500, A; Figure 5-14), consistent with Sf9 cell lysis and virion release during late infection. Also consistent with higher virus production, we found that expression of rNY-ESO-1-His₆ increased in a titratable fashion, becoming saturating at a dilution of 1:5000 as determined by visualisation of a an expected ~22kDa band on SDS-PAGE analysis (Murphy et al., 2005) (B; upper panel; Figure 5-14). Because infection at 1:5000 allowed greater Sf9 cell survival than 1:2500, and hence a higher intracellular virion yield, this dilution was used during future expression experiments. The identity of the ~22kDa band was confirmed via western blot using the monoclonal α-NY-ESO-1 antibody ES121, which is not cross-reactive with LAGE-1 (Jungbluth et al., 2001) (B; lower panel; Figure 5-14). When the total, soluble and insoluble Sf9 lysate fractions were separated, we found that when using a sodium phosphate lysis buffer almost all rNY-ESO-1-His₆ remained in the insoluble fraction, confirmed by SDS-PAGE (C; upper panel; Figure 5-14) and western blot (C; lower panel; Figure 5-14). This suggested that either the pH or detergent conditions were inappropriate to maintain rNY-ESO-1-His₆ in solution, or that the Sf9 cells lacked the appropriate molecular chaperones or chaperonins to produce correctly folded, soluble rNY-ESO-1-His₆.
5.3.4.2. Optimisation of rNY-ESO-1-His<sub>6</sub> expression by Sf9 cells.

![Graph](image1)

**Figure 5-14 Optimisation and validation of rNY-ESO-1-His<sub>6</sub> by Sf9 cells.**

Sf9 cells in exponential growth phase were left uninfected or were infected with P3 baculovirus- rNY-ESO-1-His<sub>6</sub> stock at dilutions indicated. 

**A:** The effect of titrated infection on Sf9 cell viability was assessed by trypan blue exclusion. Viable cells are shown as the mean ± SEM of triplicate samples expressed as a % of uninfected cell numbers. Cell death is caused by excessive virion production and release in late infection. 

**B:** 2.5ml culture samples of Sf9 cells uninfected (V) or infected (40 – 2.5) as above were lysed under non-denaturing conditions, and protein content analysed via paired SDS-PAGE (upper panel) and western blot with monoclonal antibody ES121 (lower panel). Arrow indicates an ~22kDa rNY-ESO-1-His<sub>6</sub> band detectable in all infected cell samples at ≥1x10<sup>3</sup> by SDS-PAGE, and in all infected samples by western blot. 

**C:** 2.5ml culture sample infected at 1:5000 was partitioned into total (T); soluble (S) and insoluble (I) fractions and rNY-ESO-1-His<sub>6</sub> content analysed via paired SDS-PAGE (upper panel) and western blot (lower panel).
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5.3.4.3. Optimisation and scale-up of rNY-ESO-1-His<sub>6</sub> extraction from Sf9 cells.

To investigate whether rNY-ESO-1-His<sub>6</sub> could be restored via altering the pH or detergent content of the lysis buffer used, we extracted small total soluble and insoluble protein from small Sf9 cultures infected at 1:5000 using lysis buffers at pH 7.2, 8.0 and 9.6, and containing either non-ionic (Nonidet P-40 and Tx-100) or anionic (SDS) surfactants. Interestingly, we found that this insolubility was not dependent on pH or detergent used, as in all conditions tested the rNY-ESO-1-His<sub>6</sub> band remained in the insoluble fraction (Figure 5-15).

![Image](image-url)

**Figure 5-15 rNY-ESO-1-His<sub>6</sub> insolubility is pH- and detergent-independent.**

1ml culture samples of Sf9 cells uninfected (UI) or infected with P3 rNY-ESO-1-His<sub>6</sub> baculovirus (1:5000) were pelleted and lysed using non-denaturing lysis buffers at pH and with detergent content indicated. Total soluble (S) and insoluble (I) fractions were separated and analysed via paired SDS-PAGE.

Urea or guanidine-HCl are commonly used to solubilise intracellular protein inclusion bodies. We investigated the incorporation of titrated concentrations of urea in our standard pH 7.2 lysis buffer, and found that at concentrations > 4M, and particularly at 6-8M, the majority of rNY-ESO-1-His<sub>6</sub> could be found in the soluble fraction of the Sf9, as confirmed by SDS-PAGE (A; left panel; Figure 5-16) and matched western blot (B; left panel; Figure 5-16). As such, 6M urea was included in Sf9 lysis buffer for larger-scale protein expression.
Figure 5-16 Soluble rNY-ESO-1-His6 can be isolated from Sf9 cells under denaturing conditions.

1ml culture samples of Sf9 cells uninfected or infected with P3 rNY-ESO-1-His6 baculovirus (1:5000) were pelleted and lysed using standard lysis buffer, or lysis buffer supplemented with 1-8M urea. Total soluble (S) and insoluble (I) fractions (resuspended in nuclear solubilisation buffer) were separated and analysed via paired A: SDS-PAGE and B: western blot using monoclonal ES121 antibody.

5.3.4.4. Scale-up of rNY-ESO-1-His6 production in Sf9 cells.

Having defined lysis conditions that allowed extraction of soluble (denatured) rNY-ESO-1-His6, we proceeded to large-scale expression, utilising 200ml Sf9 cultures inoculated with 1:5000 P3 baculovirus stock. Cell pellets were processed as described in section 2.2.19.2, and filtered soluble lysate applied to an equilibrated Histrap™ HP (GE Healthcare) metal ion affinity column attached to a peristaltic pump. After extensive column washing rNY-ESO-1-His6 was eluted by application of an escalating imidazole gradient. We found that rNY-ESO-1-His6 could be eluted from the column at >300mM imidazole (C; Figure 5-17). Although SDS-PAGE analysis of fractions taken at each stage of the elution appeared to contain contaminating protein bands at MW >25kDa (A; Figure 5-17), paired western blot analysis using ES121 demonstrated that many of these apparent contaminants were dimers and other aggregates (B; Figure 5-17). Aggregation of urea-denatured r-NY-ESO-1-His6 has also been reported in E.coli expression systems (Lowe et al., 2011; Murphy et al., 2005).

Interestingly, these contaminants were less prevalent in the initial total lysate (T) or filtered soluble fractions (SF) (A; Figure 5-17), presumably because these fractions were taken from large volumes and immediately diluted in SDS-PAGE loading buffer, suggesting that the formation of urea-mediated protein aggregates was time- or concentration-dependent.
Figure 5-17 Large-scale expression, isolation and validation of rNY-ESO-1-His$_6$.

rNY-ESO-1-His$_6$ was extracted from infected Sf9 cell lysate as described (section X) and isolated via IMAC. C: rNY-ESO-1-His$_6$ was bound to a HisTrap column and eluted at >300mM imidazole. Standardised Sf9 lysate, column washes and elution fractions were analysed via A: SDS-PAGE and B: western blot with monoclonal antibody ES121 to validate the presence of rNY-ESO-1-His$_6$. A: B: L: ladder; T: total lysate; I: insoluble fraction; S: soluble fraction; SF: soluble fraction, filtered; FT: column flowthrough; W: column wash; E$_1$-E$_7$: elution fraction 1-7 as annotated in C.

We attempted to further purify the major ~22kDa rNY-ESO-1-His$_6$ protein band through size-exclusion chromatography (SEC). Protein-containing elution fractions were pooled and applied to a Sephadex®-75 gel filtration column. Interestingly, although the rNY-ESO-1-His$_6$ did elute from this column in a relatively narrow peak, only a minor increase in purity was observed, primarily a loss of the ~70kDa contaminating band, (Figure 5-18), suggesting that the presence of the urea and the denatured state of the protein(s) present was interfering with their size-mediated differential passage through the resin bed. As such, all rNY-ESO-1-His$_6$ fractions were pooled and concentrated to a minimal volume to be used in cell culture assays. When protein concentration was assessed using the BCA assay, we found that a typical 200ml Sf9 culture yielded between 100-500µg rNY-ESO-1-His$_6$. 

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Figure 5-18 Size-exclusion chromatography (SEC) clean-up of rNY-ESO-1-His6.

rNY-ESO-1-His6 was applied to a Sephadex®-75 gel filtration column attached and total protein content in elution fractions analysed by ABS 280nm. Collected fractions were analysed by SDS-PAGE. L: ladder; P: pre-SEC; A-G: elution fractions A-G as noted on absorbance trace.

5.3.5. NY-ESO-1 SLP are more easily processed and cross-presented to CD8+ T cells than full-length, denatured rNY-ESO-1

Having isolated pure rNY-ESO-1-His6 we sought to compare SLP and denatured recombinant protein to determine which was more easily processed and presented by our professional APC lines, in order to determine which antigen source would be optimal for subsequent memory T cell stimulation. In these experiments we utilised: SSP NY-ESO-1157-165 (SLL); SLP NY-ESO-1151-169; SLP NY-ESO-1153-180; and rNY-ESO-1-His61-180. T cell activation was determined using clone M56 SLL 2F2 G3 (IL-7/21) generated in chapter 3, as described in section 5.2.5. T2 and LCL were incubated in Rs5 supplemented with SSP; SLP; or protein for 16h at three 5-fold dilutions covering 0.1 – 2.5µM. As only rNY-ESO-1-His6 was solubilised in a potentially cytotoxic (due to hypotonicity) urea-containing buffer (section 5.3.4.3; final urea concentration in r-NY-ESO-1-His6 loaded samples reached 20mM) we also incubated T2 and LCL with SLP NY-ESO-1151-169 at 2.5µM in the presence of an equivalent concentration of urea-containing buffer to assess any effects on cell viability and SLP processing and presentation. We found that at each concentration tested, and for both cells lines used, each SLP gave significantly greater T cell activation than r-NY-ESO-1-His6 (A, B; Figure 5-19; ***p<0.001, 2-way
ANOVA with Bonferroni post-test). T2 and LCL elicited detectable T cell activation with each SSP and SLP treatment – by contrast T2 cells could only elicit ≥50% T cell activation at 2.5µM r-NY-ESO-1-His6, while LCL elicited negligible T cell activation at any concentration tested (A, B; Figure 5-19). Interestingly, we again saw that T cell activation is not dependent on SLP length, as SLP NY-ESO-1_{153-180} was better processed and presented than SLP NY-ESO-1_{151-169}, especially by LCL. The presence of urea buffer did not have any significant effect on T2 or LCL viability (C; Figure 5-19) or on processing and presentation of SLP NY-ESO-1_{151-169}. (D; Figure 5-19) Taken together, and considering the relative difficulty of producing rNY-ESO-1-His6, these data suggested that SLP represented a more versatile and appropriate stimulus for peptide-mediated T cell expansion. The application of an NY-ESO-1-derived SLP was further investigated in Chapter 6.
Chapter 5 – Results III

Figure 5-19 Differential processing of NY-ESO-1 SLP and rNY-ESO-1 by professional APC.

A: T2 cells or B: M56 LCL were incubated overnight in medium alone or in the presence of peptide or rNY-ESO-1 at titrated concentrations, and then washed three times and incubated for 26h with CellTrace™ Violet-stained clone M56 2F2 (SLL9-responsive) at a ratio of 3:1. Activation of M56 SLL 2F2 was assessed by flow cytometric detection of CD137. C: To assess the effect of the rNY-ESO-1 urea storage buffer on APC survival, T2 and LCL viability in the presence or absence of urea storage buffer (final concentration 20mM) was determined by trypan blue exclusion after overnight incubation but prior to presentation assay. D: To assess the effect of the urea storage buffer on processing and presentation, activation of M56 2F2 by T2 and LCL loaded overnight with 2.5µM p151-169 in medium alone or supplemented with urea storage buffer (20mM) was assessed. All data show triplicate sample mean + SEM. A; B: ***p <0.001 two-way ANOVA and Bonferroni post-test, comparing each SLP to rNY-ESO-1 at each concentration.
5.3.6. Optimisation of MoDC-independent SLP-mediated memory CD8+ T cell expansion.

Previous work in this chapter demonstrated that SLP were more easily processed and presented, in a time-dependent fashion, than rNY-ESO-1-His6 and that professional APC lines (T2/LCL) and non-professional APC (T cell libraries) were able to process and present SLP to CD8+ T cells, albeit with different efficiencies. PBMC contain several cell types that could potentially internalise and present SLP, including B-cells, monocytes and blood-borne dendritic cells. As our intention was to apply SLP to PBMC in order to rapidly expand memory T cells in the absence of matured MoDC, we asked whether modulating SLP incubation time might improve memory T cell expansion. As in vivo memory T cells are known to be dependent on both IL7 and IL-15 for their homeostasis, we modified our T cell cloning expansion protocol to include IL-15 (detailed further in Figure 6-1). Interestingly, IL-15 has also been shown to direct the maturation of monocytes into Langerhans-like dendritic cells in vitro (Mohamadzadeh et al., 2001; Saikh et al., 2001), and to increase their expression of the co-stimulatory ligands CD80 and CD86, which may aid T cell expansion (Agostini et al., 1999). We utilised our previously described pp65-derived ILA32 and CAG49 SLP and measured the extent of an NLV9-specific T cell expansion in four donors. Prior to expansion NLV9-specific precursor frequency was determined by pentamer staining – we found that two donors had borderline undetectable precursor populations (<0.05%), one donor had a detectable 0.1% population and one donor had a large (1%) population. We expanded T cells using two different peptide-loading protocols. In both cases PBMC were incubated for 2h with 40µM peptide in a minimal volume (200µl) to maximise loading. PBMC were then either washed (‘pulse/wash’) and resuspended in a total of 2ml for culture in 24wp-wells, or simply had their total volume made up to 2ml to give a final concentration of 4µM. In the latter case free peptide was only removed through subsequent media exchange at d3 and d7 (‘continuous’). T cell outgrowth was supported by administration of IL-2 and IL-12 at d0 only, and by IL-7 and IL-15 every ≤72h (Figure 6-1). When we assessed NLV9-specific T cell frequency within the CD8+ T cell population at d14, we found that notable expansion was dependent on clear detection of a precursor population, consistent with this protocol being suboptimal for the priming and expansion of naïve T cells (C; Figure 5-20). In two donors that did exhibit a clearly detectable NLV9-specific precursor population, we found that greater expansion was detected in the ‘continuous’ condition within each peptide treatment (B, C; Figure 5-20). Both peptides were able to elicit T cell expansion but typically CAG49 mediated greater expansion than ILA32. Based on these data, all future memory T cell expansions were performed by loading PBMC under ‘continuous’ conditions.
Figure 5-20 Long-term incubation with SLP enhances memory T cell expansion in the absence of mature APC.

PBMC from four donors was incubated in Rs5 + 10µM ILA$_{32}$ or CAG$_{49}$ for 2h and then washed to remove free peptide, or incubated in Rs5 + 10µM ILA$_{32}$ or CAG$_{49}$ without peptide being removed until media exchange at 96h. Rs5 was supplemented with IL-2 and IL-12 (d0 only) and IL-7 and IL-15 every 3 days (all 10ng/ml) to support expansion. At D14 all samples were stained for CD3, CD8 and pentamer-NLV, and pentamer-specific cells expressed as % of total CD8$^+$ T cells. 

A: mock expansion with DMSO did not alter NLV-specific T cell frequency. B: Data shown as representative plots for each peptide and loading condition for one donor. C: Donors exhibited varying NLV-specific precursor frequencies ranging from <0.05 to ~1 of CD8$^+$ T cells. Data shows pre-expansion and post expansion pentamer-NLV frequencies for each condition. Data shown as mean +/- SEM of duplicate samples per condition per donor.
5.4. Chapter summary.

In this chapter we investigated the expression kinetics of candidate T cell activation markers following anti-CD3/CD28 stimulation. We defined HLA-DR and CD56 as markers not responsive to CD3/CD28-mediated signalling in both CD4+ and CD8+ T cells. We found that within an 18-36h window post-stimulation, the markers that best defined CD4+ T cells were CD25, CD69, CD71, CD134 and ICOS. The markers that best defined CD8+ T cells were CD25, CD69, CD71 and CD137. We demonstrated that equivalent CD25, CD69 and CD137 kinetics could be observed when CD8+ T cell clones received a true TCR:pMHC stimulus, and activation-marker expression could be used to isolate antigen-specific cells via FACS with high sensitivity.

We next investigated the kinetics of synthetic short peptide (SSP) and synthetic long peptide (SLP) processing and presentation by professional and non-professional APC lines. We found that presentation of SSP was rapid, while SLP processing and cross-presentation was time-dependent but TAP-independent. Further, SLP processing and MHC I epitope generation was not SLP-length-dependent. We found that non-professional APC (T cells) were able to process and present SLP to high-affinity T cell clones, but they did so inefficiently compared to professional APC (T2 and LCL).

We produced full-length recombinant NY-ESO-1 using a transgenic baculovirus vector and an Sf9-cell expression system. NY-ESO-1 proved insoluble in this system, and was isolated in a denatured form in urea. We compared processing and presentation of SLP and full-length denatured protein, and found that SLP were a more tractable system to allow T cell activation. Based on these data, we investigated the optimal conditions for expansion of a pre-existing memory T cell population within PBMC in the absence of in vitro generated mature MoDC, and found that memory T cells could be efficiently expanded, but that this expansion was dependent on retention of SLP in culture. As such, in this chapter we developed the elements of a protocol for expanding, activating and isolating antigen-specific CD4+ and CD8+ T cells of undefined peptide specificity. Technical aspects defined in this chapter were applied in chapter 6, and the wider significance of these results is discussed in detail in chapter 7.
Chapter 6. T cell activation marker-guided cloning and epitope discovery

6.1. Introduction.

In chapters 3 and 4 we defined the optimal conditions for expansion of CD8\(^+\) T cell clones, including a cytokine regimen that allowed retention of co-stimulatory and SLO-homing markers on the cell surface. In chapter 5 we defined optimal conditions for synthetic long peptide (SLP) processing and SLP-mediated memory T cell expansion. Further, we investigated the TCR/CD3-dependent expression of candidate T cell ‘activation markers’ (AM), and identified surface markers that reliably allowed flow cytometric identification of activated T cells at a defined time post-stimulation.

In this chapter we aimed to validate peptide-mediated upregulation of our chosen AM, following peptide-mediated expansion of memory T cells as defined in chapter 5. We also aimed to prime peptide-specific naïve T cell responses using MoDC and isolate these cells following secondary expansion, in order to broaden the application of our activation-marker guided FACS protocol.

Our final aim was to apply these conditions to the expansion, isolation and cloning of CD4\(^+\) and CD8\(^+\) T cells specific for the cancer-testis antigen NY-ESO-1. We aimed to expand T cell clones and map their minimal peptide epitopes (MPE) and the MHC class I or II context in which those epitopes were presented, in order to define novel, therapeutically relevant TCR:pMHC restrictions.

NY-ESO-1 is highly immunogenic, stimulating both humoral and cell-mediated immunity. It is expressed in many cancers as a result of global hypomethylation (Simpson et al., 2005) and expression is typically correlated with metastasis, advanced staging and cancer de-differentiation (Velazquez et al., 2007a). NY-ESO-1 has been widely targeted in clinical vaccination protocols (Cebon et al., 2010), in trials utilising adoptive transfer of NY-ESO-1 specific T cell clones (Hunder et al., 2008), and in trials utilising adoptive transfer of T cells expressing a genetically engineered NY-ESO-1-specific TCR (Morgan et al., 2006a; Robbins et al., 2011). A summary of previously described MHC I and MHC II peptide epitopes within NY-ESO-1, and their presence within available SLP, is presented in tables 6-1 and 6-2.
**Chapter 6 – Results IV**

**Table 6-1** Naturally processed and presented MHCI-restricted epitopes within NY-ESO-1.

<table>
<thead>
<tr>
<th>SLP</th>
<th>MHCI Restriction</th>
<th>Epitope</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>A*02</td>
<td>1-11</td>
<td>(Aarnoudse et al., 1999)</td>
</tr>
<tr>
<td>-</td>
<td>A*31</td>
<td>18-27</td>
<td>(Wang et al., 1998)</td>
</tr>
<tr>
<td>-</td>
<td>A*31</td>
<td>53-62</td>
<td>(Ebert et al., 2009)</td>
</tr>
<tr>
<td>79-116</td>
<td>C*06</td>
<td>80-88</td>
<td>(Gnjatic et al., 2000)</td>
</tr>
<tr>
<td>79-116</td>
<td>A*24:02</td>
<td>91-101</td>
<td>(Eikawa et al., 2013)</td>
</tr>
<tr>
<td>79-116</td>
<td>C*03</td>
<td>92-100</td>
<td>(Gnjatic et al., 2000)</td>
</tr>
<tr>
<td>79-116</td>
<td>B*07</td>
<td>60-72</td>
<td>(Eikawa et al., 2013)</td>
</tr>
<tr>
<td>79-116</td>
<td>B*35:01</td>
<td>92-102</td>
<td>(Benlalam et al., 2003)</td>
</tr>
<tr>
<td>79-116</td>
<td>B*35</td>
<td>94-102</td>
<td>(Jager et al., 2002)</td>
</tr>
<tr>
<td>79-116</td>
<td>C*03</td>
<td>96-104</td>
<td>(Jackson et al., 2006)</td>
</tr>
<tr>
<td>79-116</td>
<td>C*12:02</td>
<td>96-104</td>
<td>(Eikawa et al., 2013)</td>
</tr>
<tr>
<td>118-143</td>
<td>B*52:01</td>
<td>96-104</td>
<td>(Jackson et al., 2006)</td>
</tr>
<tr>
<td>118-143</td>
<td>C*03</td>
<td>124-133</td>
<td>(Knights et al., 2009)</td>
</tr>
<tr>
<td>118-143</td>
<td>B*49</td>
<td>124-135</td>
<td>(Matsuzaki et al., 2008)</td>
</tr>
<tr>
<td>151-174</td>
<td>A*02</td>
<td>157-165</td>
<td>(Jäger et al., 1998)</td>
</tr>
</tbody>
</table>

**Table 6-2** Naturally processed and presented MHCII-restricted epitopes within NY-ESO-1.

<table>
<thead>
<tr>
<th>SLP</th>
<th>MHCII Restriction serotype</th>
<th>Epitope</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>-</td>
<td>DR3</td>
<td>14-33</td>
<td>(Slager et al., 2004)</td>
</tr>
<tr>
<td>-</td>
<td>DR15</td>
<td>37-50</td>
<td>(Hasegawa et al., 2006)</td>
</tr>
<tr>
<td>-</td>
<td>DQ7 (DQB1*03)</td>
<td>49-72</td>
<td>(Karbach et al., 2006)</td>
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<tr>
<td>79-116</td>
<td>DR2</td>
<td>86-97</td>
<td>(Chen et al., 2004)</td>
</tr>
<tr>
<td>79-116</td>
<td>DR9 (DRB1*09:01)</td>
<td>87-100</td>
<td>Mizote, 2010</td>
</tr>
<tr>
<td>79-116</td>
<td>DP4</td>
<td>87-111</td>
<td>(Mandic et al., 2005)</td>
</tr>
<tr>
<td>79-116</td>
<td>DR1</td>
<td>87-111</td>
<td>(Chen et al., 2004)</td>
</tr>
<tr>
<td>79-116</td>
<td>DR4</td>
<td>87-111</td>
<td>(Chen et al., 2004)</td>
</tr>
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<td>79-116</td>
<td>DR7</td>
<td>87-111</td>
<td>(Mizote et al., 2010)</td>
</tr>
<tr>
<td>79-116</td>
<td>DQ4 (DQB1*0401)</td>
<td>95-107</td>
<td>(Jäger et al., 2000)</td>
</tr>
<tr>
<td>118-143</td>
<td>DRB4*01</td>
<td>119-138</td>
<td>(Ayyoub et al., 2010)</td>
</tr>
<tr>
<td>118-143</td>
<td>DR1</td>
<td>119-143</td>
<td>(Zeng et al., 2000)</td>
</tr>
<tr>
<td>118-143</td>
<td>DR4</td>
<td>121-130</td>
<td>(Ayyoub et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Antigen</td>
<td>Start-Stop</td>
<td>Reference</td>
</tr>
<tr>
<td>-------</td>
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<tr>
<td>118-143</td>
<td>DR7</td>
<td>123-137</td>
<td>(Zarour et al., 2002)</td>
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<tr>
<td>118-143</td>
<td>DR11</td>
<td>123-137</td>
<td>(Zarour et al., 2002)</td>
</tr>
<tr>
<td>118-143</td>
<td>DR52b</td>
<td>123-137</td>
<td>(Bioley et al., 2009)</td>
</tr>
<tr>
<td>118-143</td>
<td>DR8</td>
<td>124-134</td>
<td>(Mizote et al., 2010)</td>
</tr>
<tr>
<td>118-143</td>
<td>DR8</td>
<td>125-134</td>
<td>(Mizote et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>DR4</td>
<td>139-156</td>
<td>(Jäger et al., 2000)</td>
</tr>
<tr>
<td>151-174</td>
<td>DP4</td>
<td>157-170</td>
<td>(Zeng et al., 2001)</td>
</tr>
</tbody>
</table>

6.2.1. Cells.

PBMC and PBMCexp were cultured in sterile Rs5, as described in sections 2.3.1 and 2.3.2. Rs5 was supplemented with cytokines as described in text. During expansion and maintenance, Rs5 and supplementary cytokines were replenished as described in text. LCL were generated as described in section 2.3.6 and maintained in RF10. T2 cells were maintained in RF10. MoDC were isolated and matured as described in section 2.3.9.

6.2.2. Flow cytometry for surface markers and intracellular cytokines.

Surface expression of CD3, CD4, CD8, CD25, CD38, CD56, CD69, CD71, CD134, CD137, CD154, ICOS and HLA-DR was analysed via flow cytometry as described in section 2.5.1.1 and intracellular flow cytometry for IFNγ was carried out as described in section 2.5.1.5 using antibodies and doses listed in Table 2-5. Live/Dead discrimination was carried out using DAPI or Live/Dead™ fixable dyes (section 2.5.2). Typically, positive expression gates were drawn based on FMO controls, and median fluorescence intensity (MFI) was calculated by subtraction of FMO background fluorescence.

6.2.3. Activation marker-guided live-cell sorting for CD4+ and CD8+ T cells.

Tetramer/Pentamer staining was carried out on fresh or frozen PBMC as described in section 2.5.1.4. Activation-marker-guided T cell sorting was carried out as described in section 2.5.3.2 and T cell clones were expanded as described in section 2.3.10. All activation marker expression was assessed within the CD3+CD4+; CD3+CD8+ or CD4+CD56−; CD8+CD56− fractions of cell cultures. CD56− exclusion gating was utilised to exclude any NK or NKT cells from analysis and collection gates.
6.2.4. Expansion of naïve and memory antigen-specific T cells.

A general overview of our memory T cell expansion (CLG426-434 and NY-ESO-1 SLP) and naïve T cell priming and expansion (PRAME SSP and ELA26-35) protocols is shown below. IL-2, -12, -21 provided at 10ng/ml. IL-7 and I-15 provided at 5ng/ml. MoDC (B; Figure 6-1) generated as described in section 2.3.9. In all expansions, IL-12 was provided during at d0 only, and IL-2 was provided only at d0 and d3. IL-15 was provided from d6-14/25, and IL-7 and -21 were provided d0 – d14/25.

Figure 6-1 Peptide-mediated expansion strategies for memory T cell expansion using PBMC alone, and naïve T cell priming using MoDC and PBMC.
6.2.5. HLA haplotypes for donors and APC lines used in chapters 4-6.

Table 6-3 Donor and cell line HLA haplotypes.

<table>
<thead>
<tr>
<th>Donor/Cell line</th>
<th>MCH I</th>
<th>MHC II</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M56</em></td>
<td>A<em>01:01; A</em>02:01 / B<em>27:05 // C</em>01:02; C*07:01</td>
<td>DRB1<em>01:01; <em>15:01 // DPB1</em>04:01 // DQB1</em>05:01</td>
<td>Ludwig Institute and NZ Blood; HLA-C*03 SSP-PCR carried out in this study</td>
</tr>
<tr>
<td><em>P062.004</em></td>
<td>A<em>02:01; A</em>03:01//B<em>62//C</em>03:03; C*03:04</td>
<td>DRB1<em>04 // DPB1</em>04:01; <em>0402 // DQB1</em>03</td>
<td></td>
</tr>
<tr>
<td><em>P062.005</em></td>
<td>A<em>02:01 // B</em>27:05; B<em>15:01// C</em>02:02; C*03:03</td>
<td>DRB1<em>0403; <em>11:03 // DPB1</em>04:02 // DQB1</em>03</td>
<td></td>
</tr>
<tr>
<td><em>P062.006</em></td>
<td>A<em>01:01 // B</em>44 // C:ND (C*03- by PCR)</td>
<td>HLA-DR/DP/DQ - ND</td>
<td>(Lorente et al., 2011)</td>
</tr>
<tr>
<td><em>T2</em></td>
<td>A<em>02 // B</em>51 // C*01:02</td>
<td>HLA-DR/DP/DQ - ND</td>
<td>(Battle et al., 2013)</td>
</tr>
<tr>
<td><em>THP-1</em></td>
<td>A<em>02 // B</em>15:08; B<em>15:11 // C</em>03</td>
<td>DRB1*01:01; <em>15:01 // DPB1 - ND // DQB1</em>01:05; *01:06</td>
<td>(Gebreselassie et al., 2006)</td>
</tr>
<tr>
<td><em>U937</em></td>
<td>A<em>03:01 // B</em>18:01; B<em>51:01 // C</em>01:02; C*07:02</td>
<td>HLA-DR/DP/DQ - ND</td>
<td></td>
</tr>
</tbody>
</table>

6.2.6. T cell clone expansion.

All CD4+ and CD8+ T cell clones isolated were stimulated using PHA in the presence of irradiated LCL and expanded in the presence of IL-12, -2, -7 and -21, as described in section 2.3.10 and previously shown in section 3.2.3.

6.2.7. T cell clone validation by IFNγ ELISPOT.

CD4+ and CD8+ T cell clones generated via activation-marker guided cell sorting were validated for peptide specificity by IFNγ ELISPOT (BD). CLG9 and ELA9-specific clones were screened against peptide-loaded T2 cells (as CLG9-responsive clones would recognise EBV-transformed LMP-1+ LCL independent of peptide loading). NY-ESO-1 SLP-specific clones were screened against peptide-
loaded autologous LCL lines. Typically, for poorly surviving clones 50% of well pellet was used for screening, while for large clone pellets (1-5x10^5 cells) 5ul of clone culture was used per ELISPOT well.

6.2.8. HLA-C*03 profiling via SSP-PCR.
Melanoma and LCL genomic DNA was extracted as described in section 2.2.4, and PCR conducted as described in section 2.2.3.1, using a protocol adapted from (Bunce and Welsh, 1994).

6.2.9. NY-ESO-1 expression screening via RT-PCR.
Melanoma cell line cDNA was produced as described in sections 2.2.6 - 2.2.7, and PCR conducted as described in sections 2.2.2 - 2.2.3.

6.2.10. rAAV2/2 and rAAV2/6 eGFP and NY-ESO-1 vector construction.
rAAV_eGFP and rAAV_NY-ESO-1 vector construction was carried out as described in sections 2.1.15 and 2.2.22 with the technical assistance of Dr James Ussher. rAAV stocks were quantified as described in section 2.2.22.5.
6.3. Results.

6.3.1. Expansion and detection of CD8\(^+\) antigen-specific populations.

In chapter 5 we demonstrated that synthetic peptides could induce memory T cell expansion within a 14-day timeframe when applied to PBMC (section 5.3.6) and that, in a contrived system where a known clonal T cell population was seeded into autologous PBMCexp, surface expression of activation markers following peptide challenge could be used to collect a pure peptide-specific population via FACS (section 5.3.2.2). Our initial aim was to expand these observations and apply them to the isolation of peptide-specific T cell clones following peptide-mediated expansion of memory T cells in PBMC, and naïve T cells following a MoDC-dependent prime/PBMC boost regimen (Figure 6-1), with the eventual aim of applying these techniques to the isolation and expansion of NY-ESO-1-specific T cell clones.

6.3.1.1. Peptide-specific expansion of pre-existing memory cells within PBMC.

As NLV\(_9\)-specific cells often outgrew to make up >20% of CD8\(^+\) T cells following a single SLP-mediated expansion (Figure 5-20), we investigated expansion of T cells specific for the nonameric epitope CLG\(_{426-434}\) (CLG\(_{9}\)) derived from the sub-immunodominant Epstein-Barr Virus protein LMP-1 (Table 2-10). We utilised three donors shown to contain small but detectable CLG\(_{9}\)-specific CD8\(^+\) T cell populations (<0.05%, left panel; Figure 6-2). After a single round of peptide exposure (A; Figure 6-1) we found that CLG\(_{9}\)-specific cells in these donors expanded to 0.2 – 3.8% of all CD8\(^+\) T cells (middle and right panels, Figure 6-2).

![Figure 6-2 Peptide-specific expansion of memory CD8\(^+\) T cells within PBMC.](image)

Memory T cells specific for peptide CLG\(_9\) were expanded through a single application of 10\(\mu\)M peptide to PBMC as described in Figure 5-20. CLG-specific cells were detected by staining for IFN\(\gamma\) production after peptide re-challenge as described in sections 2.5.1.5 and 2.5.1.6. CLG\(_9\)-specific cells were detected pre-stimulation (left panel) and at d12 following stimulation (middle panel). In three donors exhibiting <0.05% CD8\(^+\) CLG\(_9\) -specific precursor cells prior to expansion, expansions of up to 3.8% of total CD8 T cells were generated (right panel).
Following peptide-specific memory T cell expansion and screening, we aimed to isolate CLG$_\gamma$-specific cells based on activation marker expression following 24-26h peptide recall, as previously shown in Figure 5-7. We investigated the co-expression of CD25, CD137 and CD69 (defined as optimal activation markers in sections 5.3.2.1 - 5.3.2.2) on both our CLG$_\gamma$-specific expanded memory cells (A; Figure 6-3), and on PBMC treated with the same cytokine regimen and then spiked with an ELA$_\gamma$-specific T cell clone to ~10% of total cells (B; Figure 6-3). The ELA$_\gamma$-clone-spiked sample served as both a control for activation marker expression, and a control for the efficacy of our SSP recall. We found that the activation marker$^+$ population in this sample (20% of CD8$^+$ events, which constituted ~50% of total cells at d12) did approximate all clonal cells added, indicating that the peptide recall had been saturating. We assessed co-expression of each of the three activation marker pairs (A, B; Figure 6-3: CD69/CD25 (top panels); CD137/CD25 (middle panels); CD137/CD69 (bottom panels) within the CD8$^+$CD56$^-$ fraction of unstimulated and stimulated expansion samples, using expression gates defined by fluorescence-minus-one (FMO) controls for each marker, and then assessed the expression of the remaining marker within each double-positive gate, in order to assess whether one marker combination was superior or if triple$^+$ gating was necessary for stringent sorting. We found that in spiked clone samples each marker combination defined cells that were 93-100% positive for the remaining marker (B; Figure 6-3), but that this specificity was only observed when gating on CD137$^+$CD25$^+$ cells in the CLG$_\gamma$-peptide expansions (A, middle panels; Figure 6-3) which were typically >95% CD69$^+$ across all three donors. By contrast, gating on CD69$^+$CD25$^+$ or CD137$^+$CD69$^+$ minor CD137$^+$ and CD25$^+$ populations were observed, respectively, indicating that triple-marker gating may confer superior specificity (A, right panels; Figure 6-3). Based on these data individual CD25$^+$/CD69$^+$/CD137$^+$ events were sorted and clones expanded as described in sections 2.5.3.2 and 2.3.11. Interestingly, although across three donors the appearance of T cell clones was similar to that seen following pentamer-mediated T cell sorting (27-50% of wells seeded) no clones formed large, rapidly metabolising pellets, and many died prior to d22, indicating that peptide recall stimulation followed by reactivation with PHA within 24h may be deleterious for T cell survival. Clones that did survive to d22 were analysed for CLG$_\gamma$ specificity via IFN$\gamma$-ELISPOT (C; Figure 6-3) and CD137 expression (D; Figure 6-3) in response to CLG$_\gamma$-loaded T2 cells, and specificities of 50, 67 and 75% were observed across the three donor clone sets tested (overall 16/25 tested clones). These results are summarised in Table 6-4.
Figure 6-3 Validation of activation-marker guided CD8+ T cell sorting from PBMC.

A: Following a single round of PBMC-CLG9-mediated memory T cell expansion (Figure 6-1 and Figure 6-2) expansions from three donor were re-challenged with 1µM CLG9 (recall, middle panels) or DMSO (unstimulated, left panels) and at 26h post-challenge were stained for CD3, CD8, CD25, CD69 and CD137 (section 2.5.1.1). Collection gates were drawn based on CD25, CD69 and CD137 expression in unstimulated samples (left panels) and were also based on FMO controls and Dynabead®-activated positive control (not shown). Within each paired collection gate (CD69+/CD25+; CD137+/CD25+; CD137+/CD69+) expression of the remaining marker was assessed (right panels). CD8+CD25+CD69+CD137+ events were sorted and T cell clones generated based as described in sections 2.5.3.2 and 2.3.10. Data shown is representative of three donors.

B: As an additional control, PBMC spiked with an autologous ELA26-35-specific T cell clone to ~20% of total CD8+ T cell were stimulated with 1µM ELA26-35 and equivalent nested gates drawn.

C: Surviving d22 T cell were screened for responsiveness against T2 loaded with no peptide (NP) or CLG9 (Pep) by IFNγ ELISPOT (section 2.4.4). Clones exhibiting 'Pep' IFNγ production above 'NP' well were considered to be specific (+). Positive and negative control well sets, containing T2 only (-) and T2 loaded with no peptide or with ELA26-35 and incubated with an ELA26-35-specific T cell clone (+) are also shown.

D: As low levels of background IFNγ could be seen in some NP wells, three clones exhibiting this pattern in (C) were incubated with T2 loaded with no peptide or with CLG9 and stained for surface CD137 expression. In all cases this confirmed that clones were CLG9-specific.
6.3.1.2. Sequential MoDC-mediated priming and PBMC-mediated secondary expansion of naïve CD8+ T cells.

Having demonstrated that activation-marker guided sorting could be used to isolate antigen-specific memory T cells following a single PBMC:peptide-mediated expansion and recall, we aimed to expand our protocol to allow isolation of primed and expanded naïve T cells. As naïve T cell priming is fundamentally dependent on CD28- and CD27-mediated co-stimulatory signals to allow T cell activation and expansion, initial presentation in these assays was carried out using matured, peptide-loaded MoDC, following a protocol adapted from (Wolfl and Greenberg, 2014) (B; Figure 6-1).

Interestingly, MoDC have been found to be sub-optimal for secondary expansion of primed T cells, potentially because they present too potent a stimulus, leading to activation-induced cell death (Ho et al., 2006), as such secondary expansion was conducted using peptide-loaded PBMC introduced to primary expansions at a 1:5 ratio. We primed and expanded naïve T cells from two healthy donors over several assays using SSP derived from two therapeutically relevant targets: ELA26-35 derived from Melan-A (as described in chapter 3) and a suite of three SSP from the cancer-testis antigen ‘Preferentially expressed antigen on melanomas’ (PRAME) 100-108; 425-433 and 435-443 (Kessler et al., 2001; Quintarelli et al., 2008).

Naïve ELA26-35-specific T cells can typically be found at an unusually high precursor frequency (>0.01%) due to a high thymic output (Pittet et al., 1999; Zippelius et al., 2002). This population is extremely heterogeneous in terms of Vβ usage (Zippelius et al., 2002) and exhibits a profound capacity to expand following MoDC-priming (Wolfl et al., 2011). Consistent with these previous reports, we found that ELA26-35-specific T cells could be rapidly expanded following a single priming incubation, typically reaching 10-50% of total CD8+ T cells (one example is discussed in Figure 6-5). These ELA26-35-specific expansions were used to validate activation-marker-guided T cell cloning from naïve expansions (Figure 6-5, below By contrast, PRAME-specific T cells were typically undetectable pre-priming in healthy donors (B, left panel; Figure 6-4) and required both a round of MoDC-mediated priming to elicit a small detectable population (B, right panel; Figure 6-4), and a subsequent secondary expansion mediated by PBMC loaded with PRAME100/425/435 to allow outgrowth of a large, easily detectable population (C; Figure 6-4). Following secondary expansion we were able to map peptide-specific responses and found that in this donor T cell responses were directed against PRAME100 and 435, but not PRAME425 (C,D; Figure 6-4). Although these expansions were not used a source for activation-marker-guided T cell cloning, this magnitude and pattern of response was consistent over several experiments and confirmed that our MoDC-prime/PBMC-boost expansion protocol was effective in expanding naïve T cell populations and allowing detection of a peptide-response hierarchy.
Chapter 6 – Results IV

Figure 6-4 MoDC-mediated peptide-specific expansion of naïve T cells from HLA-A2+ PBMC.

T cells specific for preferentially expressed antigen in melanoma (PRAME) were expanded via sequential MoDC-peptide-mediated priming and secondary PBMC stimulation in healthy donors as described in section 2.3.9 and Figure 6-1. B-D: PRAME-peptide specific CD8+ T cells were detected by staining for IFNγ production after peptide re-challenge as described in sections 2.5.1.5 and 2.5.1.6. A: One representative IFNγ assay showing isotype control (red) and IFNγ-stained (black) samples in a contrived assay whereby PBMC were spiked with an ELA26-35-specific T cell clone to ~2%. B: MoDC-mediated priming with a PRAME100/425/435 peptide pool expanded a detectable specific T cell population. C; D: Secondary stimulation with PRAME100/425/435-pulsed PBMC allowed expansion of this specific population to levels where individual peptide responsiveness could be determined. Results are representative of one expansion of three. This donor developed responses against PRAME100 and 435 only. An equivalent protocol was used to expand ELA26-35-specific T cells in healthy donors.

Naïve ELA26-35-specific cells could be readily expanded from an ~\leq 0.05 CD8+ precursor frequency (A; Figure 6-5) to form a major component of the total CD8+ T cell pool following a single MoDC-mediated stimulation. To assess whether our activation-marker-guided T cell cloning strategy could also be applied to these populations, we measured the ELA-pentamer+ fraction of total CD8+ T cells at d12 of expansion (B, left panels; Figure 6-5) and then stimulated expansions by addition of 1\muM ELA26-35. At 26h, we analysed CD25, CD69 and CD137 expression within the CD8+CD56- fraction of
unstimulated (B, right panels; fig x) and peptide-stimulated (B, central panels; Figure 6-5). We found that the activation-marker* population typically approached, but did not match, the pentamer+ population – this may have been because, as seen in Figure 4-5, some ELA26-35-specific T cells are of insufficient TCR avidity to recognise peptide below 10µM-loading, even on professional APC. As in section 6.3.1.1 and Figure 6-3, gating was defined based on unstimulated expression levels and FMO controls. Individual CD8+ CD25+CD69+CD137+ events were sorted and stimulated as described in sections 2.5.3.2 and 2.3.11. Similar to outgrowth-efficiency results observed in sections 3.2.3.3 and 6.3.1.1, 13/30 (43%) of seeded wells yielded T cell blasts, and 9 clones remained viable at d22 to allow validation via IFNγ ELISPOT against ELA26-35-loaded T2. All clones tested were IFNγ+. These cloning results are summarised in Table 6-4, and provide further proof of the sensitivity and specificity of this activation-marker guided system.

Figure 6-5 Validation of activation-marker guided CD8+ T cell sorting from primed naïve expansions.  
A: Prior to MoDC-peptide-priming, donor PBMC were screened for ELA26-35.precursor frequency (section 2.5.1.4). All CD8+ events are shown in the absence (FMO, left) or presence (right) of ELA-pentamer. B: Following a single round of MoDC-mediated priming and expansion (B, Figure 6-1), this donor exhibited up to 15.4% ELA26-35-specific T cells within the CD8+ population (Pentamer, D12, two replicates expansion shown). PBMC were re-challenged with 1μM ELA26-35 (recall) or DMSO (unstimulated) and at 26h post-challenge were stained for CD3, CD8, CD25, CD69 and CD137 (section 2.5.1.1). Collection gates were drawn based on CD25, CD69 and CD137 expression in unstimulated samples (right panels) and were also based on FMO controls and Dynabead®-activated positive control (not shown). CD8+CD25+CD137+ events were sorted and T cell clones generated based as described in sections 2.5.3.2 and 2.3.10 C: Following clonal expansion, surviving T cells were screened for responsiveness against HLA-A2+ LCL loaded with no peptide (NP) or ELA26-35 (Pep) by IFNγ ELISPOT (section 2.4.4). Clones exhibiting ‘Pep’ IFNγ production above ‘NP’ well were considered to be specific (+) Negative control wells containing LCL only are also shown (top left, ‘-’).
6.3.2. Activation-marker-guided sorting of CD4+ and CD8+ T cells responsive to NY-ESO-1 SLP.

Having demonstrated that activation-marker guided T cell sorting following expansion and peptide recall could be used to isolate CD8+ T cell clones with high levels of specificity, we then sought to expand our approach to isolate both CD4+ and CD8+ T cells responsive to epitopes derived from long NY-ESO-1 polypeptides. We previously demonstrated that SLP were more efficiently processed and presented than full-length recombinant protein (Figure 5-19). Further, several studies have demonstrated that the majority of currently known CD4+ and CD8+ T cell epitopes exist within three highly hydrophobic regions within NY-ESO-1, spanning residues 74-116; 118-144 and 153-180 (Table 6-1 & Table 6-2) and (Eikawa et al., 2013; Gnjatic et al., 2006; Valmori et al., 2007). The host laboratory had access to clinical specimens from previous melanoma vaccines, validated as having ≤1% T cell responsiveness against overlapping 18-mer peptides within one or more of these regions (Sintia Winkler, personal communication). We treated patient PBMC samples with each of NY-ESO-1 SLP 79-116, 118-143 and two overlapping 18-mers spanning residues 151-174 (151-169+157-174) using the ‘continuous’ SLP application strategy optimised in section 5.3.6, and standard cytokine support (A; Figure 6-2 and section 6.2.4). At d14 IFNγ responses within the CD3+CD4+ and CD3+CD8+ T cell compartments of samples of each expansion were assessed (section 2.5.1.5), as compared to mock expanded (DMSO), matched SLP-recall samples (A; Figure 6-6). Donor/subset/peptide combinations showing notable IFNγ responses were stimulated again with cognate SLP, and stained for activation marker expressions previously described (B; Figure 6-6). As in sections 6.3.1.1 and 6.3.1.2, CD8+CD56+CD25+CD69+CD137+ events were sorted to establish CD8+ T cell clones (B, upper right panels; Figure 6-6), while CD4+ CD56+CD25+CD69+CD134+ events were sorted to establish CD4+ T cell clones (B, lower right panels; Figure 6-6). CD4+ activation-marker selection was based on results observed in section 5.3.2 and Figure 5-6). All seeded T cells were restimatulated and expanded as previously described (section 2.3.10). T cell outgrowth was scored and surviving clones screened for specificity at d22 by IFNγ-ELISPOT against autologous SLP-loaded LCL (C; Figure 6-6). Similar to previous results, CD8+ T cells showed high T cell outgrowth efficiency (40-44% of wells containing blasts across two SLP plates), but relatively poor formation of large clonal pellets and survival at d22 (Table 6-4). 1/2 SLP 79-116 and 6/8 SLP 79-116-specific T cell clones were shown to be SLP-specific via ELISPOT, and this one SLP 118-143 and three 79-116 specific clones were able to be expanded to form G2 and G3 stocks. Interestingly, although CD4+ T cells showed a similar outgrowth efficiency (30; 42 and 68% of wells contained T cell pellets across two donors and three SLP plates) survival and large pellet formation amongst these CD4+ clones was excellent, with 70-95% of all blasting clones (across three plates) surviving to analysis at d22. These clones also exhibited a high level of specificity, with 70-97% of tested clones proving IFNγ-ELISPOT+. Several G2 and G3 clone stocks recognising SLP 118-143 (two donors) and SLP 151-
169/157-174 were expanded for subsequent analysis. Given the large number of available CD4+ clone pellets, only the healthiest from each SLP-expansion were re-expanded. These data are summarised in Table 6-4, and demonstrate that CD8+, and especially CD4+ T cells specific for epitopes derived from SLP can be rapidly isolated and expanded based on activation-marker-guided FACS.

Figure 6-6 CD4+ and CD8+ activation-marker-guided cloning of NY-ESO-1 SLP-specific T cells.

PBMC from previously vaccinated melanoma patients were stimulated for 12d with SLP 79-116, 118-143, 151-174, or mock-stimulated with DMSO only. A: at D12, PBMC samples were re-challenged with peptide in the presence of GolgiStop™ for 6h (section 2.5.1.5), and IFNγ+ CD3+CD4+ and CD3+CD8+ enumerated. Two representative examples are shown, using peptides as indicated. B: Peptide-responsive cultures were then challenged again with either peptide or DMSO for 24h, and cells then stained for CD4, CD8, CD56, CD25, CD69, CD134 and CD137 (section 2.5.1.1). Cells were gated into CD4+CD56-; and CD8+CD56- fractions (left and middle panels), and within each gate CD69hi/CD25hi/CD134hi (CD4+ cells, bottom right) or CD69hi/CD25hi/CD137hi (CD8+ cells, top right) were individually sorted into cloning wells (right panels, orange). Collection gating was based on CD25/CD69/CD134/CD137 expression in matched DMSO-challenged (‘unstimulated’) samples (right panel, black). C: Following clonal expansion, healthy pellets were screened for responsiveness against autologous LCL loaded with no peptide (-Pep) or challenge peptide (+Pep) by IFNγ ELISPOT (section 2.4.4). Clones exhibiting +Pep IFNγ production above –Pep well were considered to be specific.
Table 6-4  CD8⁺ T cell activation marker-guided cloning summary.

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<th>Peptide</th>
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<th>T cell Subset</th>
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<td>13</td>
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6.3.3. Common gamma chain cytokines induce non-specific ‘activation marker’ expression.

During peptide-mediated expansion of naïve and memory T cells, we supported PBMC with a complex mixture of cytokines (section 6.2.4 and Figure 6-1), with administration timed to replicate in vivo signalling events. Specifically, IL-12 was provided at d0 to replace IL-12 production by mature DC (Merrick et al., 2005), IL-2 was provided at d0 and d3 to ensure that the small number of stimulated precursor cells received IL-2 signalling, as without reference to their starting phenotype, there was no way to be sure of their IL-2-production capacity (Mallard et al., 2004). IL-7 and IL-21 were provided throughout expansion based on their previously described beneficial effects on T cell outgrowth and phenotype (chapters 3-4), and IL-15 was provided from d5-14 of expansion, because it has been shown to be an important factor in memory T cell expansion and maintenance (Weng et al., 2002). Interestingly, although the activation markers screened in section 5.3.2 are generally considered to be indicators of TCR-mediated T cell activation, when assessing marker expression in our unstimulated d12-d14 cultures we often observed the appearance of a CD3⁺CD8⁺CD56⁺ population (too great in magnitude to represent NKT cell expansion) and a CD3⁺CD8⁺CD56 CD69⁺ population. This TCR-independent expression was consistent across several cultures (A, B; Figure
6-7) and was not observed when assessing CD137 expression (A; Figure 6-7). To investigate whether this CD56 and CD69 induction was cytokine-mediated, we cultured PBMCexp previously produced from several donors (section 3.2.2) in Rs5 supplemented with only IL-2; IL-4; IL-7 or IL-15 for 10d and analysed expression of surface CD25, CD38, CD56, CD134, CD137 and HLA-DR on the CD4+ and CD8+ PBMCexp fractions pre- and post-culture (C; Figure 6-7). We found no expression or induction of CD25 or CD137 on either PBMCexp subset (data not shown on C). We found negligible induction of CD38, CD56 or CD69 by culture in IL-7 in either subset (C; Figure 6-7). By contrast, de novo CD38, CD56 and CD69 expression was induced on both CD4+ and CD8+ PBMCexp by IL-2 and IL-15, with expression being more prominent on CD8+ T cells. Interestingly, pre-existing CD134+ and HLA-DR+ populations within the CD4+ fraction, and an HLA-DR+ population within the CD8+ fraction could be observed, and these were retained in IL-2, -7 and -15 culture, suggesting that the initial process of polyclonal expansion by Dynabeads® in the presence of sustained IL-7 and IL-21 signalling was responsible for this marker expression. Finally, culture in IL-4 not only did not induce expression of any of the activation markers screened, but also led to a loss of CD134 and HLA-DR on previously positive cells (C; Figure 6-7). These data indicated that it was most likely sustained IL-2 supply, followed by sustained IL-15 administration that was responsible for the observed CD3+CD8+CD56+ and CD3+CD8+CD56-CD69+ populations in our cultures. Interestingly, culture of PBMCexp in IL-2 and IL-15 was associated with the appearance of large (100-500µm) clusters in vitro (E; Figure 6-7), consistent with expression of cell:cell adhesion molecules, while cells cultured in IL-7 remained in a confluent monolayer (D; Figure 6-7).
Figure 6-7 Application of common γc cytokines during T cell culture and expansion induces TCR-independent ‘activation marker’ expression.

A: CD69 and CD137 induction within the CD3+CD8+ CD56- subset of unstimulated peptide expansions described in Figure 6-1. Eight expansions across 4 donors are shown. B: CD69 gating within all CD3+CD8+ CD56- cells as described in A. One example is shown, demonstrating CD8-only (FMO; black) and CD8/CD69-stained (red) events. C: PBMCexp were thawed and cultured for 12d in cytokines (10ng/ml, refreshed every 72-96h) as indicated. Surface markers were detected via flow cytometry (section 2.5.1.1) on thawing (‘original’) and at d12 of culture. D: PBMCexp cultured in IL-7 (10ng/ml) for 12d, showing single cell monolayer and rounded morphology. E: PBMCexp cultured in IL-15 (10ng/ml) for 12d, showing the formation of large T cell clusters. D; E: scale bars represent 100µm, images acquired at 100x magnification (section 2.4.2).

Given the unexpected pre-existing expression of CD134 and HLA-DR on our PBMCexp samples, we next sought to further characterise the induction of cytokine-mediated TCR on T cells isolated directly ex vivo. Total T cells were isolated from three donors by MACS (section 2.3.8) and again cultured in Rs5 supplemented with IL-2; -4; -7 or -15 for 10d. Additionally, as IL-4 had shown the potential to antagonise expression of CD134 and HLA-DR, samples were also incubated in each of IL-2; -7 and -15 supplemented with IL-4. At d12 expression of each ‘activation marker’ listed in Table 5-1 was assessed on CD4+ and CD8+ subsets by flow cytometry (Figure 6-8). Although variability across donors was high, several trends were clearly evident. We found that ex vivo T cells displayed minimal activation marker expression (<5%), with the exception of a low background ICOS^+.
population in both CD4+ and CD8+ subsets. As previously observed, particular activation markers displayed specific cytokine-induction patterns, and some cytokines induced a wide range of surface marker expression, while others either did not, or antagonised induction by other cytokines (Figure 6-8; data summarised as an expression heat map to allow visualisation of induction patterns).

Consistent with previous observations (C; Figure 6-7), IL-4 induced negligible expression of any activation marker tested. Several activation markers proved cytokine-resistant within each subset, exhibiting \(<10\%\) induction at a population level on d12 – specifically, CD56, CD137 and CD154 on CD4+ T cells, and CD134, CD137 and CD154 on CD8+ T cells. Other activation markers exhibited intermediate inducibility, typically being found on up to 30\% of all T cells within a subset. These included CD38, CD69, CD134 and HLA-DR on CD4+ T cells, and CD56 on CD8+ T cells. As previously observed on PBMCexp culture (C; Figure 6-7), CD38, CD56 and HLA-DR were mainly induced by IL-2 and IL-15 treatment, while CD134 on CD4+ T cells was induced by IL-2, IL-7 and IL-15. Interestingly, although IL-4 alone did not induce any expression of these markers, it did not always antagonise expression on co-administration with IL-2/-7/-15 – in fact co-administration: increased CD4+ CD69 and CD134 expression compared to IL-2 alone; increased CD69 expression compared to IL-15 alone; and increased CD134 expression when compared to IL-7 alone, while having no effect on CD38 expression levels. Conversely, co-administration of IL-4 decreased CD56 expression on CD8+ T cells with any other cytokine.

The remaining markers showed a high level of responsiveness to particular cytokines. Within the CD4+ population, CD25 was most inducible by IL-7 and IL-15, with IL-4 co-administration showing increasing IL-7-mediated induction (up to ~70\% of cells), while antagonising IL-15 mediated expression. CD71 and ICOS were induced on ~27-47\% of all CD4+ cells by IL-2, -7 and -15, with expression typically showing a small decrease on IL-4 co-administration. Within the CD8+ T cell population, CD25 and CD69 were moderately induced by IL-2 and were highly induced (~50-55\%) by IL-15, but expression was notably diminished on IL-4 co-administration. Conversely, CD71 and ICOS were induced by IL-2, -7 and -15 signalling, and were notably upregulated on IL-4 co-administration (up to ~70\% in the IL-2 + IL-4 condition for CD71). Finally, as observed in Figure 6-7, CD38 and HLA-DR were strongly induced by IL-2 and IL-15 (with only weak induction by IL-7) with IL-4 co-administration typically having only a weak antagonistic effect. These data demonstrate that several putatively TCR-dependent activation markers are highly inducible by common \(\gamma\)c cytokine signalling, but that induction patterns, especially in a multiple-cytokine milieu, are highly complex. It should be noted that as T cells were initially CD25-, much of the early ‘IL-2’ signalling is probably solely mediated by CD122, prior to the onset of CD25 expression. This is also consistent with the similar patterns of marker expression induced by IL-2 and IL-15, which share CD122 as part of their signalling complex.
Figure 6-8 Application of common γc cytokines during pure T cell culture and maintenance induces TCR-independent ‘activation marker’ expression.

Pan-T cells isolated from three donors as described in section 2.3.8 were cultured in Rs5 supplemented with cytokines alone or in combination as indicated (10ng/ml per cytokine, 1 x 10^5 T cells per condition per donor). Media and cytokine refreshed every 72-96h for 12d. T cells were stained for CD4 and CD8 only, or for CD4, CD8 and surface activation markers as indicated at d0 (pre) and d12 and samples analysed via flow cytometry (section 2.5). Data is shown as mean +/- SD % marker-positive CD4+ (left) or CD8+ (right) T cells for three donors, and is presented as a heat map to allow visualisation of induction patterns.
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6.3.4. Characterisation of CD8$^+$ NY-ESO-1-specific T cell clones.

In section 6.3.2 we isolated and expanded four CD8$^+$ T cell clones from donor p062.004 via activation-marker guided FACS following NY-ESO-1-SLP-mediated expansion and recall. Three clones, designated p062.004 1D7, 1F5 and 1F10, were responsive to SLP 79-116, and one, designated p062.004 1C11 was responsive to SLP 118-143. These clonal pellets were restimulated to form G2 and G3 stocks as described in section 2.3.10. Cell surface marker phenotyping and cytokine responsiveness were investigated using G2 stocks (each expanded to $>2 \times 10^7$ cells), and fine epitope and HLA restriction mapping were carried out using G3 stocks.

6.3.4.1. CD8$^+$ T cell clones retain co-stimulatory marker expression and IL-7 responsiveness.

We profiled expression of memory, co-stimulatory and SLO-homing markers on the surface of CD8$^+$ clones via flow cytometry. All clones screened were universally CD45RO$^{(hi)}$, CD45RA$^{(dim)}$ and CCR7$^-$, indicating a T$_{EM}$ phenotype (A; Figure 6-9). Further, these clones exhibited universal PD-1 expression, although this did not appear to have an impact on subsequent proliferation, as samples of each clone could be expanded to form a G3 stock, representing $>5$ further population doublings.

Given that these clones had been expanded at least once in vivo, twice in vitro prior to cell-sorting and then two further times to create G2 stocks, a highly differentiated memory phenotype was anticipated. Importantly however, G2 stocks of these clones expanded in the presence of IL-7 and IL-21 exhibited CD28 and CD62L retention. Each clone was assessed for responsiveness to common γc cytokines, following previously described survival assays protocols (sections 3.1.6 and 3.2.3.6). We found that each clone showed maintenance or a low level of homeostatic proliferation in response to IL-2 or IL-15, and clones could be maintained in IL-7 (typically 70-90% survival over 12d) and IL-4 (45-90% survival over 12d) (B; Figure 6-9). Surface IL-7Rα was detectable on all four clones (one example is shown in C; Figure 6-9), confirming that these were true memory cells rather than effector cells.
Figure 6-9 CD8+ T cell clones exhibit varying levels of co-stimulatory marker expression and homeostatic cytokine responsiveness.

A: Surface marker expression was analysed via flow cytometry (sections 2.5.1.1 and 2.5.1.3). Data shown as % of live CD8+ events that exhibited marker expression, with gating based on paired CD8-only control samples. B: Clones were washed and plated at 1x10^5/well in U-bottom 96wp in Rs5 supplemented with cytokine as indicated. Media and cytokines refreshed every 72-96h. At d12 viable cells enumerated via trypan blue exclusion and expressed as % of seeded cells. Data shown as mean + SEM for triplicate samples per clone per condition. C: Clones were washed and incubated in cytokine-free Rs5 for 48h (section 2.4.7) and the stained for surface CD8 and IL-7Rα. One clone, exhibiting high surface IL-7Rα expression (1F5) is shown. Data represent CD8-only FMO (black filled) and CD8/IL-7Rα (black line) stained samples.

6.3.4.2. T cell clones recognise three distinct minimal peptide epitopes in an HLA-C*03-restricted context.

SLP 79-116 and 118-143 contain several previously described CD8+ T cell epitopes, presented in a variety of HLA-A, B and C-contexts. In order to better define the minimal peptide epitopes (MPE) recognised by each p062.004 CD8+ clone within SLP 79-116/118-143, clones were screened for responsiveness to autologous LCL loaded with the SLP against which they were raised or with individual 18mer peptides spanning the breadth of that SLP. These 18mers exhibit an 11-amino acid overlap, allowing rapid definition of rough MPE sequences. Partial p062.004 MHC class I sequence information (at the HLA-A and B loci) was available (Ludwig Institute), while sequence-specific-primer(SSP)-PCR (described in sections 6.2.8 and 6.3.4.3, below) indicated that p062.004 was HLA-C*03+ (Table 6-3 and section 6.3.4.3). Three HLA-C*03-restricted nonameric epitopes have previously been described within the sequence of SLP 79-116: amino acids 92-100; 94-102 and 96-104 (see Table 6-1 for description and references). As such, 79-116-responsive clones p062.004 1D7, 1F5 and 1F10 were also screened against LCL loaded with these peptides in order to rapidly identify if any of these known sequences were the MPE. These data are summarised in Figure 6-10: We found that clones 1D7 and 1F5 recognised LCL loaded with 79-116, overlapping 18mers 85-102 and 91-108, and peptide 92-100, indicating that 92-100 was the cognate MPE for both clones (top panels, A;
Figure 6-10). Clone 1F10 recognised LCL loaded with 79-116, but only recognised 18mer 91-108, and peptide 96-104, indicating that 96-104 was its cognate MPE (bottom left, A; Figure 6-10). As such, CD8+ T cell responses against two distinct epitopes could be raised from within one donor by stimulation with a single long peptide.

Clone 1C11 recognised LCL loaded with 118-143, but only recognised 18mer 121-138 (bottom right, A; Figure 6-10). As 18mers 115-132 and 127-144 were not recognised by this clone, this indicated that its MPE could not lie exclusively between residues 121-132 or 127-138, i.e. the N-terminus of its MPE must lie within residues 123-126, assuming that this clone recognised an MPE of ≤11 amino acids. To further define the MPE for this clone, we designed a further set of 11mer peptides exhibiting a one amino acid frameshift and spanning residues 123-136, and measured clone 1C11 activation by LCL loaded with these peptides over a 5-log10 dilution range. We found that responses against peptides 124-134 and 126-136 were poor, while strong and closely matched responses were seen against p123-133 and 125-135, indicating that the MPE was likely to be amino acids 125-133 (B; Figure 6-10). It is possible that the extremely poor responses against p124-134 were due to exposed lysine 124 inhibiting MPE processing or binding to HLA-C*03. Based on these data we defined the MPE for clone 1C11 as the nonameric sequence 125-133, although this remains to be absolutely confirmed with a further overlapping nonameric peptide library.
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Figure 6-10 CD8+ T cell clones derived from donor p062.004 recognise three discrete peptide epitopes.

A; B: p062.004 LCL were loaded with 1µM peptide as indicated, and then incubated for 24h with CellTrace™ Violet-stained clones p062.004 1D7; 1F5; 1F10 and 1C11 as indicated. Samples were stained for CD137 expression (section 2.5.1.1), and CD137 MFI determined within all CellTrace™ Violet+ events. Data shown as mean + SEM for duplicate samples, representative of two independent experiments.

Having defined absolute or putative MPE for CD8+ clones p062.004 1C11, 1D7, 1F5 and 1F10, we assessed the ‘on-cell’ TCR:pMHC for each clone using autologous LCL loaded with 92-100, 96-104 or 125-135 across a 7-log₁₀ series, as previously described (section 4.3.1). We observed that clones 1D7 and 1F5 (MPE 92-100) exhibited saturating activation to 10nM peptide, and ~40-50% activation at 1nM peptide (Figure 6-11). Clones 1F10 (96-104) and 1C11 (125-135) exhibited approximately 10-fold lower affinity for their cognate MPE with saturating responses to 100nM peptide and ~50-70% activation at 10nM peptide loading (Figure 6-11). It should be noted that results observed for clone 1C11 may underestimate true TCR:pMHC affinity for the absolute MPE of this clone, as the 11mer used (125-135) requires processing that may delay or inhibit MPE presentation compared to short peptide alone (as seen in Figure 5-9). Interestingly, clones 1F10 and 1C11 exhibited very similar TCR:pMHC affinities for their MPE to clone ELA 2D10 for ELA26-35 A27L.
Figure 6-11 CD8+ T cell clones exhibit disparate ‘on-cell’ TCR:pMHC affinities.

p062.004 LCL were loaded with titrated doses of p92-100; p96-104 or p125-135 peptide for 2h, washed three times and then incubated for 24h with CellTrace™ Violet-stained clones p062.004 1D7; 1F5; 1F10 and 1C11 as indicated. Samples were stained for CD137 expression (section 2.5.1.1), and CD137 MFI determined within all CellTrace™ Violet+ events. Data shown as mean + SEM for duplicate samples, representative of two independent experiments.

Having characterised the MPE for clones 1D7, 1F5, 1F10 and 1C11 we sought to confirm their p:MHC-restriction using a panel of partially HLA-matched LCL lines, T2 cells, and the transformed monocytic and macrophage cell lines THP-1 and U937 (HLA alleles detailed in Table 6-3 and reiterated in figure Figure 6-12). APC were loaded with 1μM MPE for each clone, and T cell activation was determined via flow cytometric detection of surface CD137 as previously described (sections 2.5.1.1 and 4.3.1.2). The response patterns of clones 1D7, F5 and F10 to autologous and partially-matched APC lines confirmed that each of these clones was HLA-C*03-restricted (A; Figure 6-12). Clone 1C11 exhibited a more complex response pattern to APC loaded with peptide 123-133. Maximal activation was only observed on incubation with HLA-C*03+ LCL and THP-1 lines (left panel, B; Figure 6-12). Co-incidentally, each of these lines were also HLA-A2*, but no response was observed against HLA-A2+ p062.006 LCL (confirmed to be HLA-C*03 by SSP-PCR: A; Figure 6-13), demonstrating that clone 1C11 did not recognise peptide in an HLA-A2-restricted context. Interestingly, partial activation of clone 1C11 was observed against M56-LCL and U937 loaded with 123-133 (left panel, B; Figure 6-12). Neither of these lines share an HLA-B allele with each other or with p062.004, however both M56 and U937 express HLA-C*01 and -C*07, both closely related to HLA-C*03 (Bunce and Welsh, 1994; McKenzie et al., 1999). Interestingly, when we extended our
analysis by loading each APC line with the longer peptide 121-138, we found that 1C11 responsiveness to M56 LCL was retained, but was lost against U937, potentially suggesting that the U937 line lacked the ability to process and present MPE from this longer peptide (right panel, B; Figure 6-12). From these data, we inferred that an epitope similar to the 125-133 MPE could be cross-presented in either an HLA-C*01 or C*07-restricted context, with sufficient cross-reactivity to activate the typically HLA-C*03-restricted clone 1C11. To define which of HLA-C*01/C*07 was responsible, we investigated responses of clone 1C11 to T2 loaded with either 123-133 or 121-138 across a 1-log10 range. T2 express HLA-C*01 but not -C*07 (Table 6-3). We have previously shown that T2 are efficiently able to process and present SLP to CD8+ T cells in an HLA-A2-restricted context (sections 5.3.3 and 5.3.5), and others have demonstrated that T2 are also able to present HLA-C*01-restricted peptides to CD8+ T cells (Andersen et al., 1999; Stoll et al., 2015). We found that T2 loaded with either 123-133 or 121-138 showed equivalent capacity to activate clone 1C11 to M56-LCL, confirming that the partial cross-reactive activation observed was mediated by HLA-C*01 rather than C*07 (B; Figure 6-12).
### Figure 6-12 CD8+ T cell clones derived from donor p062.004 display HLA-C*03 restriction and HLA-C*01 cross-reactivity.

A: M56-, p062.004-, .00-5- LCL, THP-1 and U937 were loaded with no peptide or 1µM p92-100 or p96-104 peptide for 2h, washed three times and then incubated for 24h with CellTrace™ Violet-stained clones p062.004 1D7; 1F5; or 1F10 as indicated. B: M56-, p062.004-, .00-5- LCL, THP-1 and U937 were loaded with no peptide or 1µM p125-135 or p121-138 peptide for 2h, washed three times and then incubated for 24h with CellTrace™ Violet-stained clone p062.004 1C11. C: M56-LCL, p062.004-LCL and T2 cells were loaded with no peptide or titrated concentrations of p125-135 or p121-138 as indicated, and then washed and incubated with 1C11 as in B. A; B; C: All samples were stained for surface CD8 and CD137 expression (2.5.1.1) and CD137+ cells expressed as a % of total CD8+ CellTrace™ Violet+ events. Data shown as mean + SEM of duplicate samples, representative of two independent experiments.

### 6.3.4.3. Available melanoma lines did not co-express HLA-C*03 and NY-ESO-1.

In order to confirm whether these four NY-ESO-1-responsive clones were able to recognise their MPE presented by NY-ESO-1+ melanoma cells, we profiled HLA-C*03 and NY-ESO-1 expression on each of our available melanoma cell lines by SSP-PCR using a gDNA template, and RT-PCR using a cDNA template, respectively (detailed in sections 2.2.3.1, 2.2.4 and 2.2.7). In all cases
amplification of a β-actin amplicon was used to confirm PCR success and template quality. We found that p062.004 and p062.005 were the only LCL expressing HLA-C*03 (A; Figure 6-13). We found strong NY-ESO-1 expression by melanoma lines MZ2, DAGI, Trombelli and Me275, and weak expression by line SK-Mel-29 (B; Figure 6-13). Unfortunately, when profiling HLA-C*03 expression we found that only line Na8 (NY-ESO-1) expressed HLA-C*03 (C; Figure 6-13). As none of our available melanoma lines shared HLA-C*03 and NY-ESO-1 coexpression, we sought an alternative system with which to demonstrate endogenous processing and presentation of the MPE for each clone.

Figure 6-13 NY-ESO-1 and HLA-C*03 expression screening via RT-PCR.

A; C: Paired HLA-C*03 and β-actin PCR reactions using 10ng LCL gDNA or melanoma line gDNA template and KAPA 2G robust polymerase. Amplified products at 562 and 353bp are indicated. L: ladder; M56/62.04/62.05/62.06: donor LCL gDNA; -: HLA-C*03- gDNA; TC: no template control. B: Paired NY-ESO-1 and β-actin PCR reactions using 100ng cDNA template and Platinum Pfx Polymerase. All PCR products analysed by AGE. L: ladder; N: Na8; Z: MZ2; D: DAGI; T: Trombelli; M: Me275; 23: SK-Mel-23; 29: Sk-Mel-29; F: Fibroblast; Ts: testes; +: rNY-ESO-1 plasmid; NTC: no template control. C: Paired HLA-C*03 and β-actin PCR reactions using 100ng cDNA template and KAPA 2G robust polymerase. L: ladder; N: Na8; Z: MZ2; D: DAGI; T: Trombelli; M: Me275; 23: SK-Mel-23; 29: Sk-Mel-29; M56: HLA-C*03- control; 62.04: HLA-C*03+ control; NTC: no template control
6.3.4.4. All clones recognised endogenously derived cognate epitopes presented by 

rAAV2/6_NYESO1-transduced autologous LCL.

In order to establish a system whereby endogenous processing and presentation, rather than cross-presentation, could be assessed, we produced recombinant adeno-associated-virus (rAAV) expression vectors carrying eGFP or NY-ESO-1 transgenes under the control of a strong CAG promoter. We amplified NY-ESO-1 cDNA from testis RNA as described in section 2.2.7, using primers NYESO1_AAV_F and NYESO1_AAV_R (Table 2-1). As seen when using primers NYESO1_HTB_F/R in section 5.3.4.1, these primers also amplified LAGE-1, but NY-ESO-1 and LAGE-1 amplicons could easily be distinguished on AGE (A; Figure 6-14). NY-ESO-1 amplified with terminal PacI and ClaI restriction sites was excised, digested and ligated into plasmid vector pAM/CAG_NYESO1_WPRE_bGHpA, encoding rAAV vector genome and promoter within inverted terminal repeat packaging sequences. An equivalent plasmid vector containing encoding eGFP (pAM/CAG_eGFP_WPRE_bGHpA) was kindly provided by Dr James Ussher. The rAAV vector genome elements encoded by these plasmids were derived from a serotype 2 virus (rAAV2). Several serotypes of rAAV (1-11) exist based on the viral capsid protein, and these different serotypes are known to exhibit differential transduction of mesenchymal, epithelial and hematopoietic cells based on different capsid:cell surface receptor binding (Sheppard et al., 2013; Ussher and Taylor, 2010). As such, we produced rAAV2_eGFP and rAAV2_NY-ESO-1 packaged into two different capsid serotypes, serotype 2 (rAAV2/2) and serotype 6 (rAAV2/6), whereby the first number designates the vector genome type and the second number designates the capsid type. All rAAV vectors were produced by triple transfection of HEK293T cells by transgene expression cassette vector (pAM/CAG_NYESO1_WPRE_bGHpA or pAM/CAG_eGFP_WPRE_bGHpA), serotype-specific packaging plasmids (pAAV2/6 for serotype 6 or pNLRep for serotype 2), and the helper plasmid pFΔ6 – responsible for packaging of the transgene expression cassette into the capsid. Virions were subsequently isolated from HEK293T supernatant and cell lysate by heparin affinity chromatography (HAC) (section 2.2.22.4, following (Sheppard et al., 2013). Virion purity was assessed by SDS-PAGE and silver stain (B; Figure 6-14) and vector stocks titrated by quantitative real-time-PCR (section 2.2.22.5). We transduced several melanoma lines, LCL and MoDC generated from three donors with titrated vector genomes per cell (vgc) of rAAV2/2_eGFP and rAAV2/6_eGFP across a 2-log10 range. Consistent with previous reports, we found that rAAV2/2 was superior for transduction of melanoma cells, while rAAV2/6 was superior for transduction of hematopoietic LCL and MoDC, although melanoma cells exhibited much greater transduction rates overall (C, D; Figure 6-14).
Figure 6-14 Production and validation of eGFP- and NY-ESO-1- expressing rAAV2/2 and 2/6 vectors.

**A:** Amplification of NY-ESO-1 from testes RNA template via RT-PCR with Platinum Pfx DNA polymerase using primers NY-ESO-1_AAV_F and _R. PCR products were analysed via AGE. NY-ESO-1 major band (~550bp) and LAGE-1 minor band (~750bp) are indicated. W: water; NT: no template control; T: testes RNA template.

**B:** rAAV serotype 2/2 and 2/6 NY-ESO-1 and eGFP vectors were isolated via heparin affinity chromatography and eluted using 600mM NaCl. Elution fractions were pooled and analysed via SDS-PAGE and silver staining. rAAV capsid proteins (VP1/2/3) are indicated.

**C:** Melanoma lines SK-Mel-23, SK-Mel-29 and Trombelli; and hematopoietic LCL (one donor) and MoDC (3 donors) were transfected with rAAV2/2 and 2/6 vectors expressing eGFP for 48h at viral genome copies/cell indicated.

**D:** eGFP expression within transfected samples (black lines) was determined by flow cytometry. The eGFP+ gate for each cell type was defined based on untransfected cell background fluorescence (grey filled histogram).
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Having demonstrated that rAAV2/6 vectors were able to transduce LCL, we utilised this system to investigate endogenous NY-ESO-1 MPE presentation to clones M56 2F2 (responsive to SLL\textsubscript{167-175}) and p062.005 1D7, 1F5, 1F10 and 1C11. M56 and p062.004 LCL were transduced with empty rAAV2/6 or rAAV2/6\_NY-ESO-1 at $10^5$ vgc for 48h, to allow vector unpackaging and NY-ESO-1 transcription, or loaded with 100nM cognate MPE for 16h. We found that each clone exhibited CD137 upregulation when incubated with rAAV2/6\_NY-ESO-1 transduced autologous LCL, typically at higher levels than with peptide-loaded LCL, demonstrating that each MPE (92-100, 96-104 and 125-133) was naturally processed and presented, and that this presentation was highly efficient (Figure 6-15). No T cell activation was observed against LCL transduced with empty rAAV2/6 vector, confirming that T cell activation was NY-ESO-1 specific (Figure 6-15). The naturally processed MPE and HLA-restrictions for each CD8$^+$ T cell clone generated through activation-marker-guided FACS are summarised in Table 6-5, and relevance within the context of NY-ESO-1 immunogenicity discussed in chapter 7.

![Figure 6-15](image)

**Figure 6-15 All CD8$^+$ NY-ESO-1-specific T cell clones recognise rAAV2/6-NY-ESO-1-transduced autologous LCL.**

M56 and P062.004 LCL were transfected with rAAV2/6-NY-ESO-1 at 1x10$^5$ vgc/cell for 48h, loaded with 100nM short NY-ESO-1 peptide as indicated for 16h, or left untreated. All LCL were washed three times and then incubated with CellTrace$^\text{TM}$ Violet-labelled CD8$^+$ T cell clones as annotated (plot titles) at 3:1 for 24h. Samples were stained for surface CD8 and CD137 expression (section 2.5.1.1) and T cell clone activation was determined by flow cytometric detection of surface CD137 within the CD8$^+$ CellTrace$^\text{TM}$ Violet$^+$ gate. Representative plots of duplicate samples are shown.
Table 6-5 CD8+ T cell epitopes and MHC restrictions defined in this study.

<table>
<thead>
<tr>
<th>Peptide epitope (minimum defined range)</th>
<th>Representative clone(s)</th>
<th>MHC I Restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>92-100</td>
<td>CD8+ p062.004 1D7, 1F5</td>
<td>HLA-C*03</td>
</tr>
<tr>
<td>96-104</td>
<td>CD8+ p062.004 1F10</td>
<td>HLA-C*03</td>
</tr>
<tr>
<td>125-133</td>
<td>CD8+ p062.004 1C11</td>
<td>HLA-C<em>03 (HLA-C</em>01:02)</td>
</tr>
</tbody>
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6.3.5. Characterisation of CD4+ NY-ESO-1-specific T cell clones.

In section 6.3.2 we isolated and expanded 23 CD4+ T cell clones: 6 derived from donor M56 responsive to NY-ESO-1 SLP 151-174; 12 derived from donor M56 responsive to SLP 118-143; and 5 derived from donor p062.005 responsive to SLP 118-143, via activation-marker guided FACS. These clonal pellets were restimulated to form G2 and G3 stocks as described in section 2.3.10. Cell surface marker phenotyping and cytokine responsiveness were investigated using G2 stocks (each expanded to ≥2x10⁷ cells), and fine epitope and HLA restriction mapping was carried out using G3 stocks.

6.3.5.1. CD4+ T cell clones retain co-stimulatory marker expression and IL-7 responsiveness.

A limited phenotypic screen was carried out on eight CD4+ clones from donors M56 and p062.005, covering all SLP specificities. All clones tested were universally CD45RO+ (data not shown), and CCR7− (A; Figure 6-16) indicating a TEM phenotype. Clones screened exhibited negligible surface CD62L, but clonal populations were typically entirely CD28+ (one clone showed a biphasic CD28 expression profile) (A; Figure 6-16). All G2 clones could be maintained in IL-7 for extended periods. Surface IL-7Rα was detectable on most clones following incubation in cytokine-free medium (A, B; Figure 6-16), indicating that these were true memory, rather than effector, cells.
6.3.5.2. CD4⁺ T cell clones recognise minimal peptide epitopes presented by disparate HLA-DR/DP/DQ molecules.

In order to better define the minimal peptide epitopes (MPE) recognised by each CD4⁺ clone within NY-ESO-1 SLP 118-143 or 151-174, clones were screened for responsiveness to autologous LCL loaded with their cognate SLP or individual overlapping 18mer peptides spanning the breadth of that SLP. T cell clone activation was determined by flow cytometric detection of surface CD25 and/or CD134 expression (Figure 6-17). Each of the suite of M56 CD4⁺ 151-174 clones were found to recognise both 18mers 151-169 and 157-174, but not 145-162 or 163-180 (one exemplar shown in A; Figure 6-17). From these data, and as donor M56 was known to be homozygous for HLA-DPB1*0401 (Table 6-3) we concluded that each of these clones were specific for the well-characterised HLA-DP4-restricted epitope 151-170 (Table 6-2 and Zeng et al., (2001)). Each of the suite of M56 CD4⁺ 118-143-responsive clones were shown to exhibit responses to LCL loaded with 118-143 (data not shown) and to 18mers 115-132 and 121-138 only, suggesting that the MPE for these clones lay exclusively in the range 121-132 (one exemplar shown in B; Figure 6-17). Within the suite of p062.005 CD4⁺ 118-143-responsive clones two peptide recognition patterns emerged: all clones exhibited responses to LCL loaded with 118-143 (data not shown) but three clones exhibited responses against LCL loaded with 18mers 121-138 and 127-144 only, suggesting an MPE lying exclusively within the range 127-138 (one exemplar shown in C; Figure 6-17), while two further
clones were responsive against LCL loaded with 18mers 115-132 and 121-138 only, suggesting an MPE lying exclusively within the range 121-132 (one exemplar shown in D; Figure 6-17). As such, CD4+ T cell responses against two distinct epitopes could be raised from within one donor by stimulation with a single long peptide. Interestingly, although donors M56 and p062.005 both produced CD4+ T cells recognising a motif within 121-132, these donors were highly HLA-disparate (Table 6-3) and as such it was likely that this epitope promiscuously associated with multiple MHC class II molecules.

Figure 6-17 CD4+ clones derived from donor M56 and p062.005 recognise three discrete NY-ESO-1 epitopes.

A; B: M56 LCL were incubated for 16h with 1µM SLP or in media alone as indicated (pX-pX). LCL were then washed three times and incubated for 24h with CellTrace™ Violet-stained clone M56 1C11 and M56 1D6, respectively. C; D: p062.005 LCL were incubated and washed as described for A; B; and then incubated with clones p062.005 1E4 and 1E9, respectively. All samples were then stained for surface CD4 and CD134 expression. Data shown as mean + SEM CD134 MFI within all CellTrace™ Violet+ events; duplicate samples per condition. Experiment performed three times.
Having characterised the peptide-restriction pattern for each CD4+ clone, and identified exemplars for each peptide, we next investigated the MHC-restriction for each exemplar clone by measuring T cell activation against autologous and partially-matched LCL lines loaded with cognate 18mer-peptides for each clone, or with no peptide. In these assays, T cell activation was measured by flow cytometric analysis of surface CD25 MFI on CellTrace™ Violet-labelled clonal cells. Concurrently, we investigated whether clones were specifically HLA-DR restricted by comparing IFNγ production by each clone (section 2.5.1.5 - 2.5.1.6) on incubation with peptide loaded autologous LCL pre-treated with a blocking anti-HLA-DR antibody (2µg/ml clone L243, E; Figure 6-18) or isotype control.

We found that CD4+ clone M56 1C11 (representing M56 clones with MPE 157-170) recognised 18mer 157-174 presented by p062.004; p062.005 and M56 LCL (A; Figure 6-18). As the only shared MHC element across these three lines was expression of HLA-DPB1*0401/2, we confirmed this clone as recognising the well-characterised HLA-DP4 restricted 157-170 epitope. Incubation of peptide-loaded M56 LCL with anti-HLA-DR prior to incubation with clone 1C11 led to only a very small decrease in IFNγ production (E; Figure 6-18), indicating that in our blocking assay a small non-specific modulation of T cell activation even in a non-HLA-DR restricted setting could be expected.

CD4+ clone M56 1D6 (representing M56 clones with MPE 121-132) could be activated by autologous (M56) LCL presenting 18mer 121-138, but not by p062.004- or p062.005-LCL, indicating that this suite of clones must be restricted by an M56-exclusive MHC element (B; Figure 6-18). Further, we found that IFNγ production by clone 1D6 and by another of the same suite (M56 1D5) was decreased by ~50% on pre-incubation of peptide loaded M56 LCL by anti-HLA-DR compared to isotype control (E; Figure 6-18), demonstrating that these clones recognised peptide in an M56-exclusive HLA-DR-restricted context. M56 expresses HLA-DRB1*0101 and HLA-DRB1*1501, and these alleles are not shared by either of p062.004/5. As such, these data confirm that CD4+ clone recognise MPE 121-132 presented by either HLA-DRB1*0101 or HLA-DRB1*1501, but further work will be required to completely define the absolute MPE and restricting molecule.

CD4+ clone p062.005 1E4 (representing p062.005 clones with MPE 127-138) could be maximally activated by autologous (p062.005) LCL presenting 18mer 121-138, exhibited a very low level of CD25 upregulation on incubation with p062.004-LCL but no activation by M56-LCL (C; Figure 6-18). Further, we found that IFNγ production by clone 1E4 and by another of the same suite (p062.005 1D4) remained constant whether peptide-loaded p062.005 LCL were pre-incubated with anti-HLA-DR or isotype control (E; Figure 6-18), demonstrating that these clones did not recognise peptide in an HLA-DR-restricted context. As all LCL lines tested shared expression of HLA-DPB1*0401/2, the lack of strong T cell activation on incubation with p062.004 and M56 LCL (in stark contrast to the activation observed when incubating HLA-DP4-restricted M56 1C11 with all lines) indicated that this these clones did not recognise peptide in an HLA-DP-restricted context.
p062.005 and M56 are entirely disparate at the HLA-DQ locus, and M56 LCL cannot present peptide to clone 1E4. Interestingly, p062.005 and p062.004 share homozygous expression of HLA-DQB1*03 (Table 6-3), and a low level of CD25 upregulation was observed when clone 1E4 was incubated with peptide-loaded p062.004 (C; Figure 6-18). Further HLA sequencing (NZ Blood; Luminex) revealed that p062.005 expressed both HLA-DQA1*03 and -DQA1*05, while p062.004 expressed only HLA-DQA1*03. As such, our present knowledge indicates that clone p062.005 1E4 (and others) recognises an MPE within the range 127-138 in an HLA-DQB1*03/HLA-DQA1*05-restricted context.

CD4+ clone p062.005 1E9 (representing p062.005 clones with MPE 121-132) could be activated by autologous (p062.005) LCL presenting 18mer 121-138, but not by p062.004- or M56-LCL, indicating that this suite of clones must be restricted by a p062.005-exclusive MHC element (D; Figure 6-18). Incubation of peptide-loaded p062.005 LCL with anti-HLA-DR antibody completely abrogated subsequent IFNγ production by clone 1E9 compared to isotype control (E; Figure 6-18), demonstrating that this clone recognised MPE 121-132 in an HLA-DR-restricted context. P062.005 and M56 are entirely HLA-DR-disparate. P062.005 and p062.004 share expression of HLA-DRB1*04, indicating that this could not be the restricting element, but while p062.004 is homozygous at this locus, p062.005 also expresses HLA-DRB1*11:03. These data indicate that clone p062.005 1E9 (and others) recognises an MPE within the range 121-132 in an HLA-DQB1*11:03-restricted context. The minimal peptide epitopes and restricting MHC II molecules for each SLP-responsive clone set defined in this study are summarised in Table 6-6, and relevance within the context of NY-ESO-1 immunogenicity discussed in chapter 7.
Figure 6-18 CD4+ clones derived from donor M56 and p062.005 exhibit various p:MHC-II restrictions.

M56-, p062.004- and p062.005-LCL were loaded with either no peptide or A: p157-174 or B-D: p121-138 at 1µM for 16h. LCL were washed three times and then incubated with CellTrace™ Violet-stained clones A: M56 1C11; B: M56 1D4 C: p062.004 1E4; D: p062.004 1E9 for 24h. All samples were then stained for surface CD134 expression. Data shown as mean + SEM CD134 MFI within all CellTrace™ Violet+ events; duplicate samples per condition. Experiment performed three times. E: M56- and p062.005-LCL were loaded with either no peptide (all samples), p157-174 (M56-LCL + M56 1C11 only) or peptide p121-138 (all other samples) and washed as above. Autologous LCL were incubated with clones as indicated on x-axis in Rxs5 + Golgistop in the presence of a blocking anti-HLA-DR antibody (1µg/ml clone L243) or isotype control. After 6h cells were stained for IFNγ and acquired on the BD FACSARia™ II as described in section 2.5.1.5. Data shown as IFNγ+ cells within all CellTrace™ Violet+ events. Clone M56 1C11 is known to be HLA-DP*04-restricted (A), and acted as a negative control for blocking experiment.
Table 6-6  CD4+ T cell epitopes and MHC restrictions defined in this study.

<table>
<thead>
<tr>
<th>Peptide epitope (minimal defined range)</th>
<th>Representative clone</th>
<th>MHC Restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>121-132</td>
<td>CD4+ M56 1D6</td>
<td>HLA-DRB1<em>01:01 or HLA-DRB1</em>15:01</td>
</tr>
<tr>
<td>121-132</td>
<td>CD4+ p062.005 1E9</td>
<td>HLA-DRB1*11:03</td>
</tr>
<tr>
<td>127-138</td>
<td>CD4+ p062.005 1E4</td>
<td>HLA-DQA1<em>05//HLA-DQB1</em>03</td>
</tr>
<tr>
<td>157-69</td>
<td>CD4+ M56 1C11</td>
<td>HLA-DPB1*04</td>
</tr>
</tbody>
</table>
6.4. Chapter summary.

In this chapter we utilised synthetic short peptides (SSP) to either: expand memory T cell in a MoDC independent system directly from PBMC; or prime a naïve response using SSP-loaded mature MoDC and then promote secondary expansion of this population using SSP-loaded PBMC. We subsequently demonstrated that we could isolate expanded SSP-specific cells via FACS by detecting predicted activation-marker expression at a defined timepoint following SSP-recall. In establishing these model systems we expanded CD8\(^+\) T cells specific for therapeutically relevant viral (LMP2), tumour-associated antigen (Melan-A) and cancer-testis antigen (PRAME) targets. We were able to isolate and clonally expand these T cells with a high level of specificity.

We subsequently applied the SLP expansion protocol optimised in chapter 5 to expansion of memory CD4\(^+\) and CD8\(^+\) T cells specific for NY-ESO-1-derived SLP from PBMC of prior NY-ESO-1 vacinees. We found that we could isolate CD8\(^+\) T cells specific for SLP, but with poor overall survival. We were also able to isolate a large number of SLP-specific CD4\(^+\) T cells, exhibiting excellent survival and expansion. Based on observations that the inclusion of IL-15 in our memory T cell expansion regimen induced TCR-independent cytokine expression, we investigated induction of an array of putatively TCR-dependent activation markers, and found that many of these could be TCR-independently induced on culture in various common γc cytokines.

We characterised G2 and G3 CD4\(^+\) and CD8\(^+\) T cells expanded in IL-7 + IL-21, and found that as in chapters 3 and 4, these cells retained expression of CD28, CD62L and IL-7Rα, despite being exposed to multiple peptide-mediated stimulations in vivo and in vitro, and exhibiting a CCR7\(^-\)CD45RO\(^+\) TEM phenotype.

Finally we characterised the minimal peptide epitopes recognised by each suite of CD4\(^+\) and CD8\(^+\) clones, and determined the MHC I or MHC II context in which they recognised peptide. The CD8\(^+\) T cell clones we isolated exhibited specificity for three previously characterised epitopes 92-100, 96-104 and 125-133 (Jackson et al., 2006) restricted by HLA-C*03. However we were also able to identify cross-reactive presentation of peptide 125-133 in an HLA-C*0102-restricted context, suggesting that responses against this, or a similar epitope, could be isolated from HLA-C*01\(^+\) donors. This MHC I restriction has not previously been described for any NY-ESO-1 epitope. Further, by use of a novel rAAV expression vector, we were able to demonstrate that clones isolated in this chapter could recognise these epitopes when endogenously, rather than exogenously, derived by target cells.

The suite of 23 CD4\(^+\) T cell clones we isolated exhibited four different pattern of minimal peptide epitope: MHC II restriction. We defined clones recognising MPE 157-169 in an HLA-DPB1*04-restricted context, a well-described phenomenon (Zeng et al., 2001). We defined clones from two
donors recognising a MPE within the range 121-132, restricted by: either HLA-DRB1*01:01 or HLA-DRB1*15:01 (M56); or HLA-DRB1*11:01 (p062.005) – although MPE within this range have been described (Table 6-2) both of these combinations represent previously undescribed peptide:MHC II associations. Finally, we defined clones from one donor recognising a MPE within the range 127-138 and restricted by a specific combination of HLA-DQA1*05/HLA-DQB1*03. Again although this MPE has previously been described (Table 6-2), this represents a novel peptide: MHC II association, and to our knowledge only the third HLA-DQ-derived epitope within NY-ESO-1. The characterisation of these T cell clones validates the efficacy of our system for epitope discovery, and extends our knowledge of the immunogenicity of NY-ESO-1.
Chapter 7. General Discussion

7.1. Overview.

Previous work by our group and others has demonstrated that application of common γc cytokines in vitro can modulate T cell phenotype (Hinrichs et al., 2008; Klebanoff et al., 2004; Yang et al., 2013). Traditionally expansion of T cells in vitro has been accomplished by continuous application of the T cell growth factor IL-2, but expansion under these conditions fails to decouple proliferation from differentiation, resulting in telomere erosion, loss of polyfunctional cytokine production capacity, and susceptibility to activation-induced cell death (Restifo et al., 2012).

The use of alternative common γc cytokines IL-7, -15 and -21 have previously been shown to modulate T cell differentiation in vitro by retention of co-stimulatory and SLO homing markers when used in concert. This has typically been investigated through expansion of naïve T cells (Albrecht et al., 2011; Wolfl et al., 2011), and the ability of this regimen to modulate the phenotype of memory T cells is less well established. The production of T cell clones requires extreme in vitro expansion and represents a scenario in which the number of divisions undergone by a single cell precursor approaches or exceeds the limit normally associated with cell senescence. As the retention of co-stimulatory potential and a less-differentiated memory status has been shown to be correlated with T cell survival and persistence and with clinical efficacy (Gattinoni et al., 2012), an effective cloning protocol to develop T cells appropriate for immunotherapeutic use requires that this extreme proliferation is decoupled from differentiation and telomere loss.

The aim of this research was to investigate the application of cytokines IL-7 and IL-21 to the clonal expansion of CD4+ and CD8+ T cells. Further, to facilitate the application of cloning to novel epitope discovery, a system was developed whereby T cells responsive to synthetic long peptides containing multiple MHC I and MHC II restricted epitopes could rapidly be expanded and isolated based on temporal surface expression of proteins associated with T cell activation. This protocol was applied to the development and characterisation of T cell clones specific for the model cancer-testis antigen NY-ESO-1 in order to define novel and therapeutically relevant peptide epitopes and the MHC restriction within which they were presented.
7.2. Common γc cytokines modulate CD8+ T cell phenotype.

7.2.1. Naïve and memory T cell expansion and maintenance.

Culture of pure naïve and memory T cell populations revealed phenotypic skewing directed by particular cytokines. Both IL-2 and IL-15 promoted a loss of CCR7, CD28 and CD62L on naïve and memory T cells during culture. This loss was more pronounced on memory cells, probably because they express higher levels of the IL-2Rβ chain/CD122 (Berard and Tough, 2002). No loss of surface marker expression was observed on culture in IL-7, and co-administration of IL-7 with IL-2 or IL-15 led to partial rescue of CD28 and CD62L expression. This may be due to competition for common γc involvement in the formation of IL-7R and IL-2/15R formation (Palmer et al., 2008b). During expansion following anti-CD3/CD28 stimulation the use of IL-2 or IL-15 caused a loss of CD62L and CD28 expression (up to 50% loss at a population level) on both naïve and memory populations, while CCR7 and CD27 expression was not differentially influenced by cytokine exposure. IL-7 alone or IL-7 and IL-21 co-administration both maintained a CD27/CD28/CD62L+ phenotype following expansion, but only naïve and memory cells expanded in the presence of IL-7 + IL-21 exhibited high surface density of IL-7Ra. These data are in accord with previous observations that co-administration of IL-21 can arrest IL-15-mediated loss of CD28 (Alves et al., 2005) or CD62L (Hinrichs et al., 2008) during expansion of naïve human and murine T cells. Recently Yang et al (2013) investigated expansion of total CD8+ T cell using a similar panel of common γc cytokines. Interestingly, they found that IL-7 and IL-21 facilitated retention of CD62L and IL-7Ra expression, and they found that IL-7 and IL-21 cultured cells exhibited higher levels of transcript for transcription factors Sox2, Nanog, Oct4 and Lin28, typically considered to be involved in stem cell self-renewal. Interestingly in their analysis cells did not retain high levels of CD27 or CCR7 as observed in our study, but did retain high levels of CD28. These differences may be partly explained by the exclusion of IL-15 from their analyses. Additionally, while in this study cells were stimulated with anti-CD3/CD28 Dynabeads® and phenotype assessed at d28, Yang et al (2013) assessed phenotype at d12 following stimulation by plate-bound anti-CD3 and transduced with a lentiviral vector. As such, the provision of co-stimulatory signals and the timepoint of assessment may have contributed to the different phenotypes observed.

7.2.2. CD8+ T cell clone expansion.

The limiting factor in T cell cloning following FACS is not only clonal specificity (which may be high or low, depending on the nature of the cell labelling method), but clonal outgrowth. Protocols whereby single T cells clones are stimulated in the presence of irradiated feeder cells by anti-CD3, anti-CD3/28 or PHA typically result in outgrowth of functional T cells in only 2-40% of wells, only a
fraction of which are typically suitable for subsequent restimulation (Dunbar et al., 1999; Neller et al., 2014; Yee et al., 1999). When we investigated the relative efficiency of pentamer-guided CD8+ T cell clone outgrowth supported by IL-2 + IL-12 alone, or timed administration of IL-2, -12, -7 +/- IL-21 we found that the inclusion of IL-7 or IL-21 significantly enhanced the outgrowth of T cell blasts, and IL-21 was particularly effective at promoting the outgrowth of large clonal pellets suitable for secondary restimulation. Recent studies investigating antigen-specific CD8+ T cell outgrowth have similarly reported that inclusion of IL-21 greatly enhances the expansion of antigenic specific cells as a proportion of the total CD8+ T cell pool (Albrecht et al., 2011; Wolfl and Greenberg, 2014; Wolfl et al., 2011). Importantly, timed administration of IL-2, -12, -7 +/- IL-21 efficiently expanded naïve and memory T cells exhibiting a variety of reported (Appay et al., 2002) or demonstrated (our study) precursor phenotypes, indicating that this protocol was broadly applicable.

Retention of CD27 and CD28, telomere length and a TCM phenotype have been demonstrated to be correlates of in vivo persistence and anti-tumour efficacy for in vitro-generated T cell products in pre-clinical (Berger et al., 2008; Klebanoff et al., 2005) and clinical studies (Rosenberg et al., 2011; Zhou et al., 2005). In this study, despite profound expansion (22-27 population doublings) in the presence of IL-7 and IL-21 T cell clones derived from virus-specific memory precursors retained CD27, CD28 and partial CD62L/CCR7 expression, while being CD45RO+ - that is, they represented an early mixed TCM/TEM pool. Importantly, all clones generated using IL-7 + IL-21 retained IL-7Ra expression and were highly responsive to homeostatic cytokines, characteristics that were not found on clones expanded in IL-2 alone. T cell clones generated from a naïve pool in the presence of IL-7 and IL-21 (specific for MART-1 and NY-ESO-1 derived epitopes) exhibited a unique phenotype and rounded morphology similar to that of naïve cells. These cells could be retained in culture for several months in the presence of IL-7. These clones exhibited a CD45RA+ CD45RO+/CD27+ CD28+ CD62L+ CD95+ CD122+ CCR7+ IL7Ra+ phenotype and could be repeatedly restimulated. The least-differentiated T cell subset – stem cell memory T cells (TSCM) - have been variously described as being CD45RA+ CD45RO+ CD27+ CD28+ CD62L+ CD95+ CD122+ CCR7+ IL7Ra+ (Cieri et al., 2013) and CD45RA+ CD45RO CD27+ CD28+ CD62L+ CD95+ CD122+ CCR7+ IL7Ra+ CXCR3+ Bcl-6+ and have been detected in humans, mice and non-human-primates (Gattinoni et al., 2011; Gattinoni et al., 2009b; Libri et al., 2011). These cells were originally generated in vitro by Gattinoni et al (2009b) by application of a GSK3β-antagonist (TWS-119) following anti-CD2/CD3/CD28 stimulation of naïve cells. This system promoted signalling through the Wnt/β-catenin pathway. By contrast Cieri et al (2013) generated CD45RO+ TSCM in vitro through expansion of naïve cells in IL-7 + IL-15 following anti-CD3/28 stimulation. Interestingly, Cieri et al did not find that application of TWS-119 allowed TSCM development. In this study T cell clones were expanded following PHA stimulation. PHA is known to act in part through CD2 ligation (OFlynn et al., 1986), and the contribution of CD2 signalling to T cell clone phenotype may bear further investigation. To our knowledge, the suite of T
cell clones developed in chapter 3 may represent the first description of highly expanded T cell clones exhibiting a T_{SCM} phenotype, especially given that they exhibit a CD45RA^{HI} CD45RO^{DIM} phenotype, although expression of CXCR3 and Bcl-6 remain to be absolutely confirmed.

7.2.3. Parallel expansions from identical precursor populations.

The development of these ELA_{26-35}-specific T_{CM/SCM} offered a relatively unique opportunity to investigate the influence of common γc on expansion of genetically identical T cells. On re-stimulation of G2 stocks in paired IL-7 + IL-21 mediated or IL-2 only expansions, G3 (7/21) cells retained the same T_{CM/SCM} phenotype and homeostatic cytokine responsiveness as their G2 precursors. By contrast, exposure to chronic IL-2 signalling during expansion resulted in partial or total loss of CD28, CD62L, CCR7, and IL7Rα expression. G3 (IL-2) stocks also exhibited lower CD45RA and higher CD45RO and CD95 (Fas) surface density. Interestingly we again observed that CD27 was not modulated by cytokine exposure, suggesting that its expression may be epigenetically fixed as ‘on’ in these cells. Conversely, we noted that PD-1 expression was not detected in any G2 or G3 stock, even following anti-CD3/CD28 stimulation, suggesting that it may be constitutively repressed in our G2 stocks and that this state may be retained through subsequent stimulation. A protocol for the reliable development of PD-1^- T cells would be of benefit in immunotherapy, given the profound immunosuppression mediated by this molecule (Ahmadzadeh et al., 2009a; Mumprecht et al., 2009) and in light of recent clinical successes using anti-PD1 monoclonal antibodies (Sharma and Allison, 2015).

G2 and G3 ELA_{26-35}-specific clone stocks exhibited poor immediate cytotoxicity due to being perforin+ but granzyme B-. Granzyme B production capacity was not impaired, but was dependent on TCR:pMHC stimulation. Interestingly, although IL-21 has previously been described as promoting effector function (Zeng et al., 2005) this was not apparent in these G3 (7/21) clone stocks. The pfna gene is transcribed following TCR-mediated stimulation and has been shown to contain a STAT-binding upstream enhancer responsive to STAT5 (induced by IL-2 and IL-7 signalling) and STAT3 (induced by IL-21 signalling) (Yu et al., 1999) – as such both of our cytokine expansion regimens may have retained perforin expression. A lack of pre-formed granzyme B has been demonstrated to be characteristic of both naïve and central memory T cells (Takata and Takiguchi, 2006).

Several recent studies have addressed the mechanism by which T cell memory heterogeneity is produced following antigen exposure, and found that single gene-marked naïve T cells, when stimulated by vaccination, produce daughter cells that reconstitute short-lived effector populations and rest as both T_{CM} and T_{EM}. This has demonstrated that T_{CM} and T_{EM} are not derived from different sources during an immune response, although the point at which daughter cells are directed into
effector senescence and death, or survival as T\(_{\text{CM}}\) or T\(_{\text{EM}}\) remains to be answered. (Gerlach et al., 2013; Graef et al., 2014). Further, Stemberger et al (2014) have recently shown that in mice the CD62L\(^+\)IL-7R\(\alpha\)^+ T\(_{\text{CM}}\) population is capable of both self-renewal and heterogeneous differentiation on serial transfer. In these experiments they demonstrated that single T\(_{\text{CM}}\) OT-I transferred into normal hosts were protective against acute Listeria-OVA infection, and that T\(_{\text{CM}}\) OT-I expanded to produce effector, T\(_{\text{CM}}\) and T\(_{\text{EM}}\) populations. They found that on serial isolation and transfer of these ‘generation 2’ T\(_{\text{CM}}\) the same result was achieved, and repeated in through three iterations. In each case single-cell transfer was protective and resulted in detection of mixed effector, T\(_{\text{CM}}\) and T\(_{\text{EM}}\) progeny. The authors concluded that the T\(_{\text{CM}}\) population is the seat of T cell memory self-renewal, especially given the extremely low reported frequency of T\(_{\text{SCM}}\) in various models (Lugli et al., 2010). In this study parallel G3 expansions indicated that on continued exposure to IL-7 and IL-21 our G2 clones were able to self-renew \textit{in vitro}, but interestingly we did not see evidence of notable effector and T\(_{\text{EM}}\) formation under these conditions. Instead, all cells retained their T\(_{\text{CM/SCM}}\) phenotype. By contrast, on continuous exposure to IL-2 these G3 clones failed to re-express IL-7R\(\alpha\), a hallmark of rested memory cells (Kaech et al., 2003) and appeared to persist in an unusual effector phase characterised by mixed retention of co-stimulatory markers but a constitutive glycolytic metabolism. It should be noted that although surface marker modulation appeared to be robust and cytokine-mediated, the apparent phenotype of these G3 (IL-2) clones does not neatly fit into a defined effector/T\(_{\text{EM}}\) schema.

In order to more accurately determine the kinetic modulation of surface markers we tracked the phenotype of expanding cells from one G2 stock stimulated with either anti-CD3/CD28 or PHA and supported by wither IL-2 or IL-7 and IL-21. Although these data should be interpreted with caution, as they are only representative of a single clone, in these experiments all activated cells did enter an effector phase between d0 and d7 exhibiting loss of surface CD3, shedding of CD62L and rapid media acidification. On all samples CCR7 levels declined progressively to d10 – this finding reinforces the importance of only applying memory nomenclature to T cells that are not actively dividing and have reached a stable phenotype. Our data suggested that following this effector phase subsequent cytokine exposure, rather than proliferation level or method of stimulation, was the determining factor in surface CCR7, CD28 and CD62L expression. Interestingly T cell activation by PHA in the presence of irradiated LCL was found to be a far more potent proliferative stimulus than anti-CD3/CD28. It is possible that this is due to CD27 ligation of CD70 (expressed on LCL and on activated T cells). CD27 signalling has been shown to promote proliferation while preventing T cell differentiation (Carr et al., 2006). All samples treated with PHA exhibited a decrease in surface CD27 expression at 24h, with subsequent re-expression, although whether this immediate internalisation was due to direct CD27 ligation by PHA or by interaction with CD70 on adjacent LCL was not elucidated.
7.3. Synthetic long peptide and recombinant protein processing and presentation.

Recombinant NY-ESO-1 was produced in Sf9 cells using a baculovirus expression vector produced during this study. Several groups have described the production of recombinant NY-ESO-1 in *E.coli* to GMP grade, but in all cases the protein forms inclusion bodies and is purified as a denatured product (Karbach et al., 2011; Lowe et al., 2011; Murphy et al., 2005). Similarly in this study rNY-ESO-1-His<sub>6</sub> proved insoluble in SF9 cells and was purified using 6M urea. Following production and purification of rNY-ESO-1-His<sub>6</sub> the ability of TAP-deficient professional APC (T2 cells) and TAP-proficient proficient (LCL) to process and present synthetic short peptides (SSP), synthetic long peptides (SLP) and recombinant protein to CD8<sup>+</sup> T cell clones was assessed. Processing and presentation of SLP was TAP-independent, as T2 cells were a more potent APC than LCL, but was time-dependent as efficient T cell activation typically required 16h of SLP processing. SLP were heat- and serum stable, and processing and presentation was mediated by cellular uptake. Interestingly, efficiency of SLP processing was not determined by SLP length, and may be related to either SLP primary sequence or secondary-structure-mediated aggregation and phagocytic uptake. Non-professional APC (PBMC<sub>exp</sub>) were able to process and present some SLP to T cell clones, but did so inefficiently and required clones to exhibit high TCR:pMHC affinity. Nonetheless this result was intriguing, as to our knowledge primary non-activated primary T cells have not previously been demonstrated to be cross-presenting APC, although T cell blasts, stimulated by OKT3 or PHA are known to competently present SSP and facilitate memory T cell expansion (Foster et al., 2007). In this study SLP were found to be more efficiently processed and presented by both T2 and LCL than full-length recombinant denatured protein, and SLP rather than protein were used in subsequent T cell expansions. These results are in accord with those seen by Rosalia et al. (2013) when comparing SLP to protein processing and presentation by primary mouse and human DC.

Analysis of peptide processing was limited to comparisons of T cell activation and an investigation of processing time in this study, and processing pathways were not specifically assessed. Several groups have investigated SLP and protein cross-presentation to CD8<sup>+</sup> T cells by T2, LCL and primary human cell subsets utilising specific proteasomal and endosomal inhibitors. Interestingly, these studies have revealed that SLP processing and cross-presentation is highly SLP- and epitope-specific. Zandvliet et al. (2012) found that in both T2 and LCL C-terminal processing of *endogenously* expressed peptides (encoded by minigenes) to reveal minimal peptide epitopes was proteasome-dependent, but that N-terminal processing was both proteasome and TAP-independent. By contrast neither C-terminal nor N-terminal processing of *exogenously* derived SLP was TAP- or proteasome-dependent, suggesting that SLP may enter a vacuolar alternative presentation system and subsequently encounter empty MHC I trafficking to the surface post-ER. It has also been suggested that highly hydrophobic SLP
may be proteasomally processed following phagocytosis but then traffic into the ER in a TAP-independent manner, potentially through membrane integration (Tey and Khanna, 2012). By contrast Rosalia et al (2013) and Gnjatic et al (2003) demonstrated a requirement for proteasomal processing of SLP derived from ovalbumin and NY-ESO-1, respectively. Other studies have found that proteasomal processing is required for cross-presentation of Melan-A/MART-1 but not gp100 by MoDC (Faure et al., 2009) and that even within one protein different nested epitopes may be proteasome-dependent (for example peptide 60-72 within NY-ESO-1) or –independent (for example peptide 92-100 within NY-ESO-1) when processed by the same cells (Robson et al.). This picture is further complicated by results showing that antigen aggregation, formulation in a lipid carrier, or denaturation in urea may direct proteins and SLP to a proteasome- and TAP-independent vacuolar system (Barabas et al., 2008; Bricard et al., 2005; Faure et al., 2009; Robson et al., 2008).

Having demonstrated that SLP processing and cross-presentation was not strictly dependent on professional APC or on a classical TAP-mediated presentation system, we hypothesised that PBMC, adequately supported by exogenous cytokines, may be sufficient to stimulate memory T cell outgrowth following SLP incubation, but that this outgrowth would be dependent on prolonged SLP incubation in culture. This did prove to be the case, as PBMC from several donors exhibited a large expansion of NLV_{495-503}-specific T cells following incubation of PBMC with the longer SLP ILA_{32} and CAG_{49}. The scale of this expansion was dependent on continued incubation of SLP in culture, as brief exposure followed by SLP removal led to lesser expansion within each peptide condition. These findings were subsequently applied to the expansion of CD4+ and CD8+ memory T cells specific for SLP derived from NY-ESO-1 as described in section 7.5.

7.4. T cell activation marker expression.

Following T cell activation through TCR:pMHC, T cells increase in cytoplasmic volume, transition to a blasting state and undergo both mitochondrial biogenesis and a switch to glycolytic metabolism. This is followed by the onset of cell division, typically within 48h (Jelley-Gibbs et al., 2000). Prior to the onset of division, T cells temporally up- or down-regulate ‘activation markers’. These are surface markers important in: T cell co-stimulation for survival and proliferation; nutrient acquisition or secondary messenger generation; cell:cell adhesion and ECM degradation and trafficking. The availability of these markers on the cell surface makes them amenable to detection by flow cytometry if the timing of their expression can be predicted. A list of the activation markers assessed in this study can be found in Table 5-1, although the array of markers investigated here is by no means exhaustive.
T cells activated by a particular antigenic stimulus have also been isolated based on cytokine production (typically IL-2, IFNγ or TNFα) (Neller et al., 2014), or by evidence of amide-reactive dye dilution due to antigen-induced proliferation (Mannering et al., 2005).

### 7.4.1. Anti-CD3/CD28 induced activation marker expression.

This study used Dynabeads® to characterise activation marker expression patterns and kinetics on CD4+ and CD8+ T cells specifically mediated through TCR transduction machinery. Dynabeads® were selected as they proved superior to CD3 cross-linking or plate-bound anti-CD3 at eliciting expression of the model activation marker CD137 at 24h and because this bead system allowed specific analysis of activation markers induced by ligation of TCR signal transduction machinery . The aim of this work was to define activation markers that exhibited negligible expression on non-activated T cells, but that were expressed at high surface density within an ‘activation window’ amenable to in vitro peptide recall experiments 24-32h post-TCR stimulation. Interestingly, HLA-DR, previously reported to be a marker of T cell activation following PHA or SEB stimulation (Salgado et al., 2002) was not found to be expressed following Dynabead® stimulation on CD4+ or CD8+ T cells, nor was the NK cell marker CD56. Although most activation markers were expressed by at least a subset of T cells throughout the 72h tracking experiment, those that were expressed at the highest population level within an activation window were CD25, CD69 and CD71 on both CD4+ and CD8+ T cells, CD134 on CD4+ T cells only and CD137 on CD8+ T cells only. The co-expression of CD25, CD69 and CD137 on ELA26-35-specific T cell clones within this activation window was subsequently assessed. TCR:pMHC-mediated expression was confirmed to follow the same kinetics as Dynabead®-mediated expression, and ELA26-35-specific T cells could be recovered by activation-marker-guided FACS with absolute specificity. Interestingly CD154, previously shown to be a marker of activated CD4+T cells (Chattopadhyay et al., 2006) was not detectable on >60% of CD4+ T cells at any timepoint in this study, although it is possible that the early peak of CD154 expression was missed. Similar to our results, Wehler et al (2008) also failed to detect CD154 and CD137 co-expression at 24h. In subsequent experiments we also failed to observe CD154 up-regulation at 24h on CD4+T cell clones stimulated by peptide. One of the major advantages of activation-marker guided detection of T cell activation is that, unlike cytokine production, marker expression is not T cell subset delineated. In one recent study comparing activation marker expression to cytokine-mediated cell capture, Wolf et al (2007) found that naïve CD8+ T cells specific for Melan-A or WT-1 could not be isolated based on IFNγ production but that this could be achieved via CD137+ selection.
7.4.2. Common γc induced activation marker expression.

Following selection of candidate cell-surface markers for activation-marker guided cell sorting, model SSP-mediated memory and naïve T cell expansion protocols were established. In these expansions, T cell outgrowth was supported by timed administration of IL-2 and IL-12 (early), IL-15 (late) and IL-7 + IL-21 (continuous), based on observations in chapters 3 and 4. The validation of activation-marker guided sorting from these cultures is discussed below, but one unanticipated consequence of this 12d expansion regimen was the induction of CD69 and CD56 expression on a fraction of cultured T cells. The emergence of these populations suggested that one or more of the administered cytokines was able to induce TCR-independent expression. This was initially investigated by culture of PBMCexp generated in the presence of IL-7 and IL-21. These were found to upregulate CD69 and CD56 and form large cell clusters in the presence of IL-2 or IL-15 but, unexpectedly, high levels of HLA-DR and CD134 expression were observed on both CD4+ and CD8+, and on CD4+ cells, respectively, suggesting that chronic IL-7 or IL-21 exposure could also induce or retain expression of activation markers. Extension of this investigation to ex vivo T cells and a wider array and combination of common γc cytokines showed that IL-2 and IL-15 were potent non-specific inducers of several ‘activation markers’, notably CD38, CD69, CD71, HLA-DR and ICOS. This induction was most exaggerated on CD8+ T cells, potentially suggesting differential expression of the IL-2/IL-15 receptor complex, but was also present on CD4+ T cells. Notably, HLA-DR, which was not induced by anti-CD3/CD28 stimulation in our hands, was strongly induced by common γc cytokine signalling. Interestingly, it has recently been reported that apparent T cell activation, indicated by CD38 and HLA-DR expression in conditions of chronic viral disease (Kestens et al., 1992) is actually mediated by responses to serum cytokines (Bastidas et al., 2014). Our in vitro findings corroborate these in vivo observations. Further, as most studies investigating surface protein expression following TCR/CD3-mediated stimulation provide concurrent IL-2 (or other γc cytokines) to promote T cell survival, in many instances the relative contribution of TCR/CD3- and cytokine-mediated signalling to de novo surface protein expression has not been disentangled. Interestingly, culture of T cells in IL-4 alone did not induce any TCR-independent activation marker expression on either subset in vitro, although co-administration of IL-4 with other cytokines led to a non-intuitive increase, decrease, or no change in expression for various activation markers. Based on these findings it is possible that IL-4 could be applied alone late in a peptide-mediated in vitro expansion to minimise background expression of activation markers and improve specificity of peptide-recall-mediated activation marker signalling, although this requires further investigation and optimisation.

IL-4 has been studied for its role during naïve CD4+ T cell priming, where it induces a ‘T_{H2}’ phenotype characterised by continued IL-4, -5 and -13 production. IL-4 produced by T_{H2} cells proximal to the germinal centre of an activated lymph node plays an important role in facilitating immunoglobulin class-switching by B-cells, particularly promoting IgE production (Shimoda et al.,
The role of IL-4 signalling in CD4+ and CD8+ T cell activation has been less extensively characterised. The IL-4Rα chain is constitutively expressed on naïve T cells, and IL-4 signalling promotes increased IL-4Rα expression in a positive feedback loop (Boursalian and Bottomly, 1999; Geginat et al., 2001). Unlike the IL-2Ra, -7Ra, CD122 or the γc receptor, IL-4Rα cytoplasmic domain recruits STAT6 (Rochman et al., 2009). IL-4 is able to induce equivalent Bcl-2 and Bcl-XL to that observed on IL-2 or IL-7 signalling in vitro, and is more potent than either IL-2 or IL-7 at protecting T cells from activation-induced cell death on SAg challenge in vivo (Vella et al., 1997; Vella et al., 1998). Several studies have demonstrated that IL-4 directly and indirectly affects T cell activation and memory formation. Provision of IL-4 during T cell activation promotes expression of both EOMES and TBX21, and enhances IFN-γ production and secretion (Oliver et al., 2012). Huang et al. (2000) compared the effect of providing IL-2 alone, or IL-2 supplemented with IL-12 or IL-4, to naïve antigen-specific CD8+ T cells (2C-TCR mice) during initial priming on their subsequent phenotype and persistence in vivo following transfer into a congenic B10 murine model. T cells activated in the presence of IL-2 or IL-12 maintained an effector phenotype and failed to persist in vivo beyond 1 month. By contrast, T cells primed and activated in the presence of IL-4 persisted beyond 6 months and formed a stable CD44+CD62LHiT CM population. Interestingly in this study IL-4 treated cells exhibited markedly elevated levels of CD122 – as such it is possible that the differential inhibition or exacerbation of AM expression observed in our culture system is facilitated by altered CD122 expression and thus altered sensitivity to the co-administered cytokine (as IL-4 does not require or utilise CD122 for signalling). Although little is known about the direct role of IL-4 in promoting AM expression on T cells, IL-4 has been demonstrated to reduce CD38 protein levels on the human ‘Farage’ B cell line in vitro (Shubinsky and Schlesinger, 1996). Further, IL-4 is known to enhance HLA-DR protein expression on immature MoDC in vitro (Wolfl and Greenberg, 2014) and interestingly, several murine studies have demonstrated that NKT and CD4+ T cell-derived IL-4 is essential for dendritic cell maturation and subsequent CD8+ T cell priming and development into effector cells capable of rejecting implanted tumours following prophylactic vaccination (Geginat et al., 2001; Geginat et al., 2003b).

7.5. Activation-marker guided T cell cloning and characterisation of NY-ESO-1 specific T cells.

In this study memory T cells were expanded using SSP or SLP applied directly to PBMC, and naïve T cells were primed by SSP-loaded, matured MoDC and then expanded by addition of autologous peptide-loaded PBMC, following a protocol adapted and modified from those in Wolfl & Greenberg (2014) and Ho et al (2006). The former protocol was applied to the expansion of memory T cells specific for the immunodominant Cytomegalovirus pp65-derived epitope NLV495-503 and the subimmunodominant Epstein-Barr Virus latent membrane protein 2 (LPM2)-derived epitope
CLG426-434. The latter priming/recall protocol was applied to the expansion of naïve T cells specific for the Melan-A derived ELA26-35 A27L epitope and the expansion of naïve T cells specific for SSP derived from the cancer-testis antigen PRAME. Activation-marker-guided FACS allowed the isolation and expansion of CD8+ T cell clones specific for ELA26-35 A27L and CLG426-434. On post-expansion assessment, 9/9 ELA26-35 A27L and 16/25 CLG426-434 clones were found to be antigen-specific, demonstrating that our activation marker selection had been robust and that activation-marker guided cloning was tractable. These validation experiments revealed that gating on CD25+CD69+CD137+ events facilitated more stringent and accurate selection than gating on a combination of only two markers. In all expansions tested, activation-marker* T cell numbers were closely aligned with IFNy* or pentamer* frequencies when tested post-expansion. These results echo those seen by Wolfl et al. (2007).

Memory T cells of unknown epitope specificity were expanded from PBMC of prior NY-ESO-1 vaccinees using SLP comprising residues 79-116, 118-143 and 151-174. Both CD4+ and CD8+ T cell clones were isolated and characterised. Interestingly, although CD8+ T cells exhibited a normal frequency of T cell blast emergence (~40-50%), survival to secondary restimulation was extremely poor. This echoed results observed in the ELA and CLG-specific validation experiments and suggested that this protocol needs further optimisation to avoid CD8+ T cell death. All CD8+ clones that survived to d28 were capable of secondary and tertiary restimulation, as such the issue appeared to lie with initial survival. As previous pentamer-guided cell sorting of highly differentiated NLV495-503-specific clones yielded excellent outgrowth under the influence of IL-7 and IL-21, it is unlikely that the memory status of these NY-ESO-1-specific T cells, or the lack of IL-15 administration during outgrowth, is the issue – rather, it is likely that the multiple restimulations required within a short time-frame causes activation-induced cell death in these cells. As such, it is possible that survival and outgrowth may be improved by a ‘rest’ period of 24-48h post T cell deposition. This could be addressed in a polyclonal fashion by repeatedly non-specifically stimulating T cell pools in vitro, mimicking the activation dynamics inherent in a peptide prime/recall protocol, and subsequently FACS-sorting and cloning individual T cells at 24h, 48h and 72h post-activation, in the presence or absence of IL-15. Further, as CD8+ T cell clones isolated through this experiment were PD-1+ as is typically observed in tumour-specific T cells isolated from patients (Rizvi et al., 2015c; Topalian et al., 2012) and feeder LCL used are PD-L1+, it is possible that survival may be improved by administration of an anti-PD-1 antibody. Similarly, as AICD in CD8+ T cells following repeated antigen-specific stimulation is often Fas-mediated, utilisation of a Fas-blocking antibody during the early stages of clonal restimulation may be beneficial (Varadhachary et al., 1997). These technical considerations will need to be addressed through further experimentation. Unlike CD8+ T cell clones isolated in this study, CD4+ T cell clones could be expanded using IL-7 and IL-21 with extremely high efficiency, and the relative survival advantage of the CD4+ T cell clones over the CD8+ clones bears
Chapter 7 – General Discussion

further analysis. Some groups have demonstrated that CD8+ T cells are more likely to die following IL-2 withdrawal than CD4+ T cells (Bosque et al., 2007; Tham and Mescher, 2002), however in our hands, and in studies by other groups (Mannering et al., 2005) continued IL-2 provision is also highly disadvantageous for clonal outgrowth. A more complete investigation of the early intracellular signalling events in polyclonally expanded CD4+ and CD8+ T cells may inform future cloning and the differential survival observed in our study – in particular the relative expression of anti-apoptotic Bcl family members and pro-apoptotic Bim, Bad and Bax (Li et al., 2004; Wojciechowski et al., 2006).

Phenotypic characterisation of these T cell clones demonstrated a universal TEM phenotype, as is typically observed in expanded T cell products from melanoma patients (Restifo et al., 2012). Unusually however, retention of CD28, CD62L, IL-7Rα and homeostatic cytokine responsiveness was observed on all clones. Mapping of minimal peptide epitopes and MHC I/II restrictions exhibited by representative clones (summarised in Table 6-5 and Table 6-6) demonstrated that application of single SLP could promote T cell outgrowth against two individual epitopes within that SLP. Three CD8+ T cell clones recognising previously described HLA-C*03-restricted peptide epitopes were isolated (Jackson et al., 2006). TCR:pMHC affinity titrations revealed an equivalent affinity hierarchy to that observed by Jackson et al. Uniquely, however, based on observed cross-reactivity to peptide presented by allogeneic APC lines a previously undescribed HLA-C*0102 reactivity for peptide 125-133 was detected. Further, peptide/MHC II mapping of several suites of similarly reactive CD4+ T cell clones revealed the production of clones specific for the well-characterised HLA-DP*04-restricted peptide 157-170, but also revealed that three sets of T cell clones exhibited novel MHCII restriction against known peptide epitopes. CD4+ clones were characterised recognising peptide 121-132 in an HLA-DQB1*11:03-restricted context; peptide 121-132 presented by either HLA-DRB1*0101 or HLA-DRB1*1501; and peptide 127-138 in an HLA-DQB1*03/HLA-DQA1*05. The discovery of these novel MHC II restrictions is important because it broadens the potential pool of cancer patients in which immunotherapeutic intervention can be attempted and because it demonstrates that even within an extremely well-studied therapeutic target this T cell cloning protocol is an effective means of epitope discovery. The rapid isolation of a large suite of CD4+ clones specific for several different epitopes is of particular importance, as several studies have demonstrated that CTA- or neoantigen-specific CD4+ T cell products can induce durable remissions (Hunder et al., 2008; Tran et al., 2014).

Interestingly, a marked difference in PD-1 expression emerged in this study when comparing pentamer-isolated and Am-isolated T cell clones. ELA9-specific clones isolated from a naïve precursor pool and serially expanded remained constitutively PD-1+, and even on CD3/28 stimulation exhibited only a minor upregulation of PD-1 at 24h (Figure 4-13). By contrast, NY-ESO-1-specific CD8+ T cells isolated form a previously vaccinated melanoma patient exhibited a constitutively PD-1HI phenotype. Constitutive PD-1 expression is a common feature of TIL and circulating CTA-
specific T cells in a melanoma setting, and PD-1 ligation by tumour-expressed PD-L1 is one of the major mediators of transient intratumoural T cell dysfunction (Ahmadzadeh et al., 2009a; Daud et al., 2016; Matsuzaki et al., 2010). In both murine models and human trials administration of anti-PD1 or anti-PD-L1 antibodies, or removal of TIL from the tumour microenvironment and ex vivo expansion alleviates this inhibition of effector function (Brahmer et al., 2012; Curran et al., 2010; Hirano et al., 2005; Rosenberg et al., 2011; Topalian et al., 2012). Comparative tracking of pre- and post-REP antigen specific T cells within melanoma TIL has demonstrated that pre-existing PD-1 expression is not altered by REP and, interestingly, that PD-1HI expression is typically observed on CD28+ TIL (Li et al., 2010). This study also suggested that CD28+ PD-1+ cells exhibited the highest proliferative potential among isolated TIL, reinforcing the fact that PD-1 expression in and of itself does not indicate an anergic state. This expression pattern and proliferative capacity matches that seen in our study. Bennett et al. (2003) have demonstrated that supra-physiological IL-2, IL-7 or IL-15 can overcome PD-1-mediated signalling in a STAT5-dependent manner, and it is interesting to note that we can serially expand PD-1+ T cell clones using PHA and PD-L1+ feeder cells in vitro. Interestingly, some reports have suggested that culture of PBMC ex vivo in IL-2; -7; -15 or -21 induces surface PD-1 expression on T cells (Kinter et al., 2008; Zhang et al., 2015), but this phenomenon was certainly not observed in our suite of serially expanded and maintained ELA-specific clones. The genetic and epigenetic regulation of the PDCD1 locus is complex and multifactorial, as discussed in section 1.2.5. As several methylation sites exist in the PDCD1 promoter (Bally et al., 2016), it will be of benefit to undertake a methylomic analysis of both the constitutively PD-1- and PD-1+ clones generated in this study, to gain insight into the relative contribution of epigenetic methylation to the observed PD-1 expression patterns. Recent studies investigating the relative methylation status of PDCD1 in HIV patients and HIV-negative controls have demonstrated that in naïve HIV-specific cells PD-1 expression is silenced by promoter methylation, while in uncontrolled infection ‘exhausted’ HIV-specific T cells exhibit promoter demethylation. Interestingly though, in patients undergoing active antiretroviral therapy, with a concomitant control of viral titre, HIV-specific T cells remain PD-1+ and exhibit continued promoter demethylation, suggesting that at some point in an effector response the methylation status of the PDCD1 promoter is fixed (Youngblood et al., 2013a; Youngblood et al., 2013b). Conversely, the PD-1- status of serially expanded TCM/SCM clones generated from naïve precursors using our IL-7 + IL-21 technique provides an opportunity to adoptively transfer these cells, or equivalently expanded cells recognising other melanoma antigens, into a humanised murine model of melanoma (Brehm et al., 2010) in order to investigate whether these cells would exhibit de novo PD-1 expression on homing into seeded tumours, and to investigate the capacity of these T cells to exert an anti-tumour effect in combination with a vaccine regimen or checkpoint blockade inhibitors.

Several aspects of this study were limited in their investigative power or require further refinement. In particular, the timing of activation-marker-guided cell isolation and restimulation may need to be refined, in order to maximise T cell outgrowth and retention. The application of IL-15 was not investigated in this study, and may aid in promoting memory cell outgrowth, although care would need to be taken to avoid the phenotypic alterations observed on IL-15 administration in T cell culture. Although our observations that differential expansion of identical clones in IL-2 or IL-7 and IL-21 yielded differential phenotypes was robust across a large suite of clones, these were specific for only one epitope and further investigation will be required to demonstrate that this is a universal phenomenon. Further, we were able to generate sufficient pentamer-specific G2 clones grown in IL-2 alone to robustly compare the phenotypes generated by earlier chronic IL-2 exposure. Finally, this study investigated T cell clone phenotype at the protein level only, specifically focusing on a relatively limited suite of surface-expressed proteins known to be important in T cell memory differentiation and effector function (Appay et al., 2008; Lanzavecchia and Sallusto, 2005; Sallusto et al., 2004a). A more detailed analysis of the transcription factors and other regulatory genetic elements known to be involved in T cell differentiation is warranted in these clones, in particular expression of ‘stem’-related molecules such as Tcf7, Lef1 and Sca-1 (Jeannet, 2010; Yi, 2011). Several studies have investigated T cell memory heterogeneity at a transcriptional level in mice and humans, and microarray analyses played an important role in distinguishing the T_{SCM} T cell subset and demonstrating its intermediate differentiation status between T_N and T_{CM} (Gattinoni, 2009; Gattinoni, 2011; Hinrichs et al., 2008) Further, comparative microarray analysis of cytokine-polarised paired clonal populations, in the context of defined naïve and memory comparators, will provide a wealth of information as to the position of these clones on an established T cell memory spectrum and may suggest novel targets for further investigation. In particular, given the clear modulability of co-stimulatory (CD27/CD28) and chemokine receptors (CCR7) by differential γc cytokine treatment, the opportunity exists to profile all such known receptors at a transcriptional level in order to more comprehensively identify the co-stimulatory and cell-adhesion differences across memory subsets. Finally, the ability to reliably generate paired, polarised T_{CM/SCM} and T_{EM} allows for a comparison of these subsets in mediating tumour rejection in humanised mouse models of cancer, without the need for in vitro retroviral vector TCR transduction or βcatenin pathway modulation (Gattinoni et al., 2011).

The development of highly expanded but relatively undifferentiated antigen-specific T cells is of direct relevance for T cell mediated immunotherapy, as less-differentiated cells have been shown to endow superior anti-tumour protection and mediate regression in murine models and in clinical studies. In this study antigen-specific cells were derived from naive precursors and shown to retain a
The development of T cell clones that exhibit a stable and ‘rested’ (non-proliferative) phenotype in culture provides avenues for studying the cell biology of surface proteins, in particular CD62L, CCR7, CD27 and CD28. Most importantly, the ability to polarise individual clones into paired protein+ and protein− populations via differential cytokine exposure allows any investigations to be undertaken using cells with a genetically identical background. The availability of CCR7+ may be useful in probing the nature of memory T cell migration and the role of chemokines themselves in facilitating T cell activation and effector function. CCR7 is known to be crucial in mediating T\textsubscript{N} and T\textsubscript{CM/SCM} homing into the lymph node and localisation in the paracortex (Munoz et al., 2014). CCR7 binds two chemokines – CCL19 and CCL21. Interestingly, although being biochemically similar, the two ligands exert subtly different effects on T cells, as CCL19 induces CCR7 internalisation while CCL21 does not (Bardi et al., 2001). Further, while both CCL19 and CCL21 can induce chemotactic migration, CCL21 is also able to induce haptotactic migration, via binding to extracellular matrix (ECM) protein substrates (Shamri et al., 2005). ECM binding is mediated by a charged C-terminal domain of ~32 amino acids, not present in CCL19 (Love et al., 2012). Comparative investigation of CCR7+ and CCR7− clones exposed to CCL19 or CCL21 in a transwell or under-agarose assay, or video microscopy tracking system, will allow quantification or visualisation of the effect of these cytokines on memory T cell motility. Interestingly, CCL21 is also able to bind to glycosaminoglycan groups on APC, and this labelling has been implicated in promoting antigen-independent T cell:APC coupling in mice (Friedman et al., 2006). Utilisation of CCR7+ clones will allow investigation of this coupling in a human setting, and any role it may play in prolonging T cell:APC contact time or enabling subsequent antigen-specific T cell activation using peptide-loaded and CCL21-coated APC. Further, as cancer cells have also been shown to express surface GAG (Afratis et al., 2012), it will be
of value to investigate whether CCL21-coating of antigen-expressing cancer cell lines promotes CCR7+ T cell-mediated killing or effector cytokine release. Finally, the adoptive transfer and tracking of CCR7+ T cell clones into a humanised mouse model will demonstrate whether in vitro CCR7 expression translates into effective in vivo SLO trafficking and residence, which may have implications for anti-tumour vaccination protocols.

The ability to reliably generate CD27+ CD28+ antigen-specific T cell clones, and accurately and sensitively assess their peptide:MHC affinity, allows important investigation into the role of costimulatory signalling in human memory T cells. Early murine infection models suggested that although CD28-mediated signalling was essential for naïve T cell priming and expansion on initial pathogen exposure, it was entirely dispensable for subsequent protective memory T cell function (Croft, 1994; Croft et al., 1994). Subsequent murine studies, however, demonstrated (utilising CD80− CD86− mice, or stable CD28-blocking moieties) that in the absence of CD28 signalling CD8+ memory T cell secondary expansion and protective function was impaired in herpes simplex virus (HSV)-1 infection; and that CD4+ memory T cells failed to produce IL-2 and clear virus on secondary exposure to Influenza (Borowski et al., 2007; Ndejembi et al., 2006), suggesting that CD28 may play an important role in memory T cell function. Interestingly, comparatively little is known about the role of CD27 in T cell memory in murine models. Constitutive CD27 and CD28 expression will allow us to investigate the role of costimulation, provided in cis on CD70+/80+/86+ APC, or in trans via activating antibodies, in human memory T cell activation, cytokine production and degranulation. Interestingly, murine work, utilising Vβ2-TCR T cells non-specifically stimulated using a Staphylococcal SAg, has shown that in trans CD28 costimulation can lower the threshold of TCR molecules required to be ‘triggered’ for complete T cell activation (from ~8000 to ~1500 in this system) (Viola and Lanzavecchia, 1996). The suite of T cell clones developed through this thesis will allow us to extend these observations to various antigen-specific settings using human cells, and will allow us to determine both the modulation of peptide-response curves by CD27 and CD28 ligation, and establish a hierarchy of effector function thresholds in human memory cells.
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