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In vitro generation and programming of human CD8⁺ memory T cells

Yu-Yu Joyce Ho

A thesis submitted in complete fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences, The University of Auckland, 2013.
Abstract

Durable and effective immunity requires the generation of persistent and functional memory CD8⁺ T cells. The integration of a multitude of signals during naïve cell activation plays an important role in the programming of CD8⁺ T cells responses. However, knowledge of the regulation of naïve cell programming in humans is limited. Therefore, we aimed to expose naïve human CD8⁺ T cells to cytokines during their activation and measure their effects on the resultant memory populations. We first established a unique culture system for the stimulation and expansion of human naïve CD8⁺ T cells that allowed us to generate rested memory cells in vitro, supported by IL-7. Utilizing this system, we found that three-day cytokine exposure during priming distinctively programmed the cytokine secretion profiles and cellular phenotypes of the memory populations 21 days later. Priming in the presence of IL-12 was observed to programme enhanced effector cytokine production, and a more differentiated cell surface phenotype; while IL-21 exposure was found to programme reduced functional capacity, but a less differentiated phenotype. We then combined IL-12 and IL-21 exposure during priming, and found memory populations with an early differentiation status could be generated with superior effector function, establishing an expansion protocol with potential implications for adoptive immunotherapy. With this priming regime, a short duration of IL-2 supplementation was found to augment the expansion of naïve CD8⁺ T cells without significantly altering cellular attributes. Transcription factor expression was also examined in these experiments and novel changes were observed upon cytokine exposure, TCR stimulation and CD8⁺ T cell differentiation. Priming naïve cells with IL-4 strongly upregulated GATA-3 expression following secondary stimulation, confirming that the programming we observed in this model was acting via transcription factors. Transcription factor expression profile of the in vitro-generated memory cells also confirmed an early differentiation status with Tcf-1, Lef-1 and FoxO1 expression, and an absence of T-bet and Eomes. Collectively, we have established a culture system that enables the exploration of programming events during the priming of naïve human CD8⁺ T cells that can be further utilized to investigate human CD8⁺ T cell differentiation and optimal culture regimes for ACT.
Acknowledgements

2008-2012, four years of an astonishing quest on scientific exploration that led to the unveiling of one’s persistence and strength through the impossible. For it is always the process and not the outcome that cultivates and strengthens us, one holds an immense amount of humbling gratitude towards every person and experience encountered during this PhD.

First and foremost, I must acknowledge and thank my supervisors Professor Rod Dunbar and Dr. Anna Brooks with profound gratitude for all of their guidance and support throughout this journey. Rod has always offered a tremendous wealth of knowledge, creativity and passion for science that has guided me to aim for excellence with persistence and patience. His understanding nature and faith provided me with influential doses of reassurance and confidence that saw me through the challenges. As someone that has been there from the very beginning, Anna has provided support every step of the way with each and every technical, and metaphysical, entanglement. I cannot thank them enough for what they have done to help me achieve all that I have today.

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With eternal gratitude, I would like to thank my parents for their unlimited love, which I can never repay in any equivalent form. Their gift of life, nourishment and fundamental values and principles created who I am today. Without their tireless care and support, this journey would have been many, many times more difficult.

To all the friends that have supported me through both the bright and dark moments of this journey, a very sincere thank you for being a part of these four fascinating years and all the times that we shared. In no particular order, I would like to extend my appreciation to Nancy, Christy, Wendy, Meagen, Kay, Daniel, Ning, Maggie, John, Alice and Ivone. I am also extremely grateful to Fei Fei, Tim, Patrick and Alice, with whom our encounters have been limited, but on every occasion, they have given me a wealth of faith and courage, without which I would not have been able to have come so far.

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DNA  Deoxyribonucleic acid
dpi  dots per inch
DR  death receptor
EBV  Epstein-Barr virus
EDTA  Ethylenediaminetetraacetic acid
EM  effector memory cells
EMRA  effector memory cells with CD45RA expression
Eomes  Eomesodermin
Erk  Extracellular-signal-regulated kinase
FA  Formaldehyde
FACS  Fluorescence activated cell sorting
FAIM3  Fas apoptotic inhibitory molecule 3 (TOSO)
FBS  Fetal bovine serum
FITC  Fluorescein isothiocyanate
FoxO1  Forkhead box protein O1
FRC  Fibroblastic reticular cell
FSC  Forward scatter
g  gram
GATA-3  GATA binding protein 3
Gfi-1  Growth factor independent-1
GM-CSF  Granulocyte macrophage colony stimulating factor
GMP  Good manufacturing practice
GS  Goat serum
HCV  Hepatitis C virus
HIV  Human Immunodeficiency virus
HMG  High mobility group
hr  hours
HS  Human serum
HSV1  Herpes Simplex virus 1
ICC  Immunocytochemistry
ICFC  Intracellular flow cytometry
ICOS  Inducible T-cell co-stimulator (CD278)
ICOSL  Inducible T-cell co-stimulator ligand (CD278L)
Id  Inhibitor of DNA binding protein
IDO  Indoleamine 2,3-dioxygenase
IFN-  Interferon
Ig  Immunoglobulin
IL-  Interleukin
IU  International units
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<td>Lymphocyte function-associated antigen 1 (CD11a/CD18)</td>
<td></td>
</tr>
<tr>
<td>LIP</td>
<td>Lymphopenia-induced (homeostatic) Proliferation</td>
<td></td>
</tr>
<tr>
<td>LM</td>
<td>Listeria monocytogenes</td>
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<tr>
<td>m</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>M/C/FBS</td>
<td>Methanol-fixation, casein-blocking, FBS containing-antibody dilution immunocytochemistry protocol</td>
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<tr>
<td>M1</td>
<td>Memory cells generated in vitro after one round of stimulation</td>
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<tr>
<td>M2</td>
<td>Memory cells generated in vitro after two rounds of stimulations</td>
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<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
<td></td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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</tr>
<tr>
<td>Mbd2</td>
<td>Methyl-cpg-binding domain protein 2</td>
<td></td>
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<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence 1</td>
<td></td>
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<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>min</td>
<td>minutes</td>
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<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein-1 alpha</td>
<td></td>
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<tr>
<td>MOH</td>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>Molecular probes</td>
<td></td>
</tr>
<tr>
<td>MPEC</td>
<td>Memory precursor effector cells</td>
<td></td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Naïve cells</td>
<td></td>
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<tr>
<td>n</td>
<td>nano</td>
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</tr>
<tr>
<td>nAg</td>
<td>nuclear Antigen</td>
<td></td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
<td></td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
<td></td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
<td></td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cells</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<td>p</td>
<td>pico</td>
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PAMP Pathogen-associated molecular pattern
PBMC Peripheral blood mononuclear cells
PBS Phosphate buffered saline
PD-1 Programmed cell death protein 1 (CD279)
PD-L1 Programmed cell death protein 1 ligand 1 (CD274)
PD-L2 Programmed cell death protein 1 ligand 2
PE R-phycoerythin
PerCP Peridinin chlorophyll protein
PE-TR R-phycoerythin-texas red
PF/GS/BSA Paraformaldehyde-fixation, goat serum-blocking, BSA containing-antibody dilution immunocytochemistry protocol
PFA Paraformaldehyde
PI3K Phosphatidylinositol 3-kinase
PKC Protein kinase C
pMHC peptide-Major histocompatibility complex
Poly I:C Polyninosinic:polycytidylic acid
PRR pattern recognition receptor
R receptor
R0 RPMI supplemented with Pen/Strep/Glut
R1 RPMI supplemented with Pen/Strep/Glut and 1% human serum
RICD Restimulation-induced cell death
ROG Repressor of GATA-3
rpm revolutions per minute
RPMI Roswell Park Memorial Institute
RS5 RPMI supplemented with Pen/Strep/Glut and 5% human serum
sag surface Antigen
SCM Stem cell-like memory cells
SEM Standard error of the mean
Sgk1 Serum/glucocorticoid-regulated kinase 1
SIT SHP2-interacting trans membrane adaptor protein
SLEC short-lived effector cells
Spi6 Serine protease inhibitor 6
SSC Side scatter
STAT Signal transducers and activators of transcription protein
T-bet T-box transcription factor (TBX21)
TBS Tris buffered saline
Tcf-1 T cell factor 1
TCR T cell receptor
TGF-β Transforming growth factor-β
TIFF Tagged image file format
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
<td></td>
</tr>
<tr>
<td>TNF(-α)</td>
<td>Tumour necrosis factor (alpha)</td>
<td></td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
<td></td>
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<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
<td></td>
</tr>
<tr>
<td>TREC</td>
<td>T-cell receptor excision circle</td>
<td></td>
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<tr>
<td>VSV</td>
<td>Vesicular Stomatitis virus</td>
<td></td>
</tr>
<tr>
<td>VV</td>
<td>Vaccinia virus</td>
<td></td>
</tr>
<tr>
<td>Xbp-1</td>
<td>X-box-binding protein-1</td>
<td></td>
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<td>α</td>
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<tr>
<td>β</td>
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<tr>
<td>βc</td>
<td>common beta chain (CD122; IL-2/15Rβ)</td>
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<tr>
<td>γ</td>
<td>gamma</td>
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<tr>
<td>γc</td>
<td>common gamma chain (CD132)</td>
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This form is to accompany the submission of any PhD that contains research reported in published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Abstract.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 3, Section 3.3.3.3, Figure 3-8. assay for susceptibility to apoptosis

<table>
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<th>Nature of contribution by PhD candidate</th>
<th>Generation of cell subsets under experimental variations and interpretation of data</th>
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<td>Extent of contribution by PhD candidate (%)</td>
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<tr>
<th>Name</th>
<th>Nature of Contribution</th>
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<tr>
<td>Anna E. S. Brooks</td>
<td>Carried out the final assay to test for the apoptosis susceptibility of the cell subsets generated under experimental conditions</td>
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**Certification by Co-Authors**

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- In cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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<tr>
<td>Dr. Anna E. S. Brooks</td>
<td>[Signature]</td>
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Chapter 1: Introduction – Programming and generation of memory CD8\(^+\) T cells

Immunological memory, where memory T cells, B cells and long-lived plasma cells are produced during primary infections or immunization, is a defining characteristic of the adaptive immune system. These memory T and B cells are able to mount more rapid, sensitive, vigorous and qualitatively distinct responses upon secondary exposure to antigen, and thus provide host protection against re-infection.

CD8\(^+\) T cells, or cytotoxic T lymphocytes (CTLs), are especially important for the control of many infectious pathogens and eradication of tumours with their capability to specifically target and kill infected and transformed cells. The construction of the CD8\(^+\) T cell memory population is dependent on the priming signals received upon antigen recognition and naïve cell activation, which are then subordinately, but continually, shaped by environmental cues throughout the immune reaction. Decades of immunological research have defined the signals necessary to mount CD8\(^+\) T cell responses. Both T cell receptor (TCR) and costimulatory signals are essential for the activation of CD8\(^+\) T cells, stimulating naïve cells to proliferate and differentiate. A third signal provided by soluble factors is now also known to play an important role in directing T cell responses, not only by enabling full expansion and differentiation of the effector cells, but also by regulating the survival and quality of the memory populations. Each of these three forms of signals harbours vast potential of diversity from a wide range of receptor-ligand pairs, their associated signalling molecules and signal timing, strength and context. These factors all contribute to the final T cell response, and establish a set of memory cells with defined survival capability, reactivity and functionality. Due to the multitude and complexity of these signals, the regulation of the differentiation and optimisation of memory CD8\(^+\) T cell responses remains incompletely understood. Increasing our understanding of these processes has become increasingly important for therapeutic applications such as therapeutic vaccination and particularly adoptive cell therapy (ACT), where antigen-specific cells are selected and manipulated \textit{ex vivo} for infusion into autologous patients for the treatment of cancer and viral infections (Arens and Schoenberger, 2010; Cox, Harrington, and Zajac, 2011; Restifo, Dudley, and Rosenberg, 2012; Williams and Bevan, 2007).

The use of increasingly refined mouse models has not only provided much basic immunology knowledge but also has proven beneficial to the field of ACT. However, there are many differences between murine models and the human immune system (Davis, 2008; Mestas and Hughes, 2004). Further efforts in human immunology are required to better translate laboratory bench knowledge into clinical therapeutic approaches to realise the potential health benefits (Davis, 2008; Leslie, 2010).

In an earlier PhD project at the host laboratory, an \textit{in vitro} model was established to culture human CD4\(^+\) T cells over extended periods of time, allowing activation, expansion and resting of T cells for the formation
Chapter 1

of memory populations in vitro (Brooks, 2007). Such an in vitro model allows longitudinal study of human T cells with stringent controls over T cell signals. Therefore, we sought to confirm this established in vitro model for the study of human CD8+ T cell differentiation and molecular programming. In particular, we sought to investigate the effects of differential third signals on the activation and differentiation of human CD8+ T cells, to determine their role in controlling the attributes of effector and memory T cells. This knowledge would also allow us to determine some of the similarities and differences between murine and human T cells. Furthermore, as it is now recognized that strong interleukin (IL)-2 signalling drives CD8+ T cell differentiation (Kalia et al., 2010; Pipkin et al., 2010), which compromises CTL efficacy in vivo (Hinrichs et al., 2008; Klebanoff et al., 2005), the effects of reducing and even eliminating the use of exogenous IL-2 during in vitro expansion of human CD8+ T cells could be studied. Alternative agents were investigated for their provision of sufficient survival and metabolic signals for naive cell activation, expansion and memory cell maintenance. Refinement of this model led to T cell expansions with minimal differentiation and the generation of memory populations that could be restimulated in vitro. Information gathered from these experiments has implications for ACT, because the data relate to the manipulation of CD8+ human T cells in vitro, a process that is acknowledged to be sub-optimal in current ACT (Klebanoff, Gattinoni, and Restifo, 2012).

To put the experimental work into context, we must first consider current understanding of how naive CD8+ T cells become activated, proliferate and differentiate, and the molecular and cellular interactions involved in generating an optimal CD8+ T cell response, including current views on the nature of T cell memory.

1.1 Naïve CD8+ T cell activation and programming

CD8+ T cells, or cytotoxic T lymphocytes (CTLs), are an essential component of the adaptive immune system for protection against pathogenic infections and malignant cells. CD8+ T cells are able to eradicate infected or transformed cells by recognising non-self-peptides presented by self-major histocompatibility complex (MHC) class I molecules and exert cytotoxicity against these cells (Janeway, 2005). Upon maturation and exiting the thymus, the homeostasis of naïve CD8+ T cells requires contacts with self-MHC molecules accompanied by survival signals from members of the common gamma chain (γc) cytokine family, especially IL-7 or IL-15. Naïve CD8+ T cells express chemokine receptor CCR7 (CC chemokine receptor 7, CD197) and homing adhesion molecule CD62L (L-selectin), allowing them to continually circulate and enter secondary lymphoid organs to survey and search for cognate peptide-MHC (pMHC) molecules presented by antigen presenting cells (APCs) (Bromley, Thomas, and Luster, 2005; Kansas, 1996). Sustained APC-T cell interactions formed upon antigen recognition deliver TCR and costimulatory signals, and together with the appropriate inflammatory milieu, drive T cell activation and expansion. In the population of mature naïve CD8+ T cells, the precursor frequency that is able to detect a specific antigen has been estimated to be 1-5 cells out of 100,000 in mouse (Blattman et al., 2002), or 80 to 1200 cells per mouse using tetramer and magnetic-bead enrichment technologies (Obar, Khanna, and Lefrancois, 2008). These small precursor cell frequencies allow a broad array of potential antigenic epitopes to be recognised
by the host. However, these small frequencies also imply that very large clonal expansions of the antigen-specific lymphocytes are necessary in order to provide effective responses against specific antigens. Virus-specific naïve T cells were found to divide as many as 15 times to increase cell numbers up to 50,000-fold at the peak of expansion (Butz and Bevan, 1998; Murali-Krishna et al., 1998). During the process of clonal expansion, naïve cells undergo extensive differentiation and acquire effector functions including production of cytotoxic molecules (granzymes and perforin), cytokine secretion and migratory capabilities to peripheral tissues. The functionally active CTLs formed immediately following TCR stimulation are defined as “effector” cells and are able to eliminate pathogens by killing infected cells. As pathogens are cleared, the clonally expanded effector cells undergo contraction, whereby 90-95% of the effector population dies by apoptosis. A small fraction of antigen-specific cells survives to form a pool of memory cells that provide long term host immune protection. Memory cells are endowed with high proliferative potential, multipotency (capability to differentiate into effector cells as well as maintain memory cell characteristics) and long-term survival and self-renewal capabilities in the absence of antigen. These cardinal features of memory T cells allow enhanced control of re-infections due to the increased frequency of antigen-specific cells, their localization to peripheral sites for surveillance of infection, and more rapid and larger effector responses. This leads to reduced duration of infection, diminished severity of disease and enhanced herd immunity via decreased pathogen spread (Ahmed and Akondy, 2011; Kaech and Wherry, 2007; Kaech, Wherry, and Ahmed, 2002b). CD8+ memory T cells have been found to be maintained up to 75 years following vaccination in humans, effectively providing lifelong protection (Hammarlund et al., 2003).

Studies of immune responses against a variety of pathogens have shown that populations of antigen-experienced antigen-specific CD8+ T cells harbour vast heterogeneity in quality and function. Each pathogenic infection drives distinct profiles of T cell responses, but in general each specific profile encompasses a broad range of T cell properties. Each activated T cell can acquire different effector and memory cell qualities, resulting in a range of phenotypes that are each associated with a set of functional attributes. Although it is still uncertain how each pathogen dictates these distinct profiles of T cell attributes, there is evidence that the level and quality of TCR stimulation, costimulatory signals and the cytokine environment at the point of naïve cell activation determine, and thereby “programme”, the pathway of CD8+ T cell differentiation as well as the characteristics of the resulting responding T cells (Appay, van Lier, Sallusto, and Roederer, 2008; Jameson and Masopust, 2009).

1.1.1 TCR antigenic signal (Signal 1)

The engagement of TCR and CD8 co-receptor with cognate pMHC complex initiates antigenic stimulation and T cell responses. During the initial phase of antigen recognition, a series of transient TCR contacts take place with cognate pMHC complexes. Detection of the pMHC complexes is highly sensitive. However, a threshold of antigen density coupled to pMHC stability is required for the formation of stable TCR-pMHC interactions (Henrickson et al., 2008b), forming an immunological synapse where signalling and adhesion molecules aggregate between the interface of the two interacting cells (Friedl, den Boer, and Gunzer,
Chapter 1

2005). The formation of a stable interface after initial TCR-pMHC interactions can result either from repeated contacts with multiple APCs or from serial triggering with one T-APC contact (Bousso, 2008; Valitutti, Muller, Cella, Padovan, and Lanzavecchia, 1995). The rates of these contacts determine the time required for the establishment of immunological synapses and thus lead to the relative efficiency of T cell activation and functional development (Henrickson et al., 2008b; Stone, Chervin, and Kranz, 2009). The stable contact between APC and T cells induces TCR-mediated signalling pathway via CD3 co-receptor chain, ζ-associated protein of 70kDa (ZAP-70), and src-family kinases Lck (p56lck) and Fyn (p59fyn) to initiate T cell proliferation and differentiation (Salmond, Filby, Qureshi, Caserta, and Zamoyska, 2009). This leads to the activation of several kinase pathways including protein kinase C-θ (PKCθ) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Smith-Garvin, Koretzky, and Jordan, 2009). Co-receptor CD8 binding to MHC molecules helps to stabilize TCR-pMHC contacts and recruits the kinase Lck to the TCR complexes, thus assisting signal transduction (Davis and van der Merwe, 2003; Purbhoo, Irvine, Huppa, and Davis, 2004). Stable formation of TCR-pMHC complexes also requires multivalent binding and clustering of TCRs (Minguet, Swamy, Alarcon, Luescher, and Schamel, 2007; Stone and Stern, 2006) and hence pMHC molecules are required to be oligomerized and immobilized on a surface to allow the formation of supramolecular clusters (Chervin et al., 2009). TCR binding avidity is thus defined as the functional collective strength from multimeric TCR-pMHC stability, TCR density and MHC-co-receptor binding for a specific pair of cognate TCR-pMHC multivalent interaction (Stone et al., 2009). The accumulation of signalling events both during the initial phase of pMHC recognition and over the course of the immune responses continuously shapes the final T cell response in both quantitative and qualitative terms. While T cell activation ensues once the cumulative TCR signalling rises above a required threshold (Henrickson et al., 2008b; Stone et al., 2009), inhibitory responses are induced either by insufficient antigen stimulation (Sadegh-Nasseri, Dalai, Korb Ferris, and Mirshahidi, 2010) or by antigen persistence, such as in chronic viral infections (Shin and Wherry, 2007; Wherry, Blattman, Murali-Krishna, van der Most, and Ahmed, 2003a). Inhibited T cell responses are characterised by cell surface expression of inhibitory receptors, an absence of proliferation and effector functions upon subsequent challenges and/or an induction of antigen-specific regulatory T cells (Shin and Wherry, 2007; Virgin, Wherry, and Ahmed, 2009; Wherry and Ahmed, 2004).

Antigenic dose, duration of antigen availability, TCR affinity and antigenic epitope potency for T cell activation have all been proposed as determinants of TCR signalling and subsequent T cell activation. It has been found that the overall dose of the antigen, or the infectious pathogenic load, determines the magnitude of CD8⁺ T cells recruited into activation, clonal expansion and the subsequent memory population size (Badovinac, Porter, and Harty, 2002; Kaech and Ahmed, 2001; Wherry, McElhaugh, and Eisenlohr, 2002b). An augmented antigen dose is likely to enhance epitope presentation and increase not only the density of the pMHC complexes presented by any given APC, but also the density of APCs presenting the cognate peptide in a lymphoid organ, leading to enhanced accumulative antigen stimulation. Using two-photon time-lapse microscopy to observe intact lymph nodes ex vivo, a higher density of antigen presentation has been found to induce a larger cluster of T cells surrounding the antigen loaded dendritic
cells (DCs) (Bousso and Robey, 2003). An increase in either antigen dose or the concentrations of antigen-pulsed DC has been found to form stable DC-T cell interactions faster, leading to augmented T cell activation (Henrickson et al., 2008b), where the recruitment of precursor cells has recently been found to be an extremely efficient and non-limiting process under natural (non-TCR transgenic) conditions (van Heijst et al., 2009). Together with the augmented clonal expansion, the antigenic load also correlates with the development of CD8$^+$ cytotoxic effector function (Henrickson et al., 2008b; Wherry, Puorro, Porgador, and Eisenlohr, 1999), where the density of pMHC on DCs determines interferon (IFN)-γ expression and correlates with enhanced production of tumour necrosis factor (TNF) and interleukin (IL)-2 (Beuneu et al., 2010), leading to better CTL responses. Prolonged duration of antigenic stimulation also affects T cell responses; sustained stimulatory signals appear to favour the development of short-lived effector cells over long-lived memory populations (Intlekofer, John Wherry, and Reiner, 2006; Kaech et al., 2002b). Antigen persistence during chronic infections, which will be discussed later (1.1.6), alters many aspects of the CD8$^+$ T cell response including effector function and proliferation potential of memory cells. Investigations of differential epitopes and TCR affinities have however found similar CD8$^+$ T cell response kinetics at the cellular level, as well as similar T cell gene expression patterns once the TCR threshold has been reached (Busch, Pilip, Vijh, and Pamer, 1998; Munitic et al., 2009). Therefore, the antigen dose and the duration of antigenic stimulation are the major determinants of the scale of T cell expansion, the generation of effector cells and the establishment of memory populations.

1.1.2 Costimulation (Signal 2)

The second signal necessary for adequate T cell activation is delivered by costimulatory stimuli, which augment TCR signalling pathways in a quantitative manner to enhance signals for proliferation, survival and differentiation (Acuto and Michel, 2003; Nolte, van Olffen, van Gisbergen, and van Lier, 2009). Costimulation helps the immune system to discriminate self from non-self (Bretscher and Cohn, 1970), preventing the development of T cell tolerance in the form of either anergy or deletion (Schwartz, 2003). It is therefore especially important for the activation of naïve cells (Lenschow, Walunas, and Bluestone, 1996; Viola and Lanzavecchia, 1996). It has frequently been reported that CD28 is essential for the generation of CD8$^+$ T cell responses against infections by pathogens such as *Listeria monocytogenes*, vesicular stomatitis virus (VSV) and influenza (Andreasen, Christensen, Marker, and Thomsen, 2000; Liu, Wenger, Zhao, and Nielsen, 1997; Shedlock et al., 2003). There are rare exceptions where successful immune responses lack dependence on CD28. For example, CD28-mediated signals are not required to develop responses in *Lymphocytic choriomeningitis* virus (LCMV) infections (Shahinian et al., 1993; Suresh et al., 2001). For LCMV, it has been suggested that sufficiently high levels of TCR stimulation by viral antigen overcome the requirement for costimulatory signals (Kundig et al., 1996; Viola and Lanzavecchia, 1996). This is consistent with the idea that costimulatory signals simply serve to amplify signalling pathways initiated by TCR. In recent years, potential sources of the second signal for T cell stimulation have expanded to include many other receptor-ligand interactions that can provide synergistic signals for TCR signalling (Boesteanu and Katsikis, 2009). Most of these costimulatory receptor-ligand
pairs belong to two major families: the immunoglobulin (Ig) superfamily, which includes the CD28 subfamily, and the tumour necrosis factor receptor (TNFR) family (Crawford and Wherry, 2009). The CD28 family includes the costimulatory receptor ICOS (inducible T cell co-stimulator, CD278), while the TNFR family includes costimulatory receptors CD27, CD40L (CD40 ligand), 4-1BB (CD137) and OX40 (CD134) (Croft, 2009). However, CD28 is still regarded as the most important costimulatory receptor due to its robust enhancement of TCR signalling (Janeway and Medzhitov, 2002; Yokosuka and Saito, 2009) and its capacity to induce the expression of other costimulatory receptors upon TCR stimulation.

CD28 binds to B7-1 (CD80) and B7-2 (CD86) on mature APCs, and is constitutively expressed on naïve T cells, indicating its importance for primary T cell responses. Its ligands B7-1 and B7-2 are either absent or expressed at low levels on non-activated APCs and are rapidly upregulated upon infection, tissue injury or inflammation (Carreno and Collins, 2002; Gross, Callas, and Allison, 1992). CD28 has been found to enhance T cell survival via the NF-κB dependent Bcl-xL pathway (Boise et al., 1995). It also increases IL-2 production and CD25 (high affinity receptor of IL-2; IL-2Rα) expression (Lucas, Negishi, Nakayama, Fields, and Loh, 1995; Shahinian et al., 1993) to synergistically promote proliferation and differentiation. CD28 signalling directly facilitates cell cycle progression by allowing cell cycle entry from G0 to G1 phase and overcomes anergy by regulating the expression of p27kip1 and possibly the cyclin-D-cyclin dependent kinase (CDK)4/CDK6 complex (Appleman, van Puijenbroek, Shu, Nadler, and Boussiotis, 2002; Sears and Nevins, 2002). Another key factor contributing to robust and sustained T cell responses is the activation of glucose metabolism and macromolecule synthesis via the PI3K (Phosphatidylinositol 3-kinase)/Akt pathway and mTOR (mammalian Target of Rapamycin) activities from both CD28 and IL-2 signalling (Frauwirth et al., 2002). CD28 signalling also induces the expression of a second wave of costimulatory receptors in activated T cells, including ICOS, OX40 and CD40L. These are crucial for further engagement with ligands on APCs and the development of effector functions and memory populations (Acuto and Michel, 2003). Acquisition of cytolytic effector functions has been linked to CD28 signalling, but this may be a follow-on effect from enhanced cell survival and expansion (Suzuki et al., 2008). However, CD28 does promote production of cytokines including IL-2, IFN-γ, IL-4, MIP-1α (macrophage inflammatory protein-1 alpha), TNF and GM-CSF (granulocyte macrophage colony stimulating factor) (Herold et al., 1997; McAdam, Schweitzer, and Sharpe, 1998; Thompson et al., 1989). CD28 signalling during primary responses induces chromatin remodelling and epigenetic changes, which enable expression of genes enabling cell growth, cell cycle progression and differentiation (Acuto and Michel, 2003). CD28 signalling has also recently been recognised to be critical for secondary responses. CD28 optimises IL-2 production and promotes T cell survival in antigen-experienced cells and is required for optimal recall expansion and development of cytolytic functions (Boesteanu and Katsikis, 2009).

The second member of the CD28 family, ICOS, also promotes T cell proliferation and IL-2 production upon binding to its counter-receptor expressed on APCs, ICOS ligand (ICOSL). However, its signalling potency is much less than that of CD28 (Carreno and Collins, 2002; Sharpe and Freeman, 2002). ICOS costimulation can contribute to CD8+ T cell proliferation and formation of memory populations (Takahashi
et al., 2009; Watanabe, Hara, Tanabe, Toma, and Abe, 2005). Both CD28 and ICOS signalling pathways promote the production of cytokines such as IFN-γ, IL-4, TNF and GM-CSF (Beier et al., 2000; Riley et al., 2001; Thompson et al., 1989). Importantly, ICOS strongly induces production of IL-10 (Beier et al., 2000; McAdam et al., 2000). ICOS+ T cells are commonly found in germinal centres and surrounding T cells zones (Yoshinaga et al., 1999), suggesting it plays a more important role in the induction of type 2 responses linked to humoral immunity than in cellular responses for protection against extracellular pathogen infections (McAdam et al., 2001; Tafuri et al., 2001).

The second major group of costimulatory molecules belongs to the TNFR family and includes CD27, CD40L, 4-1BB (CD137) and OX40 (CD134), which bind to the ligands CD70, CD40, 4-1BBL and OX40L (CD252), respectively (Croft, 2009). TNFR family signalling independently regulates CD8+ T cell differentiation and the frequency of effector and memory cells generated following infection. It also complements CD28-mediated costimulatory signals with similar functional outcomes (Hendriks, Xiao, and Borst, 2003). The engagement of these costimulatory molecules enhances T cell survival, cell cycle progression and cytotoxic effector functions as well as the production of cytokines, and often amplifies inflammatory responses (Croft, 2009). This is due to the sharing of signalling pathways the between the TNFR and CD28 families. Examples include JNK (c-Jun N-terminal kinases), NF-κB and PI3K/Akt pathways.

Although CD28 provides robust and essential costimulatory signals for CD8+ T cell activation in many circumstances, under any given context of inflammation or disease, more than one costimulatory receptor ligation is commonly involved. It has been suggested that the timing, context and intensity of each of these costimulatory signals will collectively determine the final T cell response outcome (Hendriks et al., 2005; Nolte et al., 2009). The relative contribution from each costimulatory receptor may differ as the immune response progresses (Croft, 2009), and the optimal costimulatory signal requirement may also change with alterations in the antigenic experience of the specific T cells involved (Bertram et al., 2004). However, the involvement of many costimulatory receptor signalling molecules in immune reactions ultimately creates partial redundancy both within and between the two major families of costimulatory molecules. Indeed, blockade or specific gene deletion of most of the costimulatory molecules in many models have commonly caused only modest reduction in CD8+ responses (Andreasen et al., 2000; DeBenedette et al., 1999; Kwon et al., 2002; Shahinian et al., 1993; Shedlock and Shen, 2003). When more than one costimulatory pathway is disrupted, T cell activity is more severely impaired, suggesting synergistic roles for costimulatory receptors (Croft, 2009; Hendriks et al., 2003; Tan, Whitmire, Ahmed, Pearson, and Larsen, 1999).

Costimulatory signals are opposed by inhibitory receptors, often members of the same molecular superfamilies. Together with the costimulatory receptors, these inhibitory receptors balance tolerance to self-antigens against productive immune responses to foreign antigens, and dampen immune responses in chronic infections. CTLA-4 (cytotoxic T lymphocyte antigen 4; CD152) and PD-1 (programmed cell death
protein 1; CD279) are among the most studied inhibitory receptors, but others including LAG-3, 2B4 (CD244), CD160, TIM3, PIR-B and natural killer cell (NK) receptors have also been found to play a role (Crawford and Wherry, 2009). Inhibitory receptor expression is mostly found to be induced upon T cell activation. Some inhibitory receptors compete with their costimulatory counterparts for binding to a common ligand. One example is CTLA-4, which competes with CD28 to bind to B7-1 and B7-2. Others, for example PD-1, individually direct negative signalling. This receptor binds to PD-L1 or PD-L2 (programmed cell death protein 1 ligand 1 or 2), and directly downregulates CD8+ responses (Carreno and Collins, 2002; Sharpe and Freeman, 2002).

1.1.3 Soluble factors (Signal 3)

For many years, the “two-signal” model explained T cell activation requirements (Lafferty, Misko, and Cooley, 1974). “Danger” signals provided by the innate immune system via inflammatory cytokines were thought to be necessary to fully activate T cells and prevent the development of tolerance (Janeway, Goodnow, and Medzhitov, 1996; Matzinger, 1994). However, until microspheres incorporated with pMHC complexes and costimulatory ligands, or “artificial APCs” (aAPCs), were employed to stimulate naïve CD8+ T cells (Curtsinger et al., 1999), it was not known whether the inflammatory cytokines acted directly on T cells or when these signals were necessary. Murine antigen-specific naïve CD8+ T cells were found to proliferate with aAPCs, but the activated effector cells either had defects in cell survival or limited clonal expansion and developed poor effector functions. Cells that did survive were tolerised. The addition of IL-12 or type I interferons (IFN-α or IFN-β) was found to allow appropriate clonal expansion, survival, development of effector functions and establishment of competent memory populations (Curtsinger, Johnson, and Mescher, 2003a; Curtsinger, Lins, and Mescher, 2003b; Curtsinger, Valenzuela, Agarwal, Lins, and Mescher, 2005; Kolumam, Thomas, Thompson, Sprent, and Murali-Krishna, 2005). This series of murine in vitro experiments and cytokine receptor knockout systems uncovered the need for soluble factors as a third signal for optimal CD8+ T cell responses. Adoptive transfer experiments where wild type CD8+ T cells were transferred into IL-12 receptor-deficient mice also showed the direct action of the third signals in vivo (Schmidt and Mescher, 2002). Furthermore, these inflammatory cytokines were able to replace the adjuvant requirement for immunization in mice (Schmidt and Mescher, 1999) or substitute for CD4+ T cell help. This suggests that DCs activated via either toll-like receptors (TLRs) or activated CD4+ T cells are able to secrete the cytokines needed to convert an otherwise tolerising stimulus into a productive CD8+ T cell response (Ontiveros, Wilson, and Livingstone, 2011; Schmidt and Mescher, 1999, 2002; Wiesel et al., 2012). Their secretion was found to have distinct directionality in the immunological synapse between APCs and T cells, thought to increase the potency and the duration of their effects (Chen et al., 2005; Huse, Lillemeyer, Kuhns, Chen, and Davis, 2006).

In general, priming murine CD8+ naïve T cells with IL-12 or IFN-α enhances CD8+ T cell survival, clonal expansion and augments the development of effector functions including cytotoxicity, IFN-γ production, and granzyme B expression, as well as the formation of memory populations (Mescher et al., 2006). Early
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Exposure to cytokines upon TCR stimulation also influences the differentiation of CD8+ T cells, and the balance between the formation of effector and memory cell populations (Badovinac, Porter, and Harty, 2004; Cox et al., 2011). Gene expression profiles of naïve CD8+ T cells activated with or without the presence of IL-12 or IFN-α demonstrated that both cytokines led to a group of commonly regulated genes involved in effector functions, proliferation, costimulation, survival, trafficking and migration, as well as transcription factors involved in CD8+ T cell differentiation (Curtsinger and Mescher, 2010). The ability of memory cells to rapidly acquire effector functions without further direction from third signals indicated that an epigenetic inheritance mechanism is in place from the signals of the initial priming (Curtsinger et al., 1999; Curtsinger et al., 2005). Indeed, histone acetylation (Agarwal et al., 2009; Araki, Fann, Wersto, and Weng, 2008; Fann et al., 2006) and methylation (Araki et al., 2009b), and DNA methylation (Lu et al., 2003; Northrop, Thomas, Wells, and Shen, 2006) have been found to enable continual expression of a number of genes, including granzyme B, perforin, IL-2 and IFN-γ as well as transcription factor Eomesodermin (Eomes). Aside from IL-12 and type I IFNs, a few other cytokines such as IL-4 (Kemp, Backstrom, and Ronchese, 2005; Vukmanovic-Stjeic, Vyas, Gorak-Stolinska, Noble, and Kemeny, 2000), IL-21 (Alves, Arosa, and van Lier, 2005; Hinrichs et al., 2008), IL-18 (Balasubramani et al., 2010; Freeman et al., 2010) and IL-23 (Henry et al., 2010; Vanden Eijnden, Goriely, De Wit, Willems, and Goldman, 2005) have also shown some influences on CD8+ T cell responses. This is not surprising since different APC subsets are known to secrete a variety of cytokines (Hochrein et al., 2001). The activation of DCs following engagement of differential TLRs by various pathogenic products, as well as different innate immune responses, are also known to induce different cytokine profiles that lead to distinctively regulated responses (Mitsuhashi, 2010; Noble, 2009; Rajkovic et al., 2011). Each priming cytokine is presumed to provide different immune outcomes depending on the context of the immune reaction, and the quality and timing of the T cell response needed to eliminate a pathogen. However, careful interpretations are still required to dissect the detailed functions and differences between third signal cytokines such as IL-12 and IFN-α, due to complexity of the data and vast variety of experimental models used across the literature.

Following large numbers of murine studies focused on IL-12 and IFN-α as third signals, the factors responsible for the complex data in the literature are emerging. These include pathogen dependency, in vivo compensatory regulations, overlapping cytokine functions and shared receptor subunits, as well as differential experimental set-ups. Differences between the roles of IL-12 and IFN-α in different infectious models have been shown in detailed studies (Keppler and Aichele, 2011). For example, the deletion of type I IFN receptor (IFNAR) has deleterious effects on the expansion of Ag-specific T cells after LCMV infection, but has less dramatic impact after infection with Listeria monocytogenes (LM) or vaccinia virus (VV) (Harty and Badovinac, 2008; Thompson, Kolumam, Thomas, and Murali-Krishna, 2006). Originally, it was postulated that the high antigenic load in LCMV infection might contribute to the dependence of LCMV-specific CD8+ T cells on IFN-α (Huber and David Farrar, 2011). However, further investigations revealed that the large amounts of IFN-α induced during the natural response to LCMV are at the expense of IL-12 production, making the CD8+ T cell response particularly sensitive to loss of IFN-α signalling (Haring, Badovinac, and Harty, 2006). In contrast, LM and VV infections were found to be largely
dependent on IL-12 for successful immune responses (Harty and Badovinac, 2008). Concordantly, immune reactions to LM and VV were found to induce significant IL-12 production, making them especially sensitive to modulation of IL-12 signalling (Haring et al., 2006). However, there is also a certain amount of functional synergy between IL-12 and IFN-α. Thus, double knockout of both IL-12 receptor (IL12R) and IFNAR reduced the development of effector and memory cells in response to LM infections compared to single knockout (Xiao, Casey, Jameson, Curtsinger, and Mescher, 2009). Internally regulated compensatory effects have been found; IFNAR-deficiency induces upregulation of IL-12 secretion in vivo (Way, Havenar-Daughton, Kolumam, Orgun, and Murali-Krishna, 2007), which provides alternative signals to support memory development for transferred CD8+ T cells. Functional overlays between IL-12 and IFN-α are further complicated by the use of adjuvants to induce inflammatory cytokines. For example, CpG oligodeoxynucleotides and poly I:C (Polyinosinic:polycytidylic acid) induce both IL-12, type I interferons and/or IFN-γ in mice (Krieg, 2002; Manetti et al., 1995). Other important factors in the in vivo models include the choice of receptor deletions, APCs and their activation stimuli, and the use of TCR transgenics. The shared receptor subsets between IL-12 and IL-23 led to an abrogation of both IL-12 and IL-23 signalling upon the deletion of IL-12Rβ1 chain (Brombacher, Kastelein, and Alber, 2003), whereas different APCs, TLR engagements and/or CD4+ T cell help in vivo provided variations in the cytokine milieu and costimulatory receptor expression that led to differential CD8+ T cell responses (Pufnock et al., 2011; Rajkovic et al., 2011; Roses et al., 2008).

The timing of investigations also requires consideration. Effector functions are commonly assessed 3-7 days post-stimulation, either with or without restimulation (Curtsinger et al., 1999; Curtsinger et al., 2005; Hervas-Stubbs et al., 2010). At this stage cells are still engaged in the primary response, and are thus unable to provide a true indication of memory programming. The activated status of the CD8+ T cells is often accompanied by temporary changes in phenotypic expression (Akondy et al., 2009; Miller et al., 2008). Restimulations for the functional assays during these times are likely to induce AICD (Brenner, Krammer, and Arnold, 2008) as well as potentially being perceived as accumulative TCR engagements within one immune response rather than recall responses, since memory characteristics have yet to be developed. This lack of distinction between activated and rested cells has led to discrepancies in the interpretation of data. Often, cells in different functional phases of an immune reaction were being compared, and sometimes were even thought to be different memory subsets. There are also very limited data on recall responses after the formation of rested memory populations, to test the “programming” effects of the third signal cytokines. Where memory cells have been targeted for study in transgenic mouse systems, there are also difficulties in dissecting the roles of the cytokines in primary and secondary stimulations, since gene deficiencies induce systematic effects that are carried over between sequential challenges.

Reports on the effect of third signal cytokine priming for human CD8+ T cells are very limited. The use of IL-21 for the generation of optimal T cells for ACT in mice (Hinrichs et al., 2008) has led to a number of IL-21 studies on human CD8+ T cells (Albrecht et al., 2011; Kaka et al., 2009; Wölffl et al., 2010), while four
studies investigating the priming effects of IL-12 or IFN-α have been reported for human cells (Chowdhury, Ramos, Davis, Forman, and Farrar, 2011; Hervas-Stubbs et al., 2012; Hervas-Stubbs et al., 2010; Ramos et al., 2009). In vitro studies of human cells have generally avoided complexities in the interactive network of the in vivo immune system. Utilisation of anti-CD3 and anti-CD28 antibodies to stimulate CD8⁺ T cells in human studies also bypasses the variations in immune response for different pathogenic infections. However, the common gamma chain (γc) cytokines needed for in vitro studies to support T cell survival are likely to give rise to complexities in response due to the intrinsic characteristics of each γc cytokine (Mitchell, Ravkov, and Williams, 2010; Rochman, Spolski, and Leonard, 2009). Of particular importance are the induction of differentiation, proliferation and effector functions by IL-2 (Cornish, Sinclair, and Cantreli, 2006; Manjunath et al., 2001; Pipkin et al., 2010), and their combined effects with certain priming cytokines (Alves et al., 2005). Difficulties in data interpretation have also resulted from lack of consensus in T cell subset nomenclatures and molecular definitions (see later, 1.4.5), including a general lack of distinction between activated and rested cells. This is linked to variations in experimental timing, leading to comparison of cells at different phases of differentiation or functional development. Due to variations in experimental set-ups, differences between the potential third signal cytokines on human CD8⁺ T cells require further direct comparative studies.

1.1.4 CD4⁺ T cell help and IL-2 requirement

The need for CD4⁺ help during the development of CD8⁺ T cell responses has been of great interest in attempting to understand the generation of optimal CTL activity and the establishment of CD8⁺ T cell memory. Studies aiming to ascertain the role of CD4⁺ help in primary CD8⁺ T cell responses have led to an evolving understanding of the different ways CD8⁺ T cells can be optimally primed. CD4⁺ help was first observed to augment CD8⁺ T cell responses and programme activated CD8⁺ T cells to establish long-lived, protective memory (Bevan, 2004). CD40-CD40L ligation between antigen-specific CD4⁺ T cells and DCs was thought to be crucial to licence DCs, and allow them to prime CTL responses by upregulation of costimulatory molecules and secretion of inflammatory cytokines (Bennett et al., 1998; Schoenberger, Toes, van der Voort, Offringa, and Melief, 1998). Later, it was found that triggering of pattern recognition receptors (PRRs) such as TLRs to activate APCs during viral or bacterial infections could ablate the need for CD4⁺ help (Iwasaki and Medzhitov, 2004; Janeway and Medzhitov, 2002). Thus, it has been proposed that the dependency of the primary CTL response on CD4⁺ help is inversely correlated with the capability of the pathogen to generate inflammatory responses; in non-inflammatory situations, CD40 signalling on DCs is required to induce their maturation and secretion of cytokines (Williams and Bevan, 2007).

Concordantly, the role of CD4⁺ cells in inducing acute CD8⁺ effector responses appears to be minimal in acute LCMV and Listeria infections (Shedlock and Shen, 2003; Sun and Bevan, 2003), while the absence of CD4⁺ help in ovalbumin (OVA) and VV models impairs CD8⁺ T cell responses, necessitating supplement of signal 3 cytokines to restore CD8⁺ T cell effector function (Ontiveros et al., 2011; Wiesel et al., 2012). During chronic infections, however, CD4⁺ help for the primary CD8⁺ T cell responses has been found to be non-redundant, despite high levels of inflammation. Lack of CD4⁺ help or CD40L-deficiency results in an
inability to control chronic LCMV infections, and a loss in CTL activity has been found in γ-herpes virus infections and AIDS without CD4⁺ help (Altfeld and Rosenberg, 2000; Cardin, Brooks, Sarawar, and Doherty, 1996; Matloubian, Concepcion, and Ahmed, 1994).

For the generation of memory CD8⁺ T cells capable of efficient secondary responses, the need for CD4⁺ help is much more straightforward. CD8⁺ T cells primed in the absence of CD4⁺ T cells expand poorly upon secondary challenge, secrete low levels of cytokines and the memory cells generated are gradually lost over time (Badovinac, Messingham, Griffith, and Harty, 2006; Rocha and Tanchot, 2004). Similar results have been found in independent studies where CD4⁺ depletions were carried out by multiple different strategies, as well as in experiments using adoptive transfers. These eliminate carry-over effects from inefficient primary responses and antigen persistence (Janssen et al., 2005; Janssen et al., 2003; Khanolkar, Fuller, and Zajac, 2004; Shedlock and Shen, 2003; Sun and Bevan, 2003). CD4⁺ help has also been found to regulate and imprint gene expression programmes for memory CD8⁺ T cells during the primary response (Rapetti, Meunier, Pontoux, and Tanchot, 2008). This is consistent with the observation that once a memory CD8⁺ population has been established, CD4⁺ help is no longer required (Bourgeois, Rocha, and Tanchot, 2002; Sun and Bevan, 2003). CD4⁺ help has also been implicated in the prevention of death upon secondary stimulation; unhelped CD8⁺ T cells were found to express TNF-related apoptosis-inducing ligand (TRAIL) on restimulation and undergo activation-induced cell death (AICD) (Griffith et al., 2007; Janssen et al., 2005).

In summary, although some controversy still exists about the need for CD4⁺ help for primary CD8⁺ T cell responses, efficient secondary CD8⁺ T cell responses appear to be dependent on CD4⁺ help during the primary response. How CD4⁺ help enables appropriate secondary responses by CD8⁺ T cells remains unclear.

IL-2 secreted by CD4⁺ T cells was originally thought to be required for CTL proliferation or to act as a helper-dependent checkpoint upon naïve CD8⁺ T cell activation. The removal of IL-2 signalling showed IL-2 to have only modest effects on the primary CTL responses (D'Souza, Schluns, Masopust, and Lefrancois, 2002; Williams, Tyznik, and Bevan, 2006; Yu et al., 2003). However, IL-2 signalling during the primary response was later realised to be necessary for the secondary expansion of CD8⁺ memory cells (Williams et al., 2006). Therefore, it was postulated that part of the imprinting of CTL by CD4⁺ help during priming may be due to IL-2 signalling (Jameson and Masopust, 2009). Recent refinement of the mouse models that disrupt IL-2 signalling have added new detail to this concept. In this work, a strategy to specifically control IL-2 production in each relevant cell type was used rather than IL-2- or CD25-deficient mice, which manifest autoimmune and lymphoproliferative disorders. These experiments showed that CD4⁺ T cells are required to transmit CD40-CD40L signals to DCs to enable optimal CD8⁺ responses, but only CD8⁺ autocrine IL-2 production, not IL-2 secreted by CD4⁺ cells, is necessary for the development of competent CD8⁺ memory cells capable of secondary responses (Feau, Arens, Togher, and Schoenberger, 2011). However, it is still not completely understood whether CD4⁺ help provides additional signals for the
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The generation of robust secondary CD8⁺ T cell responses other than enabling the maturation of APCs and their secretion of cytokines.

Although IL-2 is not essential for the initiation of CD8⁺ T cell proliferation or the primary response, IL-2 has long been recognized for its sustained effect on clonal expansion and effector differentiation (D'Souza and Lefrancois, 2003; Manjunath et al., 2001; Van Parijs et al., 1999). Phenotypic and functional alterations are evident in CD25-deficient effector cells which exhibit an “early” differentiation phenotype, with enhanced expression of CD62L and CD127 (IL-7Rα). These cells preferentially develop into central memory cells (Bachmann, Wolint, Walton, Schwarz, and Oxenius, 2007; Obar et al., 2010; Williams et al., 2006). IL-2 has recently been shown to have an important role in the induction of effector and terminal differentiation. High dose IL-2 was found to upregulate the expression of the transcription factors Eomes and Blimp-1, leading to enhanced expression of cytotoxic molecules, granzymes and perforin (Pipkin et al., 2010), and prolonged expression of the high affinity IL-2 receptor IL-2Rα (CD25) was found to correlate with more differentiated phenotypes (Kalia et al., 2010). Furthermore, IL-2 also inhibits the activities of the quiescence-associated transcription factor FoxO1 (forkhead box protein O1) (Stittrich et al., 2010). FoxO1 induces the expression of another transcription factor KLF2 (kruppel-like factor 2), and together they are responsible for the expression of important homing molecules, CCR7 and CD62L, associated with early differentiation (Kerdiles et al., 2009; Takada et al., 2011). In summary, CD4⁺ help during the primary stimulation is crucial for the development of functional CD8⁺ memory cells, probably through interactions with APCs to induce optimal autocrine IL-2 signalling in CD8⁺ T cells. However, chronic or strong IL-2 signalling through CD25 drives effector differentiation via increased expression of Eomes and Blimp-1 to increase effector molecule expression, and suppresses FoxO1 and KLF2 which support lymph node homing.

1.1.5 The integration of signals

1.1.5.1 T cell programming

The three essential signals for naïve CD8⁺ T cell activation, namely antigen stimulation, costimulation and cytokine milieu, each contribute to CTL responses as discussed previously, and are modulated by factors such as CD4⁺ help and inhibitory receptor signalling. These signals integrate to govern the type and strength of the CD8⁺ T cell immune response (Mescher et al., 2006; Schwartz, 2003; Williams and Bevan, 2007). The concept of T cell programming describes the engagement of an autonomous programme of proliferation and differentiation pathway by each activated naïve T cell following the integration of signals received at the time of antigen stimulation (Arens and Schoenberger, 2010). Across a population of T cells, this individual programming, based on each cell’s unique experience, can generate a heterogeneous population of effector and memory cells (Kaech and Wherry, 2007). Programming is a dynamic process and the fate of the dividing cells can be continuously shaped during clonal expansion following the initial antigenic contact, for example by antigen persistence in the case of chronic infections. The integration of
signals during the encounters of naïve CD8+ T cells with one or more APCs determines the key parameters of the CTL responses. These include the extent and magnitude of clonal expansion, functional capacities of the effector population, the onset and magnitude of contraction and the survival capabilities of the resulting memory cells (Arens and Schoenberger, 2010; Joshi and Kaech, 2008; Williams and Bevan, 2007). The defined functionality of memory cells can be heritably propagated from the priming of naïve cells to the subsequent generations of clonal populations by the expression of specific transcription factors and epigenetic modifications (Wilson, Rowell, and Sekimata, 2009; Youngblood, Davis, and Ahmed, 2010). Although naïve CD8+ T cells have been shown to enter cell division and elicit specific cytolytic effector functions from as little as 2 hours of antigenic contact in vitro, when antigenic stimulation is short, proliferation aborts early and the cells do not accumulate (Kaech and Ahmed, 2001; van Stipdonk, Lemmens, and Schoenberger, 2001). Mouse models that control the duration of antigen stimulation (e.g. using Listeria monocytogenes infections with antibiotic treatments or engineered fibroblastic APCs that allow temporal control of stimulation) have shown that 24 hours of antigenic contact allow induction of an “autopilot” programme that sustained naïve CD8+ T cell expansion and robust effector function (Bevan and Fink, 2001; van Stipdonk et al., 2003; Wong and Pamer, 2001). Subsequent experimentation showed that optimal clonal expansion and memory formation are achieved when antigenic stimulation is lengthened to 2-3 days, although effector functions were not augmented with the extension of antigenic stimulation beyond one day (Curtsinger et al., 2003a; Pric, Hernandez-Hoyos, and Bevan, 2006). Following the discovery of the necessity of a third signal for T cell activation, cytokine signalling has been found to be required for optimal clonal expansion between 12-36 hours post-infection, and optimal functional development from 30 to 60 hours post-infection. Moreover, the accompanying TCR and costimulation signals are required up to 36 hours post-stimulation for the third signals to take effect (Curtsinger et al., 2003a; Curtsinger et al., 2003b; Mescher et al., 2006). Since these three signals are essential to programme T cell differentiation and functional development, this period of 36-60 hours post-infection is regarded as the critical time frame for T cell programming. It has been suggested that the onset, kinetics and magnitude of contraction are programmed during the early phases of immune reactions. Thus effector cell contraction correlates inversely with the degree of inflammation rather than the magnitude of expansion or the dose and duration of infection (Badovinac et al., 2002, 2004). In summary, the integration and timing of specific antigenic stimuli, costimulation signals and the surrounding soluble factor stimulations during the early phases of the immune reactions are critical in the determination of the outcome of the acute immune responses, and also the quality and quantity of the immunological memory produced for the protection of the host against future re-infections.

1.1.5.2 The mechanisms and regulation of T cell programming by transcriptional and metabolic control

As described above, the integration of immunological and environmental signals at T cell priming determines the outcome of immune responses not only for the acute generation of effector activities, but
also for functional attributes in memory cells. These attributes can be propagated to progeny during cell divisions via the regulation of transcription factor expression, epigenetic modifications and chromatin modifications (Youngblood et al., 2010). The influence of integrated signals at priming on heritable gene expression profiles of CD8+ T cells are only beginning to be understood, although they are much better defined for the subtypes of CD4+ helper cells, such as the expression of GATA-3 for Th2 responses and T-bet for Th1 responses (Murphy and Reiner, 2002; Wilson et al., 2009; Zhu and Paul, 2010).

Several transcription factors have been found to be important in the developmental decisions of CD8+ effector and memory cell fates. T-box factors T-bet and Eomes regulate CD8+ T cell differentiation by controlling the production of IFN-γ, granzyme B and perforin (Glimcher, Townsend, Sullivan, and Lord, 2004; Pearce et al., 2003; Sullivan, Juedes, Szabo, von Herrath, and Glimcher, 2003), as well as the expression of CD122 (IL-2 receptor β-chain, IL-2Rβ). CD122 is shared by IL-2 and IL-15 receptor complexes and thus affects both IL-2 signalling and IL-15-dependent memory cell survival (Intlekofer et al., 2005; Yeo and Fearon, 2011). CD8+ T cells deficient in either T-bet or Eomes generate anti-viral responses but produce effector cells aberrant in cytotoxic functions. However, when both transcription factors are absent, CD8+ T cells undergo anomalous type 17 differentiation, which produces effector cells that secrete IL-17 (Intlekofer et al., 2008; Intlekofer et al., 2005). This indicates that T-bet and Eomes each have specific, yet partially redundant, functions for the development of effector CTLs. Despite their apparent cooperative role in the effector functions of CD8+ T cells, T-bet and Eomes have been found to distinctively promote the development of effector or memory cells, respectively (Banerjee et al., 2010; Joshi et al., 2011; Zhou et al., 2010). These two transcription factors were found to be inversely regulated by IL-12 in murine studies (Takemoto, Intlekofer, Northrup, Wherry, and Reiner, 2006), where the levels of inflammation or the concentration of IL-12 directed graded expression of T-bet that led to terminal differentiation of T cells (Joshi et al., 2007). In contrast, the inhibition of inflammatory signalling induced the expression of Eomes, enhancing memory precursor cell generation (Rao, Li, Odunsi, and Shrikant, 2010). However, the protein expression for T-bet and Eomes is unknown in rested memory cells. Other transcriptional factors have also been found to influence the progression of terminal differentiation and direct the developmental pathways between effector and memory cells. These include Blimp-1, Bcl-6, Bmi-1 and Id2 (Angelosanto and Wherry, 2010; Cui and Kaech, 2010; Rutishauser and Kaech, 2010).

Another set of important transcription factors in CD8+ T cell differentiation is associated with the maintenance of quiescence and naïve cell characteristics. FoxO1 is responsible for naïve cell survival and, through control of the expression of CCR7 and CD62L, the capability to migrate through lymphoid organs (Kerdiles et al., 2009). Wnt signalling pathway effectors Tcf-1 (T cell factor 1, or also known as transcription factor 7, TCF-7) and Lef-1 (lymphoid enhancer-binding factor 1) are downregulated upon TCR stimulation and gradually resume expression as memory cells are generated, although overall expression levels are lowered with the progression of CD8+ T cell differentiation (Willinger et al., 2006). Recently, Tcf-1 has also been found to play a role in the generation and persistence of functional CD8+ memory cells, partly by inducing optimal Eomes expression (Jeannet et al., 2010; Zhao et al., 2010; Zhou et al., 2010).
Also, cells expressing high levels of β-catenin, Tcf-1 and Lef-1 show higher potential to form memory cells in vivo (Gattinoni, Ji, and Restifo, 2010; Muralidharan et al., 2011).

As well as transcription factor expression, DNA and chromatin modifications also directly contribute to the heritable regulation of effector function gene expression. Epigenetic modifications including DNA methylation and histone acetylation have been shown to induce rapid expression of IL-2 and IFN-γ upon recall responses in memory cells (Northrop et al., 2006; Thomas, Gao, and Wells, 2005). Also, inflammatory signals have been observed to promote histone hyperacetylation at the gene loci of Eomes and granzyme B (Agarwal et al., 2009).

Nutrient consumption and energy utilization of antigen-specific cells are also critical regulators of the immune response. This is due to the increasing metabolic demand to execute the processes of cell division and effector functions. The evolutionarily conserved mammalian target of Rapamycin (mTOR) is well known for its role in the regulation of metabolism, influencing nutrient uptake, energy generation and synthesis of macromolecules. Recently, it has become increasingly apparent that mTOR plays an important role in directing T cell activation and differentiation by centrally regulating cellular metabolism, thereby coupling T cell responses with metabolic demand and cellular processes (Pearce, 2010; Powell and Delgoffe, 2010; van der Windt and Pearce, 2012). It has been suggested that mTOR acts as a hub of signal integration, as multiple sources of signals all feed into the mTOR pathway, and subsequently direct T cell metabolism and survival. These include costimulation, inhibitory receptor signalling, cytokines, glucose and amino acid uptake (Powell and Delgoffe, 2010; Waickman and Powell, 2012). Dramatic changes in metabolic activity upon naïve cell activation involve the metabolic switch from oxidative phosphorylation (catabolic metabolism) to aerobic glycolysis (anabolic metabolism). This helps conserve cellular building blocks for proliferation as well as enhancing the uptake of glucose, amino acids and iron (Michalek and Rathmell, 2010). mTOR regulates many of these processes. Examples include protein synthesis, mitochondria biogenesis, glucose and lipid metabolism, and the expression of transporters for glucose, amino acids and iron (Powell and Delgoffe, 2010). The absence of mTOR activity leads not only to T cell anergy following TCR stimulation, but also to reduced cell size and effector cell development (Zhang et al., 2011; Zheng, Delgoffe, Meyer, Chan, and Powell, 2009). Concordantly, the less energy demanding quiescent T cell activities, including the formation of memory cells and regulatory T cells, are promoted in the absence of mTOR activity at the expense of effector cell functionality (Araki et al., 2009a; Delgoffe et al., 2009; Pearce et al., 2009). Inflammatory signals and TLR2 signalling have been found to sustain mTOR activity to promote effector functions, such as IFN-γ production, via the expression of transcription factor T-bet (Geng et al., 2010; Rao et al., 2010).

1.1.5.3 Negative feedback mechanisms for CD8+ T cell responses

To keep expanding effector cells in check, several inhibitory mechanisms operate to prevent immunopathology from uncontrolled CTL activity. Activated CD8+ T cells acquire self-regulation first during
the peak of an immune response, where fully differentiated CD8\(^+\) effector cells produce immunosuppressive IL-10 at peripheral sites to limit effector functions (Palmer, Holbrook, Arimilli, Parks, and Alexander-Miller, 2010; Sun, Madan, Karp, and Braciale, 2009; Trandem, Zhao, Fleming, and Perlman, 2011). Effector CD8\(^+\) T cells that have lost the expression of CCR7 and CD62L for secondary lymphoid organ entry can upregulate chemokine receptor CXCR3 under inflammatory conditions. In this way, they regain the capability to travel back to the lymph nodes and directly kill antigen-loaded DCs in a perforin-dependent manner to prevent further antigenic stimulation (Guarda et al., 2007; Wong and Pamer, 2003; Yang, Huck, McHugh, Hermans, and Ronchese, 2006). Proliferation and effector functions can also be directly inhibited by the expression and signalling of inhibitory receptors such as CTLA-4 or PD-1 on CD8\(^+\) T cells. These receptors may either be temporarily expressed during acute infections, or take on a more important role during chronic infections to prevent excessive tissue damage by chronic inflammation and excessive CTL activity (Crawford and Wherry, 2009). Many other cell types can also help dampen CTL functions by producing a range of immunosuppressive molecules, including the nitric oxide and IDO (indoleamine 2,3-dioxygenase) produced by mesenchymal stem cells and IL-10 and TGF-\(\beta\) (transforming growth factor-\(\beta\)) secreted by inhibitory or tolerogenic APCs such as dermal CD14\(^-\) DCs (Banchereau et al., 2012; Shi et al., 2012).

### 1.1.6 Persistent antigen and chronic infections

During the instances where immune responses are not successful in eliminating the invading pathogen upon initial insult, antigens persist and chronic infections are established. Where pathogen is present continuously, immune responses must modulate to prevent immunopathology by unrestrained inflammation and cellular effector functions in the infected tissues. Depending on the level of antigenic dose, duration of infection, availability of CD4\(^+\) help, form of chronic infection (continual replication, latent infection or genome invasion) and pathogen immune escape mechanisms, varying degrees of immune cell dysfunction occur during persistent infection. Immune cell function gradually deteriorates with the progression of the infection and sometimes result in clonal deletion, a phenomenon termed exhaustion. Mechanisms for the downregulation of immune system activities during antigen persistence include the expression of inhibitory receptors, development of regulatory T cells, alterations of APCs and production of immunoregulatory cytokines such as IL-10 and TGF-\(\beta\) (Virgin et al., 2009; Wherry and Ahmed, 2004), resulting in a sequential loss of CTL effector functions. Furthermore, CTL proliferative potential decreases and becomes dependent on the availability of continuous antigen to survive (Wherry and Ahmed, 2004). Concordantly, CD8\(^+\) T cells in this state express activation markers, suggesting continual TCR stimulation, and upregulate phenotypic markers, such as CD57, that correlate with lowered proliferative potential (Brenchley et al., 2003; Ibegbu et al., 2005).

The expression of inhibitory receptors is an important negative regulatory mechanism to limit overt CTL responses in chronic infections. These inhibitory receptors generally function to restrain IL-2 production, negatively regulate progression through cell cycle, or block CD28- and sometimes TCR-induced gene
transcription to terminate T cell responses (Crawford and Wherry, 2009; Sharpe and Freeman, 2002). Many inhibitory receptors such as CTLA-4 and PD-1 belong to the Ig superfamily and share amino acid identity with their stimulatory counterparts, CD28 and ICOS (Carreno and Collins, 2002). A range of other inhibitory receptors has recently been shown to have enhanced expression on exhausted CD8+ T cells. These receptors include LAG-3, 2B4 (CD244) and CD160 (Blackburn et al., 2009; Wherry et al., 2007). The severity of chronic infection has been correlated to intensified expression of each inhibitory receptor on any one cell and also the expression of an increased number of different inhibitory molecules. The co-expression of inhibitory receptors collaboratively suppresses immune responses, since blockade of more than one inhibitory receptor synergistically improves T cell responses (Blackburn et al., 2009; Jin et al., 2010). Some forms of exhaustion are reversible. Blockade of PD-1 and PD-L1 were found to improve CD8+ effector functions and enhance viral control (Barber et al., 2006; Velu et al., 2009) as well as deliver anti-tumour effects (Brahmer et al., 2012; Topalian et al., 2012), while CTLA-4 blockade has been found to augment anti-tumour responses (Leach, Krummel, and Allison, 1996) and CD8+ memory T cell formation and function (Pedicord, Montalvo, Leiner, and Allison, 2011). As a consequence, two human monoclonal CTLA-4 antibodies are currently under clinical development for treatment of melanoma and other cancers (Salama and Hodi, 2011). These observations have suggested promising possibilities for immunotherapy in treating persistent infections and other diseases.

1.2 Contraction of CD8+ effector populations

Following the extraordinary expansion of antigen-specific cells upon antigen recognition, it is essential that excessive effector populations are eliminated following clearance of antigen, to maintain homeostasis and integrity of the immune system and prevent immunopathology. A small population of cells persists following contraction and gradually converts from activated effectors to memory cells. These memory cells regain quiescence characteristics similar to the naïve cells, but possess high proliferative potential and immediate effector functions. They are also able to survive long term and self-renew independently of antigen to provide protection against potential future re-infections (Kaech, Hemby, Kersh, and Ahmed, 2002a; Kaech and Wherry, 2007; Wherry et al., 2003b).

The elimination of excessive effector cells upon contraction requires one of the two common pathways of apoptosis, or programmed cell death. The intrinsic pathway of apoptosis involves the permeabilization of the mitochondrial outer membrane, release of cytochrome C and the subsequent activation of apoptosome and caspases, which is regulated by the Bcl-2 family proteins (Bouillet and O'Reilly, 2009). The pro-apoptotic member of the Bcl-2 family Bim appears to play a dominant role in this process, because effector cell contractions are diminished in the absence of its expression (Hildeman et al., 2002a; Pellegrini, Belz, Bouillet, and Strasser, 2003). The activity of Bim is normally restrained by the anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xL, which are induced by cytokine survival signals such as IL-7 and IL-15 (Strasser and Pellegrini, 2004). With the expression of IL-7 receptor found to be critical for the formation and maintenance of long-lived memory cells (Carrio, Rolle, and Malek, 2007; Kaech et al., 2003; Osborne...
et al., 2007), it has been hypothesised that limited survival and growth factors during the termination of the immune responses induce apoptosis in the contracting population, so called “death by neglect” (Hildeman, Zhu, Mitchell, Kappler, and Marrack, 2002b; Jameson, 2002). However, augmented IL-7 signalling did not enlarge the subsequent pool of the memory population (Sun, Lehar, and Bevan, 2006), nor did enforced IL-7R expression prevent contraction of effector cells (Hand, Morre, and Kaech, 2007; Haring et al., 2008). This indicates that survival signals and the upregulation of Bcl-2 by IL-7 are essential to prohibit Bim activity and thus allow the transition and survival of memory cells from effectors (Carrio et al., 2007; Osborne et al., 2007). However, it is not sufficient to rescue the contracting effector population from programmed cell death, indicating that IL-7 does not select the memory population (Hand et al., 2007; Haring et al., 2008).

The second common pathway for programmed cell death, the extrinsic pathway, involves the ligation of the death receptor (DR) members of the TNFR family, including TNFRI, TNFRII and Fas (CD95). Following viral infections, CD8⁺ T cell contraction was found to be minimally altered with either TNFRI or TNFRII deficiencies. However, the effector population contractions were diminished in TNFRI and TNFRII double-deficient mice. This demonstrates the overlapping functions of the TNF receptors, and also indicates that the DR ligands, such as TNF and TRAIL, play a role in the elimination of excessive effector cells (Suresh, Singh, and Fischer, 2005), as well as involvement in the AICD following the absence of CD4⁺ help (1.1.4).

The accumulation of B and T cells, in particular double negative T cells, and the development of autoimmune diseases in Fas (CD95)- and FasL (Fas ligand)-deficient mice (Takahashi et al., 1994; Watanabe-Fukunaga, Brannan, Copeland, Jenkins, and Nagata, 1992) provide definitive evidence that Fas plays a role in peripheral T cell elimination in mice. Similar phenotypes are observed in patients with ALPS (autoimmune lymphoproliferative syndrome) caused by Fas mutations (Rieux-Laucat et al., 1995; Sneller et al., 1992) suggesting that this concept extends to humans. However, selective ablation of Fas expression in different murine cell types showed Fas expression on APCs and not CD8⁺ T cells to be the major contributor for CD8⁺ T cell deletion, and a safeguard against autoimmune diseases (Reich, Korner, Sedgwick, and Pircher, 2000; Stranges et al., 2007; Zimmermann, Rawiel, Blaser, Kaufmann, and Pircher, 1996).

More recently, it was found that Fas and Bim double deficiency caused remarkably enhanced and accelerated fatal lymphadenopathy and autoimmunity compared to Bim- or Fas-deficiency alone. This highlights the synergistic role of the two pathways for T cell elimination. The requirement of both Fas and Bim for CD8⁺ clonal deletion appears to be more apparent in chronic infections, but varies for different acute pathogenic infections (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). It has been hypothesised that differences between various pathogenic infections in factors such as TCR signalling strength, or the site of infection and virus dissemination may determine the pathways used for the elimination of CD8⁺ effector cells during contraction (Bouillet and O'Reilly, 2009). In summary, multiple pathways function concurrently to terminate T cell responses and ensure the elimination of excessive effector cells to prevent immunopathology or autoimmunity.
Other factors that also modulate the formation of memory cells following effector population contraction are those associated with the regulation of CD8$^+$ T cell differentiation. These include signals such as TCR and inflammatory stimuli, as well as their downstream molecules like mTOR and transcription factors, T-bet and Eomes (Cui and Kaech, 2010; Kaech and Wherry, 2007). Most of these factors act as double-edged swords. Sufficient signals are required for the optimal generation of effector responses and memory cell formation, but persistent antigen, augmented inflammation or intensified mTOR and T-bet activities promote expansion of the effector population and terminal differentiation or death (Jameson and Masopust, 2009). Interestingly, while heightened inflammatory signals appeared to increase the ratio between short-lived effector cells and memory precursors, the absolute numbers of memory precursors and memory cells were maintained, suggesting that increased generation of effectors does not occur at the expense of memory cell formation (Cui, Joshi, Jiang, and Kaech, 2009; Joshi et al., 2007).

### 1.3 Maintenance of CD8$^+$ memory populations

Memory populations are able to self-renew and maintain functional capabilities over long durations to provide immune protection for the host. In contrast to naïve cells, the long-term survival and homeostasis of memory CD8$^+$ T cells do not require the presence of MHC molecules or TCRs, indicating that the availability of cognate antigen is not necessary for the preservation of T cell memory (Leignadier, Hardy, Cloutier, Rooney, and Labrecque, 2008; Murali-Krishna et al., 1999). In contrast, the common γ-chain (γc) cytokines IL-7 and IL-15 are critical for the maintenance of memory cells (Surh and Sprent, 2008) and hence take part in the regulation of the transition from effector to memory populations. Mice deficient in CD132, the common γ-chain that forms the cytokine receptor complexes for both IL-7 and IL-15, do not establish memory populations despite the formation of functional effector responses following pathogen infection (Decaluwe et al., 2010). In IL-15- and IL-15R-deficient mice, antigen-specific cells are able to mount successful responses against viral infections and generate functional memory cells; however, the memory populations are unable to be maintained long term (Becker et al., 2002). IL-15-deficient mice were found to lack IL-15-dependent CD122$^+$ (IL-2/15Rβ$^+$) memory CD8$^+$ T cells and NK cells (Kennedy et al., 2000), and CD122$^+$ memory cells transferred into IL-15-deficient hosts did not homeostatically proliferate and failed to persist (Judge, Zhang, Fujii, Surh, and Sprent, 2002). In reverse experiments, injection of IL-15, or IL-15 overexpression, induced increased numbers of memory CD8$^+$ T cells (Yajima et al., 2002; Zhang, Sun, Hwang, Tough, and Sprent, 1998). Similarly, mature CD8$^+$ T cells abrogated in IL-7R and STAT-5 (signal transducers and activators of transcription 5) signalling showed no defects in the development of effector and memory cells, but the memory cells did not persist. Although these cells possess the ability to homeostatically proliferate in response to IL-15, Bcl-2 transgenic expression is required for their survival (Carrio et al., 2007; Osborne et al., 2007). These data suggest that memory cells require both IL-7 and IL-15 for long-term survival and persistence, with IL-15 being responsible for homeostatic proliferation and self-renewal. Provided IL-7 and IL-15 are available, memory cells are capable of persisting for long durations (Nanan, Rauch, Kampgen, Niewiesk, and Kreth, 2000; Van Epps et
al., 2002), perhaps providing immunological protection for a lifetime after human vaccinations (Hammarlund et al., 2003). However, upon certain heterologous infections, it has been shown that previously established CD8⁺ memory populations may be lost via a Type I or II IFN-dependent process (Dudani, Murali-Krishna, Krishnan, and Sad, 2008; Selin et al., 1999).

The sites where memory T cells acquire homeostatic signals are of considerable interest. IL-7 is produced by fibroblastic reticular cells (FRCs) in lymphoid organs (Link et al., 2007), hence the suggestion that CD62L expression and lymphoid organ homing capability are essential for the survival of naïve T cells and some subsets of memory cells (Sallusto, Geginat, and Lanzavecchia, 2004; Schuster et al., 2009). However, evidence is now accumulating that memory T cells preferentially reside in the bone marrow for both mice and humans (Herndler-Brandstetter et al., 2011; Mazo et al., 2005; Palendira et al., 2008). Here, contact with IL-7⁺ stromal cells (Tokoyoda, Hauser, Nakayama, and Radbruch, 2010) and IL-15-producing bone marrow cells directs survival and homeostatic proliferation (Herndler-Brandstetter et al., 2011; Snell, Lin, and Watts, 2012). Furthermore, the transcription factor Eomes, known to be important for memory differentiation, enables memory CD8⁺ T cells to preferentially locate to bone marrow, possibly allowing favourable conditions for memory differentiation and survival (Banerjee et al., 2010). This raises intriguing questions about the mechanism of rapid T cell responses following secondary infections, and whether “memory” populations commonly studied from the lymphoid organs and peripheral blood include all existent memory subsets, or contain only a sample of the entire memory population.

1.4 Heterogeneity and differentiation of CD8⁺ memory cells

Following naïve CD8⁺ T cell activation, cells progress through a series of changes in functional attributes and molecular phenotypes to exert cytotoxic functions and generate immunological memory for future protection, a process termed differentiation. Having discussed the factors that regulate the activation and differentiation programming of naïve CD8⁺ T cells over one round of antigenic challenge, we now must consider the heterogeneity generated within each round of antigenic challenge, as well as the fate of single cells subjected to repeated antigenic challenges.

1.4.1 The ontogeny of heterogeneous cell fates and differentiation

1.4.1.1 Generation of effector and memory cells from naïve cells

Following T cell activation and clonal expansion, 90-95% of the expanded population undergoes apoptosis following clearance of pathogen, while a small population of effector cells survive to develop into memory cells. It is tempting to speculate that pre-determined cell fates in distinct subsets of naïve cells give rise to the effector or the memory populations (Ahmed and Gray, 1996; Kaech et al., 2002b). However, every naïve T cell has been found to be capable of entering either the effector or memory cell population following activation. This has been exquisitely demonstrated by adoptively transferring single naïve CD8⁺ T
cells, which gave similar diversification of effector and memory subsets as polyclonal T cell responses following infection (Stemberger et al., 2007). The innovative method of “cellular barcoding”, where unique genetic tags are introduced into naïve cells so they may be tracked in subsequent effector and memory populations, further supports the idea that one naïve cell can differentiate along many different routes. This indicates that the differentiation pathway of each naïve cell is not pre-programmed. Instead it is specified following T cell activation (Gerlach et al., 2010; Schepers et al., 2008). Instructions upon TCR stimulation may generate heterogeneous populations of progeny via two routes: 1) concurrently establishing several subsets of cells, i.e. a branched pathway of differentiation; or 2) having individual cells progress to different degrees down the same pathway, i.e. a linear pathway of differentiation (Ahmed and Gray, 1996; Kaech et al., 2002b). Accumulating evidence from various experimental models and techniques supports the linear differentiation model in both mice and humans. According to this model, all activated T cells pass through an effector stage, and those effectors that survive the contraction phase go on to gradually develop memory cell characteristics (Ahmed and Akondy, 2011; Arens and Schoenberger, 2010; Jameson and Masopust, 2009). Further supporting evidence comes from microarray studies in mice. This work showed a gradual transition from naïve to effector to memory phenotypes (Kaech et al., 2002a), where specifically marked effector cells gave rise to the memory cell pool (Jacob and Baltimore, 1999; Opferman, Ober, and Ashton-Rickardt, 1999; Sarkar et al., 2008). Recently, the first longitudinal studies in humans have tracked the development of CD8$^+$ T cell responses following vaccination with live-attenuated virus. These also revealed uniform phenotypic changes of the antigen-specific population through activation to effector development and then to memory formation, indicating that the development of memory cell characteristics is a gradual process over time (Ahmed and Akondy, 2011; Akondy et al., 2009; Miller et al., 2008).

This gradual progression of differentiation from effector to memory cells, accompanied by progressive alterations in cellular attributes and gene expression profile (Agarwal et al., 2009; Kaech et al., 2002a), does not exclude the possibility that the activated effector population harbours cells with differential potential to preferentially develop into either short-lived, terminally differentiated effector cells or long-lived memory cells. Evidence of naïve T cells undergoing asymmetric cell division upon initial activation, unequally partitioning critical functional molecules such as CD3, CD8, IFN-γR and T-bet into the dividing daughter cells, suggest separation of the fate of daughter cells may occur early after antigenic stimulation, despite a phenotypically similar differentiation progression through an effector stage (Chang et al., 2011; Chang et al., 2007). Although the exact functional consequences of these asymmetric cell divisions and the mechanisms of the transitional processes are not completely understood, these data suggest selective survival of a certain subset of effectors is combined with longitudinal cellular conversions to generate the final memory populations (Ahmed, Bevan, Reiner, and Fearon, 2009b; Kaech and Wherry, 2007).

1.4.1.2 Repeated antigenic stimulations

As memory cells are restimulated, the process of differentiation into effector and memory populations is repeated. Rested memory cells attain activated effector characteristics once again and differentiate further.
Microarray studies of repetitive antigen stimulations (with inactive periods in between the sequential stimulations) have found differential regulation for several hundred new genes with each additional antigen challenge, indicating a stepwise diversification of the gene expression profiles. At the same time, the expression of a small group of genes and their biological pathways are expressed at similar levels across the sequentially generated memory populations (Wirth, Badovinac, Zhao, Dailey, and Harty, 2009). Interestingly, primary challenges and responses following repeated stimulations in different pathogenic infections may construct responding T cell populations with similar phenotypic and functional profiles, indicating that one general continual progression, rather than branched pathways, exists for the development of differentiation, directed by the varying, and accumulative, TCR stimulation strengths (Gett, Sallusto, Lanzavecchia, and Geginat, 2003). Although the steps of differentiation from naive to terminal differentiation are still controversial, it is expected that with enough repeated antigenic stimulations, replicative senescence may result from telomere erosion and/or unrepaired DNA damage, and functional exhaustion may take place, potentially acting as a protective mechanism for excessive immune responses (Akbar and Henson, 2011).

1.4.1.3 The generation of heterogeneity

As each individual antigen-specific cell is activated following the initiation of an immune response, it receives a distinctive set of stimuli that differs slightly from those received by other antigen-specific cells. These unique experiences of individual cells create a heterogeneous population of effector and memory cells. Upon chronic infections or repeated antigenic challenges, the variation in the accumulated TCR signals and accessory stimuli received by each antigen-specific cell increases, driving those with more antigenic experience further down the differentiation pathway, and producing a wider range of diversity across the population. Heterogeneity in the profiles of human T cell populations specific to different viruses may relate to different histories of antigenic stimulation experienced by T cells responding to different viruses (Appay and Rowland-Jones, 2004), although other factors may also be at play given that very different immune responses are necessary to control each virus. In the case of chronic infections, the responses may be tailored by both the immune evasion mechanisms of the pathogen and the host's protective measures to limit immunopathology.

Due to the complex nature of this extensively regulated differentiation process, both the ontogeny of the heterogeneous subpopulations of CD8+ memory cells and the delineation of the progressive pathway of CD8+ T cell differentiation remain incompletely understood. However, some broad agreements have been reached from studies of both mice and humans. Some general patterns in phenotypic expression and functional attributes have been repeatedly observed, from naive and “early” memory cells generated from successful acute infections to terminally differentiated cells resulting from chronic infections.
1.4.2 Changes of cellular attributes with differentiation

Naïve cells express all functional receptors necessary to initiate T cell responses. These include costimulatory receptors CD28 and CD27 as well as the lymphoid organ-homing molecules CCR7 and CD62L. Early memory cells appear to retain the expression of both the costimulatory receptors and the lymph node homing receptors, enabling them to traverse secondary lymphoid organs, obtain survival signals from lymphoid stromal cells (Bromley et al., 2005; Link et al., 2007; Schuster et al., 2009), and establish rapid T cell responses upon antigen encounters. Following repeated or chronic stimulations, a general loss in the expression of these receptors has been observed. Cells that have lost the lymphoid homing receptors are found to reside mainly in extralymphoid tissues, and home to sites of inflammation where they mediate instant cytotoxic activities upon TCR engagement (Champagne et al., 2001; Sallusto et al., 2004). The gradual loss of CD28, then CD27, suggests diminished capabilities to receive survival and cell cycle progression signals (Appay et al., 2002; Tomiyama, Matsuda, and Takiguchi, 2002). While memory cells early in differentiation readily proliferate and elicit expansion of effector cells upon antigen encounters, a loss in proliferative potential is observed for highly differentiated cells (Plunkett et al., 2007).

In humans, CD45 has also been a very important marker of differentiation status. CD45 has tyrosine phosphatase activity important for antigen receptor signal transduction and lymphocyte development as well as other modulatory functions for T cell activities. It has multiple isoforms via alternative splicing, and the high molecular weight CD45RA isoform is constitutively expressed on naïve cells (Hermiston, Xu, and Weiss, 2003). Naïve cells were observed to lose CD45RA expression upon TCR stimulation, with early memory cells instead expressing the lower molecular weight isoform CD45RO (Akbar, Terry, Timms, Beverley, and Janossy, 1988). Hence, the switch from CD45RA to CD45RO expression was originally thought to represent all antigen-experienced cells. However, it was later found that human memory T cells can regain the expression of CD45RA both in vivo and in vitro (Dunne et al., 2005; Geginat, Lanzavecchia, and Sallusto, 2003).

With recent advances in multicolour flow cytometry, the expression of numerous additional cell surface molecules has been trialled in attempt to further segregate the heterogeneous populations of memory cells. The expression of various adhesion molecules (e.g. CD11a), chemokine receptors (e.g. CCR5, CXCR4, CX3CR1) and regulatory signalling receptors (e.g. PD-1) has been examined, allowing identification of an increasing number of subpopulations (De Rosa, Herzenberg, and Roederer, 2001; Faint et al., 2001; Precopio et al., 2007; Ramos et al., 2009). However, because many markers overlap in their expression pattern across the existing categories of memory populations, consensus has yet to be reached in defining the continuum of differentiation. Nevertheless, since functions can be attributed to at least some phenotypic markers, the collective cell surface phenotype of each subpopulation implies a set of functional attributes, including the capabilities for optimal activation and survival, homing and migration, and effector functions (Appay et al., 2008).
The effector functions of CD8+ T cells are also generally observed to gradually alter with the progression of differentiation. Early differentiated cells possess little immediate cytotoxic functions, and as the CD8+ T cells differentiate, the expression of granzymes, perforin and IFN-γ increase on a per cell basis and the cytotoxic molecules become constitutively expressed (Appay and Rowland-Jones, 2004; Chattopadhyay et al., 2009; Gattinoni et al., 2005; Romero et al., 2007; Rufer et al., 2003). However, upon functional exhaustion, cytotoxic activities can be lost through inhibitory receptor signalling (Wherry, 2011; Wherry et al., 2003a). The capability of memory cells to produce cytokines such as IL-2 and TNF is also commonly compromised with differentiation and exhaustion (Gattinoni et al., 2005; Hinrichs et al., 2009; Sallusto, Lenig, Forster, Lipp, and Lanzavecchia, 1999; Wherry and Ahmed, 2004). Loss of cytokine secretion following repeated antigenic exposure occurs in a specific sequential order. The capability to produce IL-2 is always lost first, followed by TNF secretion, while the ability to secrete IFN-γ production is consistently found to persist the longest during CD8+ differentiation (Fuller, Khanolkar, Tebo, and Zajac, 2004; Seder, Darrah, and Roederer, 2008; Wherry and Ahmed, 2004; Wherry et al., 2003a). Hence, it has been proposed that the number of capabilities of antigen-specific T cells define the “quality” of the response, with multifunctional CD8+ T cells (those capable of simultaneously producing a higher number of different cytokines and cytotoxic molecules) providing the best immune protection (Seder et al., 2008). Indeed, a number of studies have demonstrated that the viral clearance or immuno-control of CMV or vaccinia infections, and long-term non-progression in HIV infections, all correlate with larger numbers of multifunctional CD8+ T cells (Betts et al., 2006; Precopio et al., 2007; Zimmerli et al., 2005). These “better quality” multifunctional cells were found to have enhanced protective activity not only due to the combined armaments, but also due to augmented expression of IFN-γ, enhanced cytolytic activities and more efficient killing of target cells (Seder et al., 2008; van Duikeren et al., 2012).

1.4.3 Proposed human CD8+ T cell differentiation models

These observations of the changes in cellular attributes have led to a number of models proposed for human CD8+ T cell differentiation. Appay et al. (2002) have proposed a pathway of differentiation based on the expression of CD28 and CD27 from studies of a range of virus-specific human CD8+ T cell populations, where the differentiation of CD8+ T cells progresses through CD28+ CD27+ → CD28− CD27+ → CD28− CD27−. The loss of these costimulatory receptors was found to correlate with an increase in expression of the cytotoxic molecules, and loss of telomere lengths and telomerase activities (Appay and Rowland-Jones, 2004; Monteiro, Evaristo, Legrand, Nicoletti, and Rocha, 2007; Plunkett et al., 2007; Romero et al., 2007; Rufer et al., 2003). Sallusto et al. (1999) proposed the classification of CD8+ T cells into central memory cells and effector memory cells according to the expression of CCR7 and CD45RA. This provided a concept for T cell compartmentalization, which later developed into a four step differentiation scheme according to progressive changes in functional capabilities as follows: CCR7+ CD45RA+ (naive) → CCR7+ CD45RA- (central memory, CM) → CCR7- CD45RA+ (effector memory, EM) → CCR7- CD45RA+ (effector memory with CD45RA expression, EMRA) (Champagne et al., 2001). The CM cells are thought to possess little or no immediate effector function. They traverse secondary lymphoid organs and provide rapid
establishment of effector populations upon antigen re-challenge. EM cells, however, having lost the homing receptor CCR7, are thought to confer immediate effector function in peripheral tissues with diminished proliferative potential (Sallusto et al., 2004). Although the higher proliferative potential and IL-2 production of the CM population were consistently observed in several studies (Gattinoni et al., 2011; Geginat et al., 2003; Sallusto et al., 1999; Wherry and Ahmed, 2004; Wherry et al., 2003b), in other work the production of IFN-γ, TNF and cytotoxic molecules were found to be nearly equivalent in the CM and EM populations (Barber, Wherry, and Ahmed, 2003; Gattinoni et al., 2011; Ravkov, Myrick, and Altman, 2003; Unsoeld, Krautwald, Voehringer, Kunzendorf, and Pircher, 2002; Wherry et al., 2003b). The functional capabilities of the originally proposed terminally differentiated population, the CCR7\(^{-}\) CD45RA\(^{+}\) effector memory (EMRA) cells, also remain controversial in the literature, where various studies have observed different proliferative potentials, survival capabilities and effector functions (Akondy et al., 2009; Carrasco, Godelaine, Van Pel, Boon, and van der Bruggen, 2006; Dunne et al., 2002; Hislop et al., 2001; Lecouroux et al., 2009; Miller et al., 2008; Monteiro et al., 2007).

Despite this complex literature, the gradual changes in cell surface phenotype and function provide an approximate outline for the general pathway of CD8\(^{+}\) T cell differentiation, defined by an association with the overall shortening of telomere lengths and diminishing number of TRECs (T-cell receptor excision circles) (Akbar, Beverley, and Salmon, 2004; Plunkett et al., 2007; Romero et al., 2007; Rufer et al., 2003). With this general progression of differentiation, the heterogeneity of CD8\(^{+}\) T cells can be portrayed as representing cells from all stages along a general continuum of differentiation, with branches of additional minor variations. At one end of the continuum, memory cells have high proliferative potential and longevity with the capability to secrete multiple cytokines, while at the other end, terminally differentiated cells possess constitutive expression of cytotoxic molecules and poor proliferative capacity (Cui and Kaech, 2010). Both ends of the continuum are also associated with specific regulators for gene expression (Angelosanto and Wherry, 2010; Rutishauser and Kaech, 2010), which will now be discussed.

### 1.4.4 Transcriptional regulation of CD8\(^{+}\) differentiation

Specific transcription factor expression has been found to correlate with the progression of human CD8\(^{+}\) T cell differentiation. Gene expression studies have revealed that transcription factors Tcf-1 and Lef-1 downregulate with differentiation of human CD8\(^{+}\) T cells, as previously discussed (1.1.5.2) (Gattinoni et al., 2011; Willinger et al., 2006; Wirth et al., 2010b), while T-bet and Blimp-1 upregulate with differentiation (Crotty, Johnston, and Schoenberger, 2010; Gattinoni et al., 2011; Joshi et al., 2011). T-bet and Blimp-1 are responsible for a number of attributes associated with terminal differentiation, such as the loss of IL-2 production, heightened expression of IFN-γ and perforin, as well as the attenuation of proliferation (Crotty et al., 2010; Gong and Malek, 2007; Joshi et al., 2007; Joshi et al., 2011; Rutishauser et al., 2009; Shin et al., 2009; Yeo and Fearon, 2011). From murine studies, a number of other transcription factors have also been implicated in the regulation of CD8\(^{+}\) T cell differentiation. These include Bcl-6 (Crotty et al., 2010), Id3 (Ji et al., 2011), and regulators of chromatic remodelling, Bmi-1 and Mbd2 (Heffner and Fearon, 2007;
Kersh, 2006), that are associated with the formation of memory precursor cells and CM cells. They also include Id2 (Yang et al., 2011), Gfi-1 (Chandele et al., 2008) and Xbp-1 (Kamimura and Bevan, 2008) that are preferentially expressed in short-lived effector cells.

Although transcription factors expressed early in differentiation (e.g. Tcf-1 and Lef-1) generally have a function in memory development (Zhou and Xue, 2012), and transcription factors that are more prominently expressed late in differentiation (e.g. T-bet and Blimp-1) have a role in effector function development (Joshi et al., 2007; Rutishauser et al., 2009), Eomes shows differential regulation. Its expression has been associated with memory generation as opposed to effector differentiation (see 1.1.5.2) (Rao et al., 2010). However, microarray studies have found Eomes transcription to gradually increase with CD8\(^+\) T cell differentiation (Gattinoni et al., 2011; Willinger, Freeman, Hasegawa, McMichael, and Callan, 2005). For all transcription factors, regulation of their expression between each cycle of antigenic challenge and differentiation is undefined, and commonly separately investigated. At the same time, transcription factor expression is often assessed by mRNA quantification, while protein expression remains unclear.

1.4.5 Complexities in the current literature

As described above, the classification of T cell subsets and their origins during cell lineage development remain incompletely understood. A number of complicating factors remain in the literature, hindering the interpretation and integration of published studies.

One overarching issue across the literature is the difficulty in reconciling results from studies across mice and humans. This is due not only to their differences in biology but also to the often quite distinct methodologies and technologies available to study T cells in these two species. Mice and human are estimated to have diverged 65 million years ago and separately evolved to acquire great differences in lifespan and dwelling environments. Many differences between mouse and human immunology have been carefully delineated by Mestas and Hughes (2004). These include differences in T cell signalling pathway components, cytokines and cytokine receptors, and costimulatory molecules expression and functions. The limited successes in the translation of therapeutic protocols from mouse models to clinical trials also provides evidence for the differences between mice and humans (Davis, 2008). CD8\(^+\) T cell responses in mice and humans have been observed from very different viewpoints. Immune responses in mice can be followed longitudinally for both acute and chronic infections, whilst human studies are mostly carried out by examining the heterogeneous population of antigen-specific T cells in chronic infections at one given time point. Cells from patients with chronic viral infections such as CMV, EBV, HCV (hepatitis C virus) and HIV (human immunodeficiency virus), have commonly been employed for studies of human CD8\(^+\) T cells (Appay et al., 2002; Champagne et al., 2001; Sallusto, 1999; Tomiyama, Takata, Matsuda, and Takiguchi, 2004; van Leeuwen et al., 2002) due to the generation of large antigen-specific populations. The range of commonly assessed phenotypic markers also differs substantially between mice and humans. This is
mostly due to the varying availability of antibodies for the assessments of expression e.g. CD45RA and CD45RO are crucial markers for human CD8⁺ memory subsets but not employed for the classification of murine memory subsets; conversely CD44 is commonly used in murine studies but not used for human subset classification.

Many T cell biology nomenclatures and definitions are inconsistent in the literature; these complexities have risen from the varying methodologies to study T cell responses as well as the nature of the gradual changes during the progression of differentiation. First, the boundaries between effectors and memory cells are indistinct due to the overlapping cellular attributes between the effector and memory cells (Jameson and Masopust, 2009), as well as the temporary alterations of memory phenotypic markers upon activation (Akondy et al., 2009; Brooks, 2007; Carrasco et al., 2006; Kaech et al., 2002a; Miller et al., 2008). The original model of T cell response defines the difference between effector and memory T cells as the amount of time elapsed following antigen stimulation and upon the clearance of antigen at the termination of immune response. However, the time point separating the two states differs in different experimental models and no consistent activation markers have been used to identify cells currently responding to antigen. “Effector” T cells can be defined as responsive cells following recent antigen stimulation, or any cell that displays effector functions \textit{ex vivo}. With late differentiation memory cells possessing immediate effector functions and early differentiation memory cells having excellent rapid effector function upon restimulation, cells of both types may be categorised as “effector” cells in different studies. “Memory” T cells are sometimes simply defined as antigen-experienced cells, or cells that possess anamnestic response capabilities, but differences in both cell phenotype and function exist between activated and rested states of memory cells, as well as between infectious contexts of antigen clearance or persistence. Although antigen-specific cells in a chronically infected host do not display typical activation markers (Appay et al., 2002), it is not clear whether these “memory” cells are truly rested whilst antigen persists.

Secondly, the molecular definitions for memory cell subset nomenclatures are also variable. Different reports employ varying combinations of phenotypic markers to specify the CD8⁺ T cell subsets both within one species and across mice and humans (Appay et al., 2002; Hikono et al., 2007; Kobayashi, Kondo, Takata, Yokota, and Takiguchi, 2006; Ramos et al., 2009; Sallusto et al., 1999; Tomiyama et al., 2002; Wang et al., 2011; Wherry and Ahmed, 2004; Wherry et al., 2003b). Although the expression of all important phenotypic markers associated with CD8⁺ differentiation downregulates with the progression of differentiation, the expression patterns do not separate distinctively. In humans, while naive cells (CCR7⁺ CD45RA⁺) express both CD28 and CD27, and CM cells (CCR7⁺ CD45RA⁻) are largely either CD28⁺ CD27⁺ or CD28⁻ CD27⁺, EM and EMRA populations are found to possess great heterogeneity in the expression of costimulatory receptors. Both these populations can be further sub-divided into four subsets according to the different combinations of CD28 and CD27 expression (Monteiro et al., 2007; Romero et al., 2007; Rufer et al., 2003). As each of these phenotypic molecules possesses a specific cellular function, the unsynchronised loss of their expression suggests that variation of cell functionality exists within each
subset of memory cells. In mice, the concept of CM and EM is also used. However, the segregation of CM and EM cells is commonly categorized by the expression patterns of CCR7, CD62L, or other markers such as CD27, CD43, CXCR3 and/or CD127 (Bachmann, Wolint, Schwarz, Jager, and Oxenius, 2005; Badovinac and Harty, 2007; Hikono et al., 2007; Kaech et al., 2003; Wherry et al., 2003b). Therefore, data across the literature must be carefully interpreted.

Furthermore, the recent discovery that bone marrow appears to be the preferred residence site for memory cells, as well as the accumulation of data supporting the existence of persistent tissue-resident memory cells that do not circulate, also implies that defined “memory” cells commonly extracted from either peripheral blood or secondary lymphoid organs may not be representative samples of the entire memory population (Casey et al., 2012; Herndler-Brandstetter et al., 2011; Jiang et al., 2012; Palendira et al., 2008).

In summary, consensus in the nomenclature and phenotypic definitions of CD8+ T cell subsets is required to enable better interpretation and compilation of data across different studies in the literature (Appay et al., 2008). For the data reported in this thesis, our definitions for effector and memory cells follow those described by a linear pathway in an acute infection (Kaech et al., 2002a), where effectors are functionally active CTLs generated immediately following TCR stimulations, and memory cells are rested cells formed after the clearance of antigen. For the subsets of memory cells, we use the terms CM, EM, EMRA with reference to rested memory cells according to their differential expression of CCR7 and CD45RA as originally defined by Champagne et al. (2001).

1.5 Adoptive immunotherapy and differentiation status

Adoptive immunotherapy, also termed ACT, is now an attractive approach to target advanced solid tumours and haematologic malignancies, and to restore immunity against chronic viral infections following bone marrow or haematopoietic stem cell transplantation, or chemotherapy (Kennedy-Nasser and Brenner, 2007; Leen, Rooney, and Foster, 2007; Restifo et al., 2012). Numerous clinical trials have now proven that ex vivo selection, manipulation and generation of highly effective and specific cells and their subsequent infusion into autologous patients can exert clinically useful effects preventing or treating disease. The recent development of techniques to genetically modify T cells for both TCR specificity and quality have increased the range of possible targets and effectiveness for ACTs (June, Blazar, and Riley, 2009; Restifo et al., 2012). These techniques use retroviruses or lentiviruses encoding a specific antigen receptor with high affinity to transduce patients’ autologous T cells for subsequent infusions, resulting in superior antigen specificity and TCR affinity (Restifo et al., 2012). Recent clinical trials have shown promising anti-tumour efficacies in a range of cancers including melanoma (Morgan et al., 2006), synovial cell sarcoma (Robbins et al., 2011), leukaemia and lymphoma (Brentjens et al., 2011; Porter, Levine, Kalos, Bagg, and June, 2011). However, ACT remains a complex therapeutic option, and there is a need to both improve the
response rates to this form of therapy and streamline methods for generating highly effective T cells ex vivo before it can be widely adopted.

An increasing number of studies are conducted to determine factors that regulate the efficacy of ACTs and to optimize the treatment regimes. An array of murine studies and clinical trials have demonstrated that reduced anti-tumour or anti-viral efficacies are correlated with properties of cultured CD8⁺ T cells associated with differentiation. These include reduced survival and proliferative capacity, reduced expression of important signalling receptors, and lowered IL-2 productivity, leading to the lack of engraftment of the transferred cells despite enhanced effector functions observed in vitro (Berger et al., 2008;Gattinoni et al., 2005; Hinrichs et al., 2009; Klebanoff et al., 2011; Zhou et al., 2005b). Interestingly, although higher IFN-γ production during the primary in vitro stimulation is commonly found to correlate with reduced anti-tumour efficacy, IFN-γ production during secondary stimulations has been found to inversely correlate with IFN-γ expression during the primary challenge, and thus directly correlate with anti-tumour efficacy (Hinrichs et al., 2009). At the same time, studies of CD8⁺ memory T cell subsets for ACT have found that naïve and stem cell-like memory (SCM) cells mediated superior in vivo persistence and anti-tumour efficacies compared to the CM and EM subsets (Gattinoni et al., 2011; Hinrichs et al., 2009; Klebanoff et al., 2011). CM cells were also found to have better potency than EM cells (Gattinoni et al., 2005; Klebanoff et al., 2005). Hence, it has been hypothesised that the capacity of CD8⁺ T cells to persist and exert efficacious functions in ACTs is inversely related to their differentiation state (Gattinoni, Powell Jr, Rosenberg, and Restifo, 2006), which is determined by both the immunological history of the transferred cells and their ex vivo manipulations.

Unfortunately, some of the key aspects in current ex vivo manipulation protocols for ACT are known to contribute to the loss of efficacy following mass clonal expansions with IL-2 and repeated stimulations. IL-2 is commonly employed during expansions for the generation of large cell populations necessary for ACTs (Chapuis et al., 2012; Heslop et al., 2010; Rosenberg et al., 2011). It is also used as a direct therapy following the infusion of CTLs in some ACT regimes (Chapuis et al., 2012; Dudley et al., 2008) due to its superb capability to enable T cell survival and growth (Miyazaki et al., 1995). However IL-2 is also a major driver of CD8⁺ T cell differentiation (Kalia et al., 2010; Pipkin et al., 2010), and sensitizes T cells to AICD (Lenardo, 1991; Refaeli, Van Parijs, London, Tschopp, and Abbas, 1998), as well as causing substantial toxicity at high doses when used directly as a therapeutic agent (Chapuis et al., 2012; Dudley et al., 2008). At the same time, repetitive strong anti-CD3 stimulations employed for expansions are found to drive CD8⁺ T cell differentiation and corrupt T cell functionality, whereas minimally cultured cells have been found to display improved anti-tumour characteristics (Besser et al., 2009; Tran et al., 2008). It is therefore unsurprising that attempts to improve preparation of less-differentiated cells for adoptive immunotherapy now often focus on the effects of γc cytokines other than IL-2 and potential modulators of T cell differentiation pathway (Klebanoff et al., 2012).
Many studies are now focusing on the generation of memory cells with early differentiation status, since this is associated with higher proliferative potential and survival capability. These attributes enhance the potential of engraftment and persistence of the adoptively transferred cells in the recipient, which is one of the current limitations for successful ACT (Berger et al., 2008; Pule et al., 2008; Zhou, Dudley, Rosenberg, and Robbins, 2005a). Such cells are also more likely to exert more potent anti-tumour activities upon the next antigen stimulation (Gattinoni et al., 2011; Hinrichs et al., 2009; Hinrichs et al., 2011; Rosenberg et al., 2011). Indeed, higher expression of CD28, CD27 and CD45RA on transferred CTLs has been correlated with the persistence of melanoma clones infused into patients (Rosenberg et al., 2011; Wang et al., 2012). In addition, activated human naïve CD8\(^+\) T cells have exhibited superior retroviral transduction efficiencies for the genetic engineering of exogenous TCRs compared to CM and EM cells (Hinrichs et al., 2011). In summary, the qualities of adoptively transferred cells associated with ACT efficacy include: a cell surface phenotype consistent with early differentiation status, an absence of markers correlated with terminal differentiation or replicative senescence, and a range of associated functional characteristics including high proliferative potential, high IL-2 secretory capability, and conversely, low persistent expression of granzyme B.

1.6 Concluding remarks

Over the last few decades, mouse models have allowed us to successfully dissect many aspects of basic immunology, including the mechanisms and regulatory factors involved in T cell activation. The combination of this knowledge with knowledge gained from studies of the heterogeneous T cell populations in human chronic viral infections has allowed broad consensus to be reached for the delineation of CD8\(^+\) T cell differentiation. However, the biological differences between mice and humans, as well as the lack of full understanding in the progression of CD8\(^+\) T cell differentiation and optimal generation of human memory cells, call for more direct efforts in human immunological studies to address knowledge gaps (Davis, 2008; Leslie, 2010; Mestas and Hughes, 2004). Enhanced understanding of the regulation of human CD8\(^+\) T cell differentiation both in vivo and in vitro is expected to enable further optimization of immunotherapeutic strategies, including both vaccination to stimulate optimal CD8\(^+\) T cell responses, and the generation of highly efficacious CTLs for ACT.

As there is now considerable evidence that the third signals are critical for the full activation of naïve CD8\(^+\) T cells and that these direct the characteristics of CD8\(^+\) T cell responses, we sought to further investigate the effects of third signals in human CD8\(^+\) T cells. It has been suggested that third signal cytokines are able to programme heritable differentiation pathways for CD8\(^+\) T cells to determine their attributes and potential for future responses. However, the effects of the third signals are commonly assessed either during the primary stimulation, or by restimulating the effector cells shortly following the primary challenge. The “programming” effects of the third signals following the generation of rested memory CD8\(^+\) T cells have not been investigated in humans. Since in vivo studies for priming CD8\(^+\) T cells in humans are impossible, we sought to develop an in vitro model that would allow the investigation of supplemented
signals during human CD8$^+$ T cell activation in a controlled environment. If the in vitro model could also allow the cells to be rested following expansion, the programming effects could be assessed by confirming establishment of “memory” for the characteristics of CD8$^+$ T cell responses following a period of time lapse since the third signal cytokine signalling. Optimising an established CD4$^+$ T cell in vitro model to make it suitable for the study of CD8$^+$ T cells will enable us to expand and generate rested memory cells for investigation of the “programming” effects produced by precise priming conditions. It will also allow us to correlate these effects to the quality of the subsequently generated memory cells, and their development of effector functions. Finally, it will permit longitudinal studies to be performed, to track the differentiation of the stimulated T cells over time, with an exact knowledge of their antigenic experience and immunological history.

Using this in vitro model of CD8$^+$ T cells, we hope to investigate the programming effects of third signal cytokines in the generation of human memory CD8$^+$ T cells, and examine the phenotypic qualities and functional effector capabilities upon secondary stimulations as well as the molecular mechanisms of the cytokine-directed programming. Insights about regulation of human CD8$^+$ T cell differentiation will provide knowledge which may be applied to the implementation of vaccination and adoptive immunotherapy, by allowing optimal in vitro priming and culture conditions to be translated into culture regimes for the generation of antigen-specific cells for ACT.
Chapter 2: General methods and validation

Methods specific for experiments performed within each chapter are described within the methods section for each individual chapter, with schematic diagrams of experimental set-ups outlined in the result sections. All other general methods, details of reagents and subsequent validations are described below.

2.1 Cell preparation and cell culture

2.1.1 Cell culture medium, conditions and maintenance

All cells were cultured in complete culture medium consisting of RPMI1640 (Gibco®, Invitrogen) supplemented with either a mixture of penicillin, streptomycin and glutamine (PSG 100x; Gibco®, Invitrogen) or penicillin, streptomycin (Pen/Strep 100x; Gibco®, Invitrogen) and glutamine separately (GlutaMAX™-I Supplement 100x; Gibco®, Invitrogen), with 5% AB human serum (heat inactivated; Invitrogen), denoted as RS5. The complete culture medium with the antibiotics and sugar but without serum was denoted as R0. Occasionally, serum was reduced to 1% for certain protocols, and this is denoted RS1. All media were filter sterilized with 0.22µM filter. All cells were cultured in a humidified tissue culture incubator at 37°C with 5% CO₂. All culture media were replaced every 2-3 days for resting cultures. Media replenishment was performed by aspirating half of the culture media and replacing with fresh, pre-warmed complete culture medium with serum. Cytokines were also replenished for the entire volume of media in culture at the same time where applicable. Cells in proliferating, or activated, cultures were subcultured whenever the cell culture well reached confluence and/or the culture media changed colour. After subcultures, fresh culture media were supplemented, as well as applicable cytokines.

2.1.1.1 Cytokine supplements

Recombinant human cytokines were supplemented in cell cultures either to support cell survival and growth or as a priming cytokine at the point of CD8⁺ T cell stimulation. All cytokines used in experimental assays and culture protocols are listed in Table 2-1.
Table 2-1 Recombinant human cytokines

<table>
<thead>
<tr>
<th>Recombinant human cytokines</th>
<th>Supplier</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>Peprotech</td>
<td>300-02A</td>
</tr>
<tr>
<td>IL-2</td>
<td>Peprotech</td>
<td>200-02</td>
</tr>
<tr>
<td>IL-4</td>
<td>Peprotech</td>
<td>200-04</td>
</tr>
<tr>
<td>IL-7</td>
<td>Peprotech</td>
<td>200-07</td>
</tr>
<tr>
<td>IL-10</td>
<td>Peprotech</td>
<td>200-10</td>
</tr>
<tr>
<td>IL-12</td>
<td>Peprotech</td>
<td>200-12</td>
</tr>
<tr>
<td>IL-15</td>
<td>Peprotech</td>
<td>200-15</td>
</tr>
<tr>
<td>IL-18</td>
<td>Bender MedSystems</td>
<td>BMS352</td>
</tr>
<tr>
<td>IL-21</td>
<td>Peprotech</td>
<td>200-21</td>
</tr>
<tr>
<td>IL-23</td>
<td>eBioscience</td>
<td>14-8239</td>
</tr>
<tr>
<td>IL-27</td>
<td>R&amp;D Systems</td>
<td>2526-IL</td>
</tr>
</tbody>
</table>

IFN, interferon; IL, interleukin.

2.1.2 PBMC extraction and preparation

Peripheral blood mononuclear cells (PBMC) were isolated from 50 or 100ml of blood drawn from healthy donors. Cells were isolated using Lymphoprep™ (Axis-Shield) and Leucosep™ tubes (Greiner bio-one). RPMI1640 and Lymphoprep™ were equilibrated to room temperature prior to cell isolation. 15ml of Lymphoprep™ was transferred into each Leucosep™ tube and centrifuged at 1000rpm for 1min. Blood was first collected into 50ml tubes (Falcon, BD) with Heparin (1000IU/ml, Sigma) and mixed thoroughly. Anticoagulated blood was separated across multiple 50ml tubes, with 15-17ml blood per tube, diluted with a 1:1 ratio of RPMI1640 and mixed by pipetting. Diluted blood was then transferred into Leucosep™ tubes containing Lymphoprep™ and centrifuged at 800g for 15min at room temperature with medium acceleration and no brakes for deceleration. The mononuclear cells formed a distinct band at the sample/medium interface after centrifugation, and were harvested using a Pasteur pipette and collected into a 50ml tube. Cells were then washed twice with 50ml of RPMI1640, pelleted by centrifugation at 400g for 10min with fast acceleration and deceleration, and the supernatant removed by aspiration. After two washes, PBMC cell pellet was resuspended in either R0 for cell enrichment procedures or RS5 for resting or cryopreservation. For CD8⁺ T cell isolations, the cell separation procedure was always carried out immediately following PBMC preparation.

2.1.3 Cell subset isolations

Specific cell subsets from PBMC were isolated using MACS magnetic bead separation techniques and/or fluorescence activated cell sorting (FACS) methodology. Naïve CD8⁺ T cells were isolated by a three-step MACS isolation protocol to enrich for CD8⁺, CD45RO⁻ and CCR7⁺ cells, while ex vivo CD8⁺ memory cell subsets were first enriched for CD8⁺ cells by MACS isolation, followed by FACS sorting (see 2.3.3).
2.1.3.1 Cell enrichment using MACS magnetic beads

Targeted cell subsets were either negatively enriched or positively selected by magnetically labelling with specific phenotypic monoclonal antibodies. Cell populations were then separated by retaining the labelled fraction within a MACS Column in the magnetic field of a MACS Separator, while the unlabelled fraction ran through the column and could be collected.

2.1.3.1.1 CD8\(^+\) T cell enrichment

CD8\(^+\) T cell enrichments were performed using the MACS Human CD8\(^+\) T Cell Isolation Kit (Miltenyi Biotec) following manufacturer’s instructions. Non-CD8\(^+\) T cells were first labelled with a cocktail of biotin-conjugated monoclonal antibodies (10µl per 10\(^7\) total cells) by an incubation at 4°C for 10min, then secondarily labelled with anti-biotin antibodies conjugated to MicroBeads (20µl per 10\(^7\) total cells) by an incubation at 4°C for another 15min. Cells were washed with chilled phosphate buffered saline (PBS)-based MACS wash buffer containing 5% fetal bovine serum (FBS; Gibco\(^\circledR\), Invitrogen) and 2mM Ethylenediaminetetraacetic acid (EDTA). Before magnetic separation, a LS MACS Column was placed in the MACS Separator and rinsed with chilled MACS wash buffer. Washed cells were resuspended in 500µl of fresh MACS wash buffer, placed in the MACS Column and the column further washed. The total effluent was collected; it contained the enriched CD8\(^+\) T cell population.

2.1.3.1.2 CD8\(^+\) naïve T cell isolation

Following CD8\(^+\) T cell enrichment, CD45RO\(^+\) cells were depleted to enrich for a pure CD45RA\(^+\) population, from which CCR7\(^+\) cells were positively selected to isolate CD8\(^+\) CD45RA\(^+\) CCR7\(^+\) naïve CD8\(^+\) T cells. CD45RO\(^+\) cells were depleted using CD45RO MicroBeads (Miltenyi Biotec) following manufacturer’s instructions, where anti-CD45RO monoclonal antibodies were directly conjugated with magnetic beads. Enriched CD8\(^+\) T cells were incubated with CD45RO MicroBeads (20µl per 10\(^7\) total cells) at 4°C for 15min, washed with MACS wash buffer and separated using a LD MACS Column as described above. The total effluent collected contained the CD8\(^+\) CD45RO\(^-\) CD45RA\(^+\) population. This population was then incubated with anti-CCR7 monoclonal antibodies conjugated with Fluorescin isothiocyanate (FITC) (20µl for every 3 million expected CCR7\(^+\) cells, R&D Systems) for 15min at 4°C, washed with MACS wash buffer and labelled with Anti-FITC MicroBeads (Miltenyi Biotec; 10µl per 10\(^7\) total cells) by another 15min incubation at 4°C, followed by further washes. Cells at this step were separated using a LD MACS Column and the labelled fraction was collected by removing the MACS Column from the magnetic field and pushing buffer through the column with a plunger to expel all positively selected cells. Isolated cells were resuspended in RS5 and counted for further experimental assays. The purity of naïve CD8\(^+\) T cells was between 90-96% in the isolated population (95% confidence interval from 22 PBMC samples).
2.1.4 Cryopreservation

Cells were routinely cryopreserved in 1ml aliquots of 10x10^6 to 20x10^6 cells for PBMC or 2x10^6 to 10x10^6 cells for in vitro-grown cell lines. Cells were prepared by resuspending them in 0.5ml RS5 per aliquot and an equal volume of freezing media containing 80% FBS and 20% DMSO (DMSO Hybrimax; Sigma Aldrich), resulting in a final concentration of 40% FCS and 10% DMSO. Cells to be cryopreserved were stored in cryovials (Nunc) and frozen at a controlled rate to -80°C by placing them in a freezing container holding isopropanol. Once frozen, cryovials were transferred to a liquid nitrogen tank for storage.

2.1.5 Thawing cryopreserved cells

Cryopreserved vials were thawed in a 37°C water bath directly from storage and warmed until all contents just liquefied. Vial contents were transferred to 15ml sterile tubes (Falcon, BD Biosciences) using sterile 3.5ml transfer pipettes and washed with 14ml of pre-warmed R0, where the first 2ml was added drop by drop then the remainder more rapidly. Cells were mixed by pipetting and centrifuged immediately at 1300rpm for 5min to remove freezing media. The supernatant was pipetted out and the cell pellet resuspended in appropriate volume of pre-warmed RS5 for subsequent manipulation. Viable cell recovery was commonly between 50-75%.

2.2 Immunological assays

2.2.1 Cell counting

2.2.1.1 Trypan blue exclusion method

Viable cells were counted by the trypan blue exclusion method, whereby senescent or dead cells take up the blue dye due to permeabilized membranes while viable cells exclude the dye (Mishell and Shiigi, 1980). Aliquots of 10µl from thoroughly resuspended culture were mixed with an equal volume of trypan blue stain (Invitrogen) and counted using a haemocytometer (Neubauer, bright-line, double ruled) under a light microscope (Leica DMI3000 B inverted microscope).

2.2.1.1.1 Cell counter

Occasionally, Countess® Automated Cell Counter (Invitrogen) was employed to aid cell counting. This also utilizes the trypan blue exclusion methodology. Similarly, samples were extracted from the cell culture, diluted to a concentration between 1x10^5 to 4x10^6 cells per ml where necessary and 10µl samples were mixed with equal volumes of trypan blue stain and pipetted into Countess® Cell Counting Chamber Slides to be read in the cell counter.
2.2.1.2 Cell counting by detection of ATP

An ATP assay, ATPLite™ (Perkin-Elmer), was employed to quantify the number of viable cells in culture by quantification of ATP, since all cells require ATP to maintain metabolic function, and the level of ATP declines rapidly as the cells undergo necrosis or apoptosis. The ATPLite™ assay detects the production of light from the reaction of ATP with added D-luciferin catalysed by firefly luciferase, and thus quantifies ATP. The assay was performed following the manufacturer’s instructions. Cells intended to be assayed were counted and plated in equal quantities in sterile black 96-well tissue culture plates with clear bottoms (Costar®, Corning). Experimental treatments were applied and the cells were cultured for a fixed period of time. At the time of assay, supernatants were partially removed to leave the necessary 100µl assay volume in each well and 50µl of mammalian cell lysis solution was added to each assay well. Plates were gently shaken at 600rpm for 5min. 50µl of substrate solution was added to each well and further mixed by gentle shaking. Plates were dark-adapted by wrapping in foil for 30min. Subsequently, plates were gently shaken for a final time prior to luminescence reading using a Wallac 1450 Microbeta plus Liquid Scintillation Counter in luminescence detection mode. Data was displayed in units of luminescence detection as a relative measure of cell counts.

2.2.2 CD8⁺ T cell stimulation and expansion

After isolation from PBMC, naïve CD8⁺ T cells were incubated with 5ng/ml of IL-7 overnight prior to stimulation, to allow the cells to recover physiologically following cell isolation procedures and gain homeostatic fitness through survival signals (Pearson, Silva, Saini, and Seddon, 2011). This pre-incubation with IL-7 for less than 24hr has been reported to have no major effect on cell proliferation and cytolytic functions for subsequent antigen stimulation (Gagnon et al., 2010). To simulate T cell activation, Dynabeads® CD3/CD28 T-cell Expander beads (also referred to as anti-CD3/CD28 beads; Dynal, Invitrogen) were employed to stimulate CD8⁺ T cells with a 1:1 ratio for 48hr at 2 x 10^5 cells per 200µl in flat-bottom 96 well plates, unless otherwise stated (protocol established in the host laboratory by Brooks (2007)). For some assays, CELLection™ Dynabeads (Dynal, Invitrogen) labelled in-house with specific concentrations of anti-CD3 or anti-CD28 monoclonal antibodies were employed, similarly at a concentration of 2 x 10^5 cells per 200µl in 96 well plates for 48hr. An 48hr incubation for CD8⁺ T cells with the stimulatory beads was required to ensure stimulation during the 8-20hr post-stimulation period, where APC-CD8⁺ T cell interactions were observed to be in a stable state by intra-vital microscopy in mice (Henrickson et al., 2008a), as well as the post-stimulatory 36 hours of accompanying TCR and costimulatory signals necessary for the 3rd signals to direct optimal clonal expansion and functional development (Curtsinger et al., 2003a; Curtsinger et al., 2003b; Mescher et al., 2006). As the CD8⁺ T cells were stimulated, a range of priming cytokines was added for differential lengths of time as per experimental design in each chapter. Meanwhile, IL-7, IL-2 or IL-15 was added throughout the culture period as indicated to support in vitro cell survival, growth and metabolic functions. Each round of expansion lasted for about 21 days. The cells expanded and rested in culture as a population of in vitro-
generated memory cells which we denoted “M1”. M1 populations could be restimulated, expanded and rested in the same manner to generate another population of memory cells which we denoted “M2”.

2.2.2.1 Stimulation with anti-CD3/CD28 beads

Dynabeads® CD3/CD28 T-cell Expander beads are uniform, superparamagnetic polymer beads similar in size to antigen-presenting cells (4.5µm) and covalently coated with an optimised, but commercially undisclosed, mixture of mouse anti-human CD3 and anti-human CD28 monoclonal antibodies on the same bead that enables simulation of in vivo T cell stimulation by APCs. Anti-CD3/CD28 beads were added to cultures at a 1:1 ratio with the cell numbers at the time of stimulation (2 x 10^5 cells/200µl for naïve cells or 5 x 10^4 to 20 x 10^4 cells/200µl for M1 restimulation functional assays), mixed by pipetting, and centrifuged at 600rpm for 3min to settle the beads and cells on the well floors of flat bottom tissue culture plates before incubation. For experimental assays in round bottom plates, culture wells were centrifuged at 1300rpm for 5min.

2.2.2.2 Stimulation with in-house labelled beads

CELLection™ Pan Mouse IgG Dynabeads® (in CELLection™ Pan Mouse IgG Kit; Dynal, Invitrogen) were coated with specific concentrations of monoclonal mouse anti-human CD3 or CD28 antibodies to stimulate CD8+ T cells, a methodology developed in the host laboratory (Brooks, 2007). CELLection Dynabeads are also uniform, superparamagnetic, polystyrene beads 4.5µm in diameter. Antibody-coated beads were then added to cultures at 1:1 ratios with the cell numbers. When both anti-CD3 labelled and anti-CD28 labelled beads were required, both were added at equivalent quantities. After addition of labelled beads to cells, the cultures were mixed thoroughly by pipetting and centrifuged at 600rpm for 3min.

2.2.2.2.1 In-house bead labelling

CELLection™ Dynabeads were first well suspended in the vial prior to extraction of the desired volume, and washed with 1ml R0 in 1.5ml eppendorf tubes to remove azide-containing storage buffer. Supernatants were removed by placing the 1.5ml tubes into a magnetic field, waiting for 1min and extracting all liquid. Beads were labelled with anti-CD3 (SPV-T3b; Zymed) or anti-CD28 (CD28.2; BD) monoclonal antibodies at indicated concentrations with a labelling volume of 80µl of RS1 per million beads. Washed beads were then resuspended in appropriate volume of RS1 in 1.5ml eppendorf tubes and the desired amount of antibodies added. Tubes were well shaken at 4°C, 1400rpm for 15min. Labelled beads were subsequently washed 3 times with R0, and either resuspended in R0 for temporary storage or RS5 for addition into culture.

2.2.2.3 Removal of antibody-bound beads from stimulated cells

Beads were removed from cultures after 48hr of stimulation. Cells were well resuspended in culture wells, transferred to a sterile 1.5ml eppendorf tube and placed in a magnetic field for 1min or until all beads were
attached to the tube wall by the magnetic force. The cell-containing supernatants were transferred to a fresh 1.5ml eppendorf tube where the procedure was repeated to remove any remaining beads. Fresh media were used to first wash the original culture well, then the 2 used eppendorf tubes with the separated beads. During the washes, the beads were thoroughly pipetted to detach any cells still linked to the beads. The two lots of cell-containing supernatants were pooled and re-plated into appropriate tissue culture wells.

### 2.2.3 Photography

Photographs of cells in culture were taken with a Leica microscope-camera system (Leica DMI3000 B inverted microscope with camera Leica DFC290, Leica Microsystem Ltd) using Leica Application Suite (LAS) software. Photographs were taken in black and white mode, and exported as TIFF files.

### 2.2.4 CFSE staining

To track their history of cell divisions, cells were labelled with Carboxyfluorescein succinimidyl ester (CFSE; CellTrace™ CFSE Cell Proliferation Kit, Invitrogen) at a concentration of 0.125µM, an optimised protocol in the host laboratory (Brooks, 2007). Targeted cells were washed with R0 to remove all serum in cell suspension and resuspended in 37°C pre-warmed, filtered-sterile PBS at a concentration of 2x10⁶ cells/ml. Lyophilized CFSE was reconstituted with molecular grade DMSO and diluted to a working stock of 500µM with pre-warmed, sterile PBS. CFSE was then further diluted to 0.25µM with PBS. An equal volume of diluted CFSE solution was added to the cell suspension and thoroughly mixed by pipetting to make up a final cell concentration of 1x10⁶ cells/ml and a CFSE concentration of 0.125µM. These cells were incubated for 10min at 37°C and immediately quenched with ice-cold RS5 at a volume of 5 times the labelling volume. The cells were incubated with RS5 for 5min on ice then centrifuged at 1300rpm for 5-10min depending on the size of total volume to remove all supernatant. The cell pellets were washed twice more with RS1 at the same volume and finally resuspended in RS5 for further experimental manipulation.

### 2.2.5 Effector functions

#### 2.2.5.1 Expression of IFN-γ by intracellular staining

5 x 10⁴ to 20 x 10⁴ cells were plated in 100µl in duplicate, for both stimulated assay wells and unstimulated controls in U-bottom 96 well tissue culture plates (BD Falcon™) with IL-7 (10ng/ml) and stimulated with a 1:1 ratio of anti-CD3/CD28 beads in appropriate wells. BD GolgiStop™ (BD Biosciences) was added to all wells 1hr after stimulation to inhibit intracellular protein transportation, and thus secretion of IFN-γ, by first making up a dilution of GolgiStop at 1.4µl/ml with RS5 and subsequently adding 100µl to each well to a final GolgiStop concentration of 0.7µl/ml. All wells were pipetted to mix, and centrifuged to pellet the cells. The plate was incubated for a further 5hr at 37°C. The cells were fixed and permeabilized with BD
Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) before intracellular staining with anti-IFN-γ monoclonal antibody and appropriate isotype control (see 2.3.1.2).

2.2.5.1.1 Acquisition of data when anti-CD3/CD28 beads were removed versus not removed

We tested to see whether the retention of anti-CD3/CD28 beads in cell suspensions would affect the detection of IFN-γ production. T cells were stimulated with anti-CD3/CD28 beads as described above (2.2.5.1) and at the end of the incubation period, the beads were either removed by magnetic means (2.2.2.3) or retained in cell suspension. All samples were stained for CD69, fixed and permeabilized, and subsequently intracellularly stained for IFN-γ. For the samples with the anti-CD3/CD28 beads retained, 3 populations were visible on the forward scatter and side scatter (FSC/SSC) profile. Only the bottom population was selected to exclude all presence of beads (confirmed by lack of signals in fluorescence channel 3, data not shown) and to match the FSC/SSC profile of stimulated cells with anti-CD3/CD28 beads removed (Figure 2–1A). Fluorescence signals from the activation marker CD69 and IFN-γ were similar between the two samples (Figure 2–1B). Hence, the anti-CD3/CD28 beads were not subsequently removed for the detection of IFN-γ.

Figure 2–1 Retention of anti-CD3/CD28 beads in cell suspensions did not affect the detection of IFN-γ
T cells were stimulated with a 1:1 ratio of anti-CD3/CD28 beads. GolgiStop (0.7µl/ml) was added to cells 1hr after stimulation and further incubated for 5hr. Anti-CD3/CD28 beads were either removed or retained in the cell suspension. Samples were stained for surface expression of CD69, fixed and permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit and then stained for intracellular IFN-γ. A. FSC/SSC profiles. B. Histograms for CD69 and IFN-γ fluorescence signals. Anti-CD3/CD28 beads removed, blue line; anti-CD3/CD28 beads retained, red line; unstained control, grey filled area.

2.2.5.2 Expression of granzyme B and perforin

5 x 10^5 to 20 x 10^5 cells were plated as 200µl duplicates, for both stimulated assay wells and unstimulated controls in flat-bottom 96 well tissue culture plates with IL-7 (10ng/ml), and activated with a 1:1 ratio of anti-CD3/CD28 beads in appropriate wells. After 3 days of incubation, the cells were fixed and permeabilized
with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit before intracellular staining with anti-granzyme B-FITC and anti-perforin-APC monoclonal antibodies with appropriate isotype controls (see 2.3.1.2).

### 2.2.5.3 Secretion of cytokines

Cytokines secreted by activated CD8+ T cells were quantified from the cell culture supernatants using BD™ Cytometric Bead Array (CBA) Human Soluble Protein Flex Set Assays (BD Biosciences) with Human Soluble Protein Master Buffer Kit (BD Biosciences). Cells were plated in flat-bottom 96 well tissue culture plates at a concentration of 2x10^5/200µl and stimulated with a 1:1 ratio of anti-CD3/CD28 beads with IL-7 (10ng/ml). 35µl to 50µl of supernatants were aspirated at 24 and 48hr after stimulation, stored in 1.2ml sample storage tubes (Scientific Specialties Inc.) with caps, and frozen at -20°C until assay. At the time of assay, lyophilized cytokine standards were made up to 100,000pg/ml with Assay Diluent, and standards of up to 10 cytokines in the assay were pooled to make up a final standard concentration of 10,000pg/ml for each cytokine standard. A 1:2 serial dilution was made up for the standards pool from 10,000pg/ml to 10pg/ml with Assay Diluent and 25µl of each dilution transferred to a U-bottom 96 well tissue culture assay plate. Supernatant samples were thawed, diluted with Assay Diluent where necessary at a ratio of 1:10, spun down at 1000rpm for 3 to 5min and 25µl of each sample transferred to the assay plates. Capture Beads mixture was made up by pooling all capture beads necessary in the assay at a volume of 0.25µl per cytokine assay per sample, with the Capture Beads stock well vortexed prior to extraction of beads. The pooled mixture was diluted with Capture Bead Diluent to make up a total volume of 25µl per sample assayed, stored at 4°C in the dark and thoroughly mixed by vortexing prior to aliquoting 25µl into each assay and standards well. The assay plates were vortexed at 500rpm for 5min and incubated for 1hr at room temperature away from light. PE Detection reagent mixture was also made up by pooling all reagents from each cytokine assayed at a volume of 0.25µl per cytokine assay per sample. The pooled reagent mixture was diluted with Detection Reagent Diluent to make up a total volume of 25µl per sample assayed and stored at 4°C assay from light until use. After the samples were incubated for 1hr with the Capture Beads, the PE Detection Reagent mixture were added to each assay well at 25µl per well. The assay plates were again vortexed at 500rpm for 5min and incubated for a further 2hr at room temperature away from light. The assay plate was centrifuged after incubation at 1300rpm for 5min, supernatants flicked off and wash with 200µl per well of Wash buffer and finally resuspended in 85µl of Wash buffer prior to transfer into microtubes for data acquisition on FACSArray™ II (BD Biosciences). Subsequently, the acquired data was analysed using FCAP Array v1.0.1 software, where the standard curves of each cytokine assay was generated and sample readings interpolated. The maximum standard concentration was 10,000pg/ml (or 100,000pg/ml if the supernatant samples were diluted at a 1:10 ratio). The concentrations above this value were still calculated by extrapolation and may not be fully quantitative. All cytokine assays employed are listed below (Table 2-2), each sampled at an optimised time point after restimulation (Appendix I) and assayed with specific dilutions of supernatants relative to assay detection range, unless otherwise stated. The limit of detection is as determined by the manufacturer by evaluating the average MFI of the negative control plus 2 standard deviations.
Table 2-2 Cytometric Bead Array (CBA) Human Soluble Protein Flex Set Assays

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Bead position</th>
<th>Catalogue Number</th>
<th>Limit of Detection</th>
<th>Time point</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>C9</td>
<td>558335</td>
<td>0.2 pg/ml</td>
<td>48hr</td>
<td>1:10</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>E7</td>
<td>558269</td>
<td>1.8 pg/ml</td>
<td>48hr</td>
<td>1:10</td>
</tr>
<tr>
<td>IL-2</td>
<td>A4</td>
<td>558270</td>
<td>11.2 pg/ml</td>
<td>24hr</td>
<td>1:1</td>
</tr>
<tr>
<td>IL-4</td>
<td>A5</td>
<td>558272</td>
<td>1.4 pg/ml</td>
<td>48hr</td>
<td>1:1</td>
</tr>
<tr>
<td>IL-5</td>
<td>A6</td>
<td>558278</td>
<td>1.1 pg/ml</td>
<td>48hr</td>
<td>1:1</td>
</tr>
<tr>
<td>IL-6</td>
<td>A7</td>
<td>558276</td>
<td>1.6 pg/ml</td>
<td>24hr</td>
<td>1:1</td>
</tr>
<tr>
<td>IL-10</td>
<td>B7</td>
<td>558274</td>
<td>0.13 pg/ml</td>
<td>24hr</td>
<td>1:1</td>
</tr>
<tr>
<td>IL-13</td>
<td>E6</td>
<td>558450</td>
<td>0.6 pg/ml</td>
<td>48hr</td>
<td>1:1</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>B9</td>
<td>558325</td>
<td>0.2 pg/ml</td>
<td>48hr</td>
<td>1:10</td>
</tr>
<tr>
<td>TNF</td>
<td>D9</td>
<td>558273</td>
<td>0.7 pg/ml</td>
<td>48hr</td>
<td>1:10 or 1:1</td>
</tr>
</tbody>
</table>

GM-CSF, granulocyte macrophage colony stimulating factor; IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor. Time point: sample extracted from cultures after stimulation for analysis of each analyte. Dilution: sample dilution for each cytokine analysis.

2.3 Flow cytometry

2.3.1 Antibody staining

2.3.1.1 Cell surface staining

Cells to be analysed by flow cytometry were transferred to 5ml FACS tubes (BD Falcon™) or U-bottom 96 well tissue culture plates for staining. Excess media were removed by centrifugation at 1300rpm for 5min to leave about 100µl for each sample. Cell suspensions were incubated on ice and antibodies added at pre-optimised doses (listed in Table 2-3). Up to 8 fluorophore-conjugated monoclonal antibodies were added per sample for data acquisition on FACSArray™ II, or up to 4 fluorophore-conjugated antibodies were used for data acquisition on FACSCalibur™ (BD Biosciences). Cells were incubated on ice for 15min protected from light, washed twice in 1ml FACS buffer (PBS with 1% FBS, chilled) with centrifugation at 1300rpm for 5min at 4°C to remove supernatant by decanting. Cell pellets were subsequently resuspended in FACS buffer for data acquisition. Cells that were stained in 96 well plates were washed with 200µl FACS buffer per well, flicked to remove supernatants and resuspended in 80-100µl FACS buffer per well for transfer into 1.2ml microtubes for data acquisition. Samples were stored on ice until analysis.
Table 2-3 Antibodies for cell surface detection on flow cytometry

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Conjugated fluorophore</th>
<th>Supplier/Brand</th>
<th>Clone</th>
<th>Cat #</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>PE</td>
<td>BioLegend</td>
<td>RPA-T4</td>
<td>300508</td>
<td>20µl (cell sorts)</td>
</tr>
<tr>
<td>CD8</td>
<td>APC-Cy7</td>
<td>BioLegend</td>
<td>RPA-T8</td>
<td>301016</td>
<td>2.5µl</td>
</tr>
<tr>
<td>CD25 (IL-2Rα)</td>
<td>APC-Cy7</td>
<td>BioLegend</td>
<td>BC96</td>
<td>302614</td>
<td>5µl</td>
</tr>
<tr>
<td>CD27</td>
<td>AF700</td>
<td>BD Pharmingen</td>
<td>M-T271</td>
<td>560611</td>
<td>2.5µl</td>
</tr>
<tr>
<td>CD27</td>
<td>AF700</td>
<td>BioLegend</td>
<td>O323</td>
<td>302814</td>
<td>2.5µl</td>
</tr>
<tr>
<td>CD27</td>
<td>APC-Cy7</td>
<td>BioLegend</td>
<td>O323</td>
<td>302816</td>
<td>10µl</td>
</tr>
<tr>
<td>CD27</td>
<td>FITC</td>
<td>BioLegend</td>
<td>O323</td>
<td>302806</td>
<td>5µl</td>
</tr>
<tr>
<td>CD28</td>
<td>PE</td>
<td>BioLegend</td>
<td>CD28.2</td>
<td>302908</td>
<td>10µl</td>
</tr>
<tr>
<td>CD28</td>
<td>PE-Cy5</td>
<td>BioLegend</td>
<td>CD28.2</td>
<td>302910</td>
<td>10µl</td>
</tr>
<tr>
<td>CD38</td>
<td>PE-TR</td>
<td>Invitrogen MP</td>
<td>HIT2</td>
<td>MHCD3817</td>
<td>1.25µl</td>
</tr>
<tr>
<td>CD45RA</td>
<td>PE-TR</td>
<td>Invitrogen MP</td>
<td>MEM-56</td>
<td>MHCD45RA17</td>
<td>2.5µl</td>
</tr>
<tr>
<td>CD45RO</td>
<td>PE-Cy7</td>
<td>BD Pharmingen</td>
<td>UCHL1</td>
<td>560608 / 337168</td>
<td>2.5µl</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-Cy7</td>
<td>BioLegend</td>
<td>HCD56</td>
<td>318318</td>
<td>5µl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20µl (cell sorts)</td>
</tr>
<tr>
<td>CD57</td>
<td>FITC</td>
<td>AbD Serotec</td>
<td>TB01</td>
<td>MCA1305F</td>
<td>10µl</td>
</tr>
<tr>
<td>CD62L (L-selectin)</td>
<td>APC</td>
<td>BD Pharmingen</td>
<td>DREG-56</td>
<td>559772</td>
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<tr>
<td>CD71</td>
<td>PE</td>
<td>BD Pharmingen</td>
<td>M-A712</td>
<td>555537</td>
<td>20µl</td>
</tr>
<tr>
<td>CD95 (Fas)</td>
<td>APC</td>
<td>BD Pharmingen</td>
<td>DX2</td>
<td>558814</td>
<td>20µl</td>
</tr>
<tr>
<td>CD98</td>
<td>FITC</td>
<td>BD Pharmingen</td>
<td>UM7F8</td>
<td>556076</td>
<td>5µl</td>
</tr>
<tr>
<td>CD127 (IL-7Rα)</td>
<td>PE</td>
<td>Beckman Coulter</td>
<td>R34.34</td>
<td>IM1980U</td>
<td>10µl</td>
</tr>
<tr>
<td>CD127 (IL-7Rα)</td>
<td>PE-Cy7</td>
<td>BD Pharmingen</td>
<td>HIL-7R-M21</td>
<td>560822</td>
<td>1.25µl</td>
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<tr>
<td>CD152 (CTLA-4)</td>
<td>APC</td>
<td>BD Pharmingen</td>
<td>BNI3</td>
<td>555855</td>
<td>20µl</td>
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<tr>
<td>CD152 (CTLA-4)</td>
<td>PE</td>
<td>BD Pharmingen</td>
<td>BNI3</td>
<td>557301</td>
<td>20µl</td>
</tr>
<tr>
<td>CD197 (CCR7)</td>
<td>FITC</td>
<td>BD Pharmingen</td>
<td>150503</td>
<td>561271</td>
<td>2.5µl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.5µl (cell sorts)</td>
</tr>
<tr>
<td>CD197 (CCR7)</td>
<td>FITC</td>
<td>R&amp;D Systems</td>
<td>150503</td>
<td>FAB197F</td>
<td>10 µl</td>
</tr>
<tr>
<td>CD244 (2B4)</td>
<td>PE</td>
<td>AbD Serotec</td>
<td>2B4.69</td>
<td>MCA2421PE</td>
<td>10µl</td>
</tr>
<tr>
<td>CD279 (PD-1)</td>
<td>APC</td>
<td>BioLegend</td>
<td>EH12.2H7</td>
<td>329908</td>
<td>5µl</td>
</tr>
<tr>
<td>Glut1</td>
<td>APC</td>
<td>R&amp;D Systems</td>
<td>202915</td>
<td>FAB1418A</td>
<td>10µl</td>
</tr>
</tbody>
</table>

Fluorophore abbreviations: AF, Alexa Fluor®; APC, Allophycocyanin; FITC, Fluorescein isothiocyanate; PE, R-Phycoerythrin; TR, Texas red. The 2 catalogue numbers for CD45RO are the same antibody product but one of which is prepared for GMP standards. All antibody doses are for analysis purposes unless otherwise stated for cell sorts and were optimised by a three dose titration. Cat #, catalogue number from supplier; MP, Molecular Probes®.

2.3.1.1.1 Live/dead staining and live cell gating

DAPI (4',6-diamidino-2-phenylindole), a fluorescence stain that binds strongly to DNA, was employed to distinguish viable versus dead cells in flow cytometry analysis. DAPI was first diluted to a working solution in FACS buffer at a ratio of 1:200 and 2µl of the working solution was added to each 100µl sample for a
final dilution of 1:10,000, and vortexed immediately prior to data acquisition. Positively stained DAPI events were then excluded from data analysis.

Alternatively, dead and doublet cells were excluded by forward scatter and side scatter (FSC/SSC) profiles of the flow cytometric data, since these were easily discernible for T cell samples. To validate this approach, in vitro-expanded T cells were stained with DAPI at a final concentration of 1:10,000. Events in the “live cell” FSC/SSC gating (Figure 2–2A) showed negligible percentages of DAPI positive cells as compared to ungated events (Figure 2–2B). Therefore, live cell gating on FSC/SSC profile accurately determines live singlet cells for typical human T cell populations. Either DAPI staining and/or FSC/SSC profile live cell gating were used for flow cytometric data analyses for the exclusion of dead cells.

**Figure 2–2 Validation of live cell gating**  
In vitro-expanded T cells were stained with DAPI at a final concentration of 1:10,000 and vortexed immediately prior to data acquisition by flow cytometry. **A.** FSC/SSC profile of live cell gating. **B.** Removal of DAPI⁺ cells by live cell gate. DAPI⁻ gate was drawn by using unstained sample as a background signal control.

### 2.3.1.2 Intracellular staining

Intracellular antigens and cytokines inhibited from secretion were detected by flow cytometry via intracellular staining. Cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) following manufacturer’s instructions. The cells to be analysed were harvested and transferred to 5ml FACS tubes or U-bottom 96 well plates, and first stained for cell surface antigens as described above, where necessary. After the cell surface stains were washed, the cells were resuspended with Fixation/Permeabilization solution at 250µl/tube or 100µl/well and incubated for 20min on ice. Two washes followed using 1x BD Perm/Wash™ buffer at 1ml/tube or 200µl/well and centrifuged at 1300rpm for 5min per wash. Pelleted cells were thoroughly resuspended in 100µl of Perm/Wash™ buffer per sample and antibodies for intracellular antigens (listed in Table 2-4) were subsequently added for an incubation of 20min at 4°C in the dark. The cells were then washed twice more...
using Perm/Wash\textsuperscript{TM} buffer, resuspended in 80-100\(\mu\)l of the same buffer, and stored on ice until data acquisition. Duplicate samples were employed for parallel staining of isotype controls (listed in Table 2-5) using doses that were equivalent to the amount of antibodies used for the target antigens.

<p>| Table 2-4 Antibodies for intracellular detection on flow cytometry |
|-----------------|----------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>ICS antibody</th>
<th>Conjugated fluorophore</th>
<th>Isotype</th>
<th>Supplier/Brand</th>
<th>Clone</th>
<th>Cat #</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granzyme B</td>
<td>FITC</td>
<td>IgG1, k</td>
<td>BD Pharmingen</td>
<td>GB11</td>
<td>558132</td>
<td>10(\mu)l</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>FITC</td>
<td>IgG1, k</td>
<td>BD Pharmingen</td>
<td>GB11</td>
<td>560211</td>
<td>10(\mu)l</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>AF647</td>
<td>IgG1, k</td>
<td>BioLegend</td>
<td>4S.B3</td>
<td>502516</td>
<td>1.25(\mu)l</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>FITC</td>
<td>IgG1, k</td>
<td>BD Pharmingen</td>
<td>4S.B3</td>
<td>554851</td>
<td>0.4(\mu)l</td>
</tr>
<tr>
<td>Perforin</td>
<td>APC</td>
<td>IgG2b, k</td>
<td>BioLegend</td>
<td>dG9</td>
<td>308112</td>
<td>5(\mu)l</td>
</tr>
</tbody>
</table>

All antibodies are anti-human mouse antibodies, unless otherwise specified. ICS, intracellular staining; Cat #, catalogue number from supplier. Fluorophore abbreviations: AF, Alexa Fluor\textsuperscript{®}; APC, Allophycocyanin; FITC, Fluorescein isothiocyanate; PE, R-Phycoerythrin. Doses were optimised by three dose titrations to maximize fluorescence signal whilst minimize unspecific staining to a fluorescence signal that is as close to the level of isotype control signal as possible.

<p>| Table 2-5 Isotype control antibodies |
|-----------------|----------------|---------|----------------|--------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Isotype control</th>
<th>Conjugated fluorophore</th>
<th>Supplier/Brand</th>
<th>Clone</th>
<th>Cat #</th>
<th>Specific QC</th>
<th>Primary antibody controlled for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG1, k</td>
<td>AF488</td>
<td>BioLegend</td>
<td>MOPC-21</td>
<td>400134</td>
<td>ICFC</td>
<td>pErk</td>
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<tr>
<td>Mouse IgG1, k</td>
<td>AF647</td>
<td>BD Pharmingen</td>
<td>MOPC-21</td>
<td>557732</td>
<td>IFN-(\gamma), GATA-3, pAkt</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1, k</td>
<td>FITC</td>
<td>BioLegend</td>
<td>MOPC-21</td>
<td>400138</td>
<td>ICFC</td>
<td>Granzyme B, IFN-(\gamma)</td>
</tr>
<tr>
<td>Mouse IgG1, k</td>
<td>PE</td>
<td>BD Pharmingen</td>
<td>MOPC-21</td>
<td>554680</td>
<td>PFA</td>
<td>T-bet</td>
</tr>
<tr>
<td>Mouse IgG2b, k</td>
<td>APC</td>
<td>BioLegend</td>
<td>MPC-11</td>
<td>400322</td>
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<td>Perforin</td>
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</tbody>
</table>

Doses employed were the equivalent amount of antibodies by weight used for the target antigens. Fluorophore abbreviations: AF, Alexa Fluor\textsuperscript{®}; APC, Allophycocyanin; FITC, Fluorescein isothiocyanate; PE, R-Phycoerythrin. Cat #, catalogue number from supplier; ICFC, (quality controlled for) intracellular flow cytometry; PFA, (quality controlled for) paraformaldehyde (fixation protocol).

### 2.3.1.3 Phospho-protein staining

Cells to be analysed were transferred into 5ml FACS tubes or U-bottom 96 well plates. Cytokine treatments were applied according to the optimized time periods for each phosphorylated protein at 37°C (see 2.3.1.3.1). For phosphorylated STAT (pSTAT) protein analysis, the cells were incubated with cytokines for 15min prior to fixation. For phosphorylated Akt (pAkt), 2min, and for phosphorylated Erk (pErk), two time points were assessed: 3min and 10min. Subsequently, 37°C pre-warmed BD Cytofix\textsuperscript{TM} Fixation Buffer (BD Biosciences) was added at a 1:1 ratio followed by an incubation at 37°C for 10min. Cells were centrifuged to remove supernatants and washed once with FACS wash buffer. Pre-chilled BD\textsuperscript{TM} Phosflow Perm Buffer III (1ml for tubes, 200\(\mu\)l for plates) was then added and incubated on ice for 30min,
followed by 2 washes with FACS wash buffer. Cells were then stained with anti-phospho-protein antibodies (listed in Table 2-6) at room temperature for 20min and subsequently washed twice more prior to data acquisition.

<table>
<thead>
<tr>
<th>Phospho-antibody</th>
<th>Conjugated fluorophore</th>
<th>Isotype</th>
<th>Supplier/Brand</th>
<th>Clone</th>
<th>Cat #</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAkt (S473)</td>
<td>AF647</td>
<td>IgG1, κ</td>
<td>BD Phosflow</td>
<td>M89-61</td>
<td>560343</td>
<td>1.25µl</td>
</tr>
<tr>
<td>pErk1/2 (T202/Y204)</td>
<td>AF488</td>
<td>IgG1</td>
<td>BD Phosflow</td>
<td>20A</td>
<td>612592</td>
<td>5µl</td>
</tr>
<tr>
<td>pSTAT-5 (Y694)</td>
<td>PerCP-Cy5.5</td>
<td>IgG1, κ</td>
<td>BD Phosflow</td>
<td>47</td>
<td>560118</td>
<td>10µl</td>
</tr>
</tbody>
</table>

2.3.1.3.1 Optimisation of treatment time length for pAkt and pErk analysis

Whole PBMC was incubated with cytokines for a series of time lengths between 2 to 10min at 37°C prior to fixation. The specimens were fixed, permeabilized and stained for pAkt and pErk as described above. A peak of pAkt signal was found after 2min incubation with cytokines, while pErk signalling appeared to be biphasic, with two peaks after 3 and 10min incubations (Figure 2–3). These time points were then used for pAkt and pErk signalling assays, respectively.

Figure 2–3 Cytokine treatment time period optimised for the detection of pAkt and pErk

Whole PBMC was incubated at 37°C with either 50ng/ml of IL-7 (pAkt) or 50ng/ml of IL-2 and IL-15 (pErk) for 2, 3, 5 or 10min. Other controls included unstained samples, isotype control for untreated samples and stained samples using cells that had no cytokine treatment. All samples were fixed immediately following the specific treatment incubation period but permeabilized and stained for the optimising phosho-proteins at the same time. Median fluorescence intensities of the phospho-protein signals were plotted.
2.3.1.4 Transcription factor staining

Cells were harvested and transferred into 5ml FACS tubes or U-bottom 96 well plates for the staining procedure. Cells were first stained with a live and dead viability dye for fixed cells as described below (2.3.1.4.3) where possible, and fixed by an addition of 100µl formaldehyde at 2% (diluted from 16% Methanol-free stock ampules with PBS; Thermo Scientific) with an incubation at 37°C for 10min. Cells were centrifuged at 1300rpm for 5min to remove formaldehyde and washed once with FACS wash buffer. Permeabilization was performed with an addition of chilled pure methanol (1ml for tubes, 200µl for plates) for an incubation on ice for 30min. Cells were again centrifuged to remove methanol and washed twice with FACS wash buffer. 2% BSA (Bovine Albumin Fraction V Solution (7.5%), Gibco® Invitrogen, diluted with PBS) was added at equal volume and incubated at room temperature for 10min. At this point, cell samples were split into different tubes for different stains. Antibodies for cell surface and nuclear antigens (listed in Table 2-7) or isotype controls were then added at the same time, and incubated for 20min at room temperature. Cells were washed twice with FACS wash buffer and resuspended for data acquisition.

<table>
<thead>
<tr>
<th>ICS antibody</th>
<th>Conjugated fluorophore</th>
<th>Isotype</th>
<th>Supplier/Brand</th>
<th>Clone</th>
<th>Cat #</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA-3</td>
<td>AF647</td>
<td>IgG1, κ</td>
<td>BD Phamingen</td>
<td>L50-823</td>
<td>560078</td>
<td>1.25ul</td>
</tr>
<tr>
<td>T-bet</td>
<td>PE</td>
<td>IgG1, κ</td>
<td>BD Phamingen</td>
<td>O4-46</td>
<td>561268</td>
<td>2.5µl</td>
</tr>
</tbody>
</table>

ICS, intracellular staining; Cat #, catalogue number from supplier. Doses were optimised by three dose titrations to maximize fluorescence signal whilst minimize unspecific staining to a fluorescence signal that is as close to the level of isotype control signal as possible.

2.3.1.4.1 Optimisation of transcription factor staining protocol

We trialled a variation of fixation and permeabilization steps to optimise the intranuclear staining of transcription factors while maintaining cell surface antigen staining signals. It is known that fixatives may destroy fluorophores if surface antigens were stained prior to the fixation step, or the antibody binding sites for the surface antigen may be altered following fixation and permeabilization. Therefore, we tested a few variations of the order of staining, fixing and permeabilizing steps in the nuclear antigen staining protocol, using formaldehyde and methanol as the fixation and/or permeabilizing agents. Unfixed samples (protocol A) and samples fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (protocol G) were used as controls for surface antigen staining. Five other protocols were tested (protocols B-F) and detailed in Table 2-8 and Table 2-9 below.
**Table 2-8 Protocols tested for the optimisation of nuclear antigen staining whilst maintaining surface antigen staining signals**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Protocol steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Unfixed control</td>
<td>sAg only</td>
</tr>
<tr>
<td>B Methanol fixation and permeabilization I</td>
<td>sAg – MOH – B – nAg</td>
</tr>
<tr>
<td>C Methanol fixation and permeabilization II</td>
<td>MOH – B – sAg/nAg</td>
</tr>
<tr>
<td>D Formaldehyde fixation and methanol permeabilization I</td>
<td>sAg – FA – MOH – B – nAg</td>
</tr>
<tr>
<td>E Formaldehyde fixation and methanol permeabilization II</td>
<td>FA – sAg – MOH – B – nAg</td>
</tr>
<tr>
<td>F Formaldehyde fixation and methanol permeabilization III</td>
<td>FA – MOH – B – sAg/nAg</td>
</tr>
<tr>
<td>G BD Cytofix/Cytoperm™ fixation and permeabilization</td>
<td>sAg – BD Cytofix/Cytoperm™ – nAg</td>
</tr>
</tbody>
</table>

sAg, staining of surface antigen; nAg, staining of nuclear antigen; MOH, fixation and/or permeabilization with methanol; B, blocking of unspecific binding sites with BSA; FA, fixation with formaldehyde; BD Cytofix/Cytoperm, fixation and permeabilization using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit. Details of each step are provided in Table 2-9.

**Table 2-9 Details of each step in the testing protocols for the optimisation of nuclear and surface antigen staining**

<table>
<thead>
<tr>
<th>Protocol step</th>
<th>Protocol step description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody staining Surface antigen (sAg)</td>
<td>Antibodies specific to surface antigen were added, cell samples were vortexed and incubated on ice for 20min. Cells were washed twice after antibody incubation with 1ml of FACS buffer and centrifuged at 1300rpm for 5min with supernatants removed by decanting. If this was the last step, cells were resuspended in 100µl of FACS buffer for data acquisition on flow cytometry.</td>
</tr>
<tr>
<td>Nuclear antigen (nAg)</td>
<td>Cell samples were vortexed after centrifugation from previous cell washes and antibodies specific to nuclear antigens and corresponding isotype controls were added. Although nuclear antigen staining after cell fixation could be done at room temperature, to equalize all antigen staining steps, nuclear antigen staining here was all carried out on ice with an incubation of 20min. 2 washes followed with FACS buffer for protocols A-F, while BD Perm/Wash was used for protocol G following the manufacturer’s protocol for the BD Cytofix/Cytoperm™ Kit. If this was the last step, cells were resuspended in 100µl of FACS buffer or BD Perm/Wash for data acquisition.</td>
</tr>
<tr>
<td>Formaldehyde fixation FA</td>
<td>16% formaldehyde (Thermo Scientific) was first diluted in PBS and added to samples at a final concentration of 2% and incubated at 37°C for 10min. If methanol permeabilization step followed, the samples were only centrifuged to remove fixative. If antibody staining step followed, the samples were washed twice with FACS buffer.</td>
</tr>
<tr>
<td>Methanol one step fixation and permeabilization MOH</td>
<td>Pre-chilled methanol was added to samples for a total volume of 1ml at a final concentration of 90% and vortexed. Cells were then incubated for 30min on ice and washed twice with FACS buffer.</td>
</tr>
<tr>
<td>Methanol permeabilization (following formaldehyde fixation) MOH</td>
<td>Pre-chilled methanol was added to samples for a total volume of 1ml at a final concentration of 90% and vortexed. Cells were then incubated for 30min on ice and washed twice with FACS buffer.</td>
</tr>
</tbody>
</table>
Cells were resuspended in FACS buffer after incubation with methanol and 2% BSA was added at equal volumes of the resuspended cell sample for a final concentration of 1% and incubated at room temperature for 10 min. Cells were then aliquoted into different tubes if the same cells were fixed and permeabilized together in one tube.

<table>
<thead>
<tr>
<th>BSA block</th>
<th>Cells were resuspended with the addition of BD Fixation/Permeabilization solution at 250 µl/tube and incubated for 20 min on ice. Two washes followed using 1x BD Perm/Wash at 1 ml/tube. Samples were then resuspended in 100 µl of BD Perm/Wash for antibody staining that pursued.</th>
</tr>
</thead>
</table>
| Cytofix/Cytoperm fixation and permeabilization | BD Cytofix/Cytoperm fixation

From protocols B-F, protocol F provided the most appropriate signals for all the fluorophores tested (Figure 2–4) and was adopted with an additional wash step added after formaldehyde incubation to ensure its complete removal. We found PerCP, PE-Texas Red and APC-Cy7 signals were almost all quenched under protocols D and E, while protocol B gave a poor separation for PE-Texas Red signal and reduced PerCP fluorescence (Figure 2–4). These results indicate that surface antigens cannot be stained prior to methanol incubation step, and the addition of formaldehyde further annihilated fluorescence signals. Although protocol C gave good fluorescence signals for PerCP and PE-Texas Red conjugated antibodies, there appeared to be unspecific staining of CD25-APC-Cy7 and provided an unusually high fluorescence signal for CD3-FITC (Figure 2–4).

![Figure 2–4 Fixation with formaldehyde coupled to permeabilization with methanol prior to both surface and nuclear antigen staining gave the most appropriate staining](image)

Expanded T cells were fixed and permeabilized by various protocols detailed in Table 2-8 and Table 2-9 for the co-staining of selected nuclear and surface antigens. Nuclear or surface antigen fluorescence signal, thick black line; isotype control, thin black line; unstained control, grey filled area.
2.3.1.4.2 Antibody staining duration

Lengthened staining durations with transcription factor antibodies were examined to determine whether an enhancement of specific fluorescence signals was possible. Whole PBMC was first stained with LIVE/DEAD® Fixable Blue Dead Cell Stain as described below (2.3.1.4.3) for the exclusion of dead cells. Subsequently, the cells were fixed, permeabilized and blocked as described above (2.3.1.4) and incubated with transcription factor antibodies at room temperature for a duration of 20, 40 or 60 min followed by final washing steps. No differences were found between the different antibody incubation periods (Figure 2–5), therefore the staining duration for transcription factor-specific antibodies remained at 20 min for the nuclear factor staining protocol.

![Staining duration](image)

Figure 2–5 Transcription factor staining for flow cytometry did not alter between 20 to 60 min of antibody incubation duration

Whole PBMC was first stained with LIVE/DEAD® Fixable Blue Dead Cell Stain to exclude dead cells. Subsequently, the cells were fixed with 2% formaldehyde and incubated at 37°C for 10 min. Permeabilization step followed by a 30 min incubation on ice with methanol. 1% BSA was added for an incubation at room temperature for 10 min to block. Antibodies were added at optimised doses for incubation at room temperature for 20, 40 or 60 min. T-bet staining is shown as a representative. Anti-T-bet fluorescence signal, black line; isotype control, grey filled area.

2.3.1.4.3 Live/dead viability staining for fixed cells

Cells were first washed with PBS. LIVE/DEAD® Fixable Blue Dead Cell Stain (Molecular Probes®, Invitrogen) diluted 1:500 with PBS was added to cells for a final ratio of 1:1000 and incubated for 30 min at room temperature protected from light. Cells were centrifuged at 1300 rpm for 5 min to remove supernatant and washed with PBS prior to continuation with fixation, permeabilization and staining procedures.

2.3.2 Acquisition of multi-colour flow cytometric data

FACS Aria™ II (BD Biosciences) with BD FACSDiva™ software (v6.1.1, BD Biosciences), or occasionally FACSCalibur™ (BD Biosciences) with CellQuest™ Pro software (BD Biosciences), was employed to acquire flow cytometric data. Prior to the acquisition of experimental samples, fluorescence compensation was analysed with FACSDiva™ using unstained and single stained controls to minimise spilled fluorescence. 20,000 to 50,000 events were commonly acquired for each experimental sample. Data were exported from FACSDiva™ as FCS3 files to be imported into analysis software. Manual compensation was carried out for experiments performed on FACSCalibur™.
2.3.3 Live cell sorting

*Ex vivo* CD8+ T cell memory subsets were separated by fluorescence activated cell sorting (FACS) after CD8+ T cell enrichment by MACS magnetic separation (2.1.3.1.1) by using CD45RA and CCR7 phenotypic expression and excluding any contaminating CD4+ and CD56+ cells left in the enriched CD8+ population. Enriched CD8+ cells were stained steriley with anti-CCR7-FITC (BD Pharmingen), anti-CD45RA-PE-TR (Invitrogen Molecular Probes), anti-CD4-PE (BioLegend) and anti-CD56-PE-Cy7 (BioLegend) monoclonal antibodies as per usual antibody staining procedure (2.3.1.1), but using phenol red-free RPMI1640 culture media and 5% human serum as wash buffer. Stained cells were resuspended in a concentration of 3-6 million cells per ml of wash buffer to sort. BD FACSARiaII cell sorter was employed with BD FACSDiva software to sort live cells using 16 purity, 16 yield sort mask, 85µm nozzle with the stream running at a pressure of 45psi and a frequency of 47 (47000 drops/sec). Cells were gated by forward and side scatter profile to include only live, small sized T cells and doublets gating to include only single cells for sorting. CD4+ and CD56+ cells were excluded and sorted into four subsets (naïve (N), CM, EM and EMRA) by their expression of CCR7 and CD45RA. Sorted cells were immediately spun down at 1300rpm for 5min to be removed from sheath fluid and resuspended in RS5 and maintained in cell culture incubator at 37°C until further experimental assay manipulation. Gating strategy and post sort validations are in Chapter 6.

2.3.4 Flow cytometric data analysis

Flow cytometry analysis software FlowJo for Windows (version 7.6.5, Tree Star) was employed to analyse and graphically present all flow cytometric data.

2.3.4.1 Gating strategy

Positive DAPI-stained cells were first excluded where the dye was included in the experiment (Figure 2–6A). FSC and SSC profiles were then employed to gate cells small in size, singular and excluding cell debris (Figure 2–6B). Cell doublets were subsequently gated out using FSC and SSC height versus width in sequence (Figure 2–6C). For the effector function assays where the stimulating beads were not removed from samples prior to flow cytometric data acquisition, the beads were gated out using any two signals from the three PE related channels: PE-Cy5, PE-Cy7 and PE Texas Red (Figure 2–6D).
2.4 Immunocytochemistry

2.4.1 Cell preparation and adherence to coverslips for staining

Approximately 200,000 cells were used for each stain. Required number of cells were washed in R0 to remove serum from cell suspension and resuspended in 50µl of R0 per staining sample. Microscope slide coverslips (22mm square, Menzel-Glaser) were placed in 6 well tissue culture plates (BD Falcon™) with pap pen (Dako) circles drawn on each coverslip. Cell samples each in 50µl aliquots were dropped into the pap pen circles on the coverslips and spread out evenly. Cells were incubated at 37°C for 20 to 30min.
2.4.2 Fixation, permeabilization and staining

Two protocols were employed for immunocytochemistry (ICC). One protocol was performed with methanol-fixation, casein-blocking and FBS-supplemented antibody dilution buffer (labelled “M/C/FBS” protocol), the other protocol was performed with paraformaldehyde-fixation, goat-serum-blocking and BSA (bovine serum albumin)-supplemented antibody dilution buffer (labelled “PF/GS/BSA” protocol). Additional sequential staining procedures were employed for four-colour staining using a fluorescence conjugated antibody. The protocols employed for each primary antibody are listed in Table 2-10. For some antibodies, both protocols could be used and have similar results (T-bet and Lef-1 are shown as examples in Figure 2–7). Secondary-only controls for each cell sample were included in every experiment to control for background autofluorescence and unspecific binding of the secondary antibodies.

2.4.2.1 M/C/FBS protocol

Cells adhered onto coverslips were washed twice with Tris buffered saline (TBS; 80g NaCl, 2g KCl and 30g Tris Base in 10L of distilled water, pH8) using transfer pipettes in the tissue culture plate wells with gentle actions. Cells were fixed using 50µl of ice-cold methanol per sample with an incubation at -20°C for 20min. Fixed cells were washed 3 times with TBS and subsequently blocked with 50µl of 0.25% casein per sample with an incubation at room temperature for 10min and washed once more. Primary antibodies were diluted at optimised doses (listed in Table 2-10) in 50µl of FBS dilution buffer (1% FBS in TBS) per sample and added to cells for a 1hr incubation at room temperature. The specimens were washed 3 times with TBS after primary antibody staining, with 5min incubations in between the two latter washes on a gentle rocker for thorough removal of excess antibodies. Secondary antibodies were centrifuged at 14,000rpm for 3min to settle any aggregates prior to extraction of required antibody for dilution at optimised doses (listed in Table 2-11) in a total volume of 50µl dilution buffer per sample. Diluted secondary antibodies were centrifuged again and subsequently applied to samples (together with DAPI diluted at 1:2000 final concentration if sequential staining is not performed) for an incubation of 30min in the dark at room temperature. Cells were washed 3 more times with TBS, with 15min rocking incubations in between the two latter washes and mounted onto frosted or plain microscope slides with ProLong Gold (Molecular Probes®, Invitrogen) warmed to room temperature and the coverslips sealed with nail polish. The slides were stored at 4°C until visualisation. Occasionally, samples were stored overnight in TBS after the 0.25% casein blocking step with the block washed off.

2.4.2.1.1 Additional sequential staining

If staining with the conjugated Eomes antibody was required, cells were further blocked with 50µl of 5% mouse serum (diluted in TBS) per sample after the secondary antibody staining. The samples were incubated for 10min at room temperature and rinsed twice using TBS with 5min rocking incubations in between the washes. Anti-Eomes-AF647 was diluted to a total volume of 50µl per sample in the same dilution buffer with an addition of Triton X-100 (1% FBS, 0.3% Triton X-100 in TBS), which was added to samples and incubated overnight at 4°C protected from light. Three more washes followed the overnight
incubation, using TBS with an addition of 0.3% Triton X-100 and 5min rocking incubations. DAPI was diluted at a ratio of 1:2000 in the same dilution buffer with Triton X-100 for a volume of 50µl per sample, added to cells and incubated for 15-30min in the dark at room temperature. The samples were subsequently washed 3 times using TBS with 0.3% Triton X-100 with 15min rocking incubations. The cells were then mounted and sealed as described above.

2.4.2.2 PF/GS/BSA protocol

After cells were adhered onto coverslips, media was aspirated and 50µl of 4% paraformaldehyde (diluted in PBS) was dropped onto each sample for a 15min incubation at room temperature to fix the cells. Paraformaldehyde was aspirated at the end of the incubation prior to three washes using PBS with 5min incubations in between each wash on a gentle rocker. Cells were subsequently permeabilized by an addition of 50µl ice-cold methanol per sample and incubated at -20°C for 10min. Three washes followed with PBS and the samples were blocked with 5% goat serum diluted in PBS and 0.3% Triton X-100 with an incubation at room temperature for 60min. Blocking solution was removed after incubation, required primary antibodies were diluted in BSA dilution buffer (1% BSA diluted in PBS with 0.3% Triton X-100) at optimised concentrations (listed in Table 2-10) for a total volume of 50µl per sample and added to cells. The specimens were incubated with the primary antibodies overnight at 4°C. Three 5min washes were performed the next morning, followed by secondary antibody dilutions at the appropriate concentrations (listed in Table 2-11) after centrifugation in the BSA dilution buffer for a total volume of 50µl per sample (with DAPI diluted at 1:2000 final concentration if sequential staining is not performed). The diluted secondary antibodies were applied to the specimens and incubated for 1hr at room temperature protected from light. The samples were rinsed in TBS with 0.3% Triton X-100 with 15min rocking incubations in between the two latter washes and mounted onto frosted or plain microscope slides with ProLong Gold warmed to room temperature and the coverslips sealed with nail polish. The slides were stored at 4°C until visualisation.

2.4.2.2.1 Additional sequential staining

If staining with the conjugated Eomes antibody was required, cells were washed twice with TBS and 0.3% Triton X-100 and twice with PBS following the secondary antibody staining. The samples were subsequently blocked with 50µl of 5% mouse serum (diluted in PBS) per sample. The blocking solution was incubated for 10min at room temperature protected from light. Two washes followed using PBS with 5min rocking incubations in between. Anti-Eomes-AF647 was diluted to a total volume of 50µl per sample in the BSA dilution buffer, applied to samples and incubated overnight at 4°C protected from light. Three more washes were performed the next morning, using TBS with an addition of 0.3% Triton X-100 and 5min rocking incubations in between the two latter washes. DAPI was diluted at a ratio of 1:2000 in TBS with 1% FBS and 0.3% Triton X-100 for a volume of 50µl per sample, added to cells, incubated for 15-30min in the dark at room temperature and subsequently washed 3 times using TBS with 0.3% Triton X-100 with 15min rocking incubations in between. The cells were then mounted and sealed as described above.
Table 2-10 Immunocytochemistry primary antibodies and their corresponding protocols performed

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Isotype</th>
<th>Supplier/Brand</th>
<th>Clone</th>
<th>Cat #</th>
<th>Dose</th>
<th>Protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eomes-AF647</td>
<td>Mouse IgG1</td>
<td>eBioscience</td>
<td>WD1928</td>
<td>51-4877</td>
<td>1:25</td>
<td>M/C/FBS; PF/GS/BSA; sequential staining</td>
</tr>
<tr>
<td>FoxO1</td>
<td>Rabbit IgG mAb</td>
<td>Cell Signaling</td>
<td>C29H4</td>
<td>2880</td>
<td>1:100</td>
<td>PF/GS/BSA</td>
</tr>
<tr>
<td>GATA-3</td>
<td>Mouse IgG1</td>
<td>BD Pharmingen</td>
<td>L50-823</td>
<td>558686</td>
<td>1:250</td>
<td>M/C/FBS</td>
</tr>
<tr>
<td>Lef-1</td>
<td>Rabbit IgG mAb</td>
<td>Abcam</td>
<td>EP2030Y</td>
<td>Ab53293</td>
<td>1:500</td>
<td>M/C/FBS; PF/GS/BSA</td>
</tr>
<tr>
<td>T-bet</td>
<td>Mouse IgG1</td>
<td>BD Pharmingen</td>
<td>O4-46</td>
<td>561263</td>
<td>1:100</td>
<td>M/C/FBS; PF/GS/BSA</td>
</tr>
<tr>
<td>Tcf-1</td>
<td>Mouse IgG1</td>
<td>AbD Serotec</td>
<td>1D2</td>
<td>MCA4168Z</td>
<td>1:100</td>
<td>PF/GS/BSA</td>
</tr>
</tbody>
</table>

All primary antibodies for immunocytochemistry were in unconjugated purified format unless otherwise stated. Cat #, Catalogue number; AF, Alexa Fluor®. All doses were optimised by titrations on positive control samples to minimize unspecific staining whilst maximizing fluorescence signal.

Table 2-11 Secondary antibodies for immunocytochemistry

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Conjugated fluorophore</th>
<th>Supplier/Brand</th>
<th>Species</th>
<th>Cat #</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-mouse IgG1</td>
<td>AF488</td>
<td>Invitrogen, MP</td>
<td>Goat</td>
<td>A21121</td>
<td>1:200</td>
</tr>
<tr>
<td>anti-rabbit IgG</td>
<td>AF555</td>
<td>Invitrogen, MP</td>
<td>Goat</td>
<td>A21434</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Cat #, Catalogue number; AF, Alexa Fluor®; MP, Molecular Probes®.

Figure 2–7 M/C/FBS and PF/GS/BSA protocols provide similar results for T-bet and Lef-1 staining.

Whole PBMC was stained for nuclei with DAPI (pseudocoloured grey) and either T-bet (green) or Lef-1 (red) using both M/C/FBS and PF/GS/BSA protocols. Overlays and single colour stains are shown. Objective magnification x40.
2.4.3 Visualisation and data acquisition

Immunocytochemistry specimens were visualised on Leica DMR microscope for all 2-dimensional images by epifluorescence with fluorescence excitation and emission filter cubes as listed in Table 2-12. These were an appropriate combination of fluorescent filter cubes chosen to avoid overlap of emission fluorescence. The two filter cubes used to visualise Alexa Fluor® 488 are the same specifications from two different companies and were thus interchangeable. However, either a bandpass filter or a longpass filter was used to visualise Alexa Fluor® 555 for different set of experiments, each thresholded with individualized secondary-only control samples for each experiment and cell specimens. The longpass filter required a slightly higher threshold, but still provided the same results (Figure 2–8). Two camera systems were employed to record the images, Leica DC 500 Camera (Leica Microsystems AG, Germany, with Leica DC500 version 1.1 driver) is able to detect the blue (DAPI), green (Alexa Fluor 488) and red (Alexa Fluor 555) fluorescence signals, where SPOT Camera (Diagnostic Instruments Inc, USA, with SPOT Basic version 4.7 driver) is able to detect the above three fluorescence colours as well as the far red signals from Alexa Fluor® 647. For each set of experiment, the same camera system was employed for consistency. AnalySIS LS Research (version 2.3, Soft Imaging System GmbH) software was employed for image acquisition. Sequential images were acquired for the different fluorescent stains for each area of visualisation and for each specimen, 5 areas were sampled.

Table 2-12 Fluorescence filter cubes used for visualisation

<table>
<thead>
<tr>
<th>Filter cube</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Supplier</th>
<th>Part number</th>
<th>Fluorescence stain employed for</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>325-375 (UV)</td>
<td>435-485 (Blue)</td>
<td>Chroma</td>
<td>31000v2</td>
<td>DAPI</td>
</tr>
<tr>
<td>EN GFP</td>
<td>450-490 (Blue)</td>
<td>500-550 (Green)</td>
<td>Chroma</td>
<td>41017</td>
<td>Alexa Fluor® 488</td>
</tr>
<tr>
<td>G/F/P</td>
<td>450-490 (Blue)</td>
<td>500-550 (Green)</td>
<td>Leica</td>
<td>11513847</td>
<td>Alexa Fluor® 488</td>
</tr>
<tr>
<td>TRITC</td>
<td>530-560 (Green)</td>
<td>590-630 (Orange/Red)</td>
<td>Chroma</td>
<td>41002c</td>
<td>Alexa Fluor® 555</td>
</tr>
<tr>
<td>N2.1</td>
<td>515-560 (Green)</td>
<td>&gt;590 (Orange/Red)</td>
<td>Leica</td>
<td>11513812</td>
<td>Alexa Fluor® 555</td>
</tr>
<tr>
<td>HQ:CY5</td>
<td>590-650 (Orange/Red)</td>
<td>663-737 (Infra-red)</td>
<td>Chroma</td>
<td>41008</td>
<td>Alexa Fluor® 647</td>
</tr>
</tbody>
</table>

The two filter cubes used for Alexa Fluor® 488 stains are completely interchangeable with the same filter wavelengths, whilst the two filter cubes used for Alexa Fluor® 555 are different for the excitation and emission wavelengths, the resulting images were proved to provide similar results. Only one or the other cube was used for each set of experiments.
2.4.4 Image analyses and presentation

All images were analysed and presented using Cytosketch software (Build 280, www.cytocode.com). Images were first superimposed for the multi-colour stains and the background autofluorescence determined by the secondary-only controls, and thresholded to zero. Subsequently, the same threshold was applied to every image for each set of stains. The brightness of the true fluorescence signals were adjusted appropriately for visualisation without raising signals in the secondary-only control. For ease of visualisation and display purposes, nuclei DAPI stains were commonly pseudocoloured to grey, whilst Alexa Fluor® 647 stains were pseudocoloured to cyan or blue. The figures were exported from Cytosketch as TIFF files with a resolution of 600dpi.

2.4.5 Nuclear localisation of transcription factors

The overlapping DAPI nuclei stains with the positive staining of the target transcription factors from epifluorescence superimposed images were confirmed to be true localisation of the transcription factors within the nuclei by confocal microscopy. Selected samples were visualised by both wide field epifluorescence and confocal microscopy on Andor Revolution spinning disc confocal microscope system (Andor Technology Ltd.). The Andor Revolution confocal microscope system employed Nikon Eclipse Ti-E inverted compound microscope (Nikon Corporation) and Andor iXon DU-885 monochrome EM-CCD Camera (Andor Technology Ltd.) with Andor iQ software (version 2.1, Andor Technology Ltd.) for confocal microscopy control and image acquisition. The images were acquired using a 60x oil objective lens and
0.23µm thick confocal sections. The confocal image files were reconstructed, analysed and presented using the Imaris software (version 7.3, Bitplane).

The cross sections of the confocal reconstructed images showed the transcription factors to be localised within the DAPI-stained nuclei through the cell centre from 3 dissection planes (Figure 2–9B), all of which had overlapping stains in epifluorescence images (Figure 2–9A). The stack sections of the confocal images revealed that the transcription factor staining appeared as the DAPI stains became apparent when the cross sections of the cells were visualized moving from the tip of the cell downwards (Figure 2–9C).

Transcription factors T-bet, Lef-1, Eomes and Tcf-1 were almost always found to be at least partially localised in the cell nuclei, if not all (Figure 2–9, Figure 2–10). The only exception was FoxO1, which could be found in the nuclei or only present in the cytoplasm. This was found to be also easily distinguishable by epifluorescence microscopy as confirmed with confocal images. Figure 2–10 shows two cells, one with FoxO1 localised only in the cytoplasm (Figure 2–10 cell a) whilst the other had FoxO1 in both the cell nuclei as well as the cytoplasm (Figure 2–10 cell b) with their corresponding epifluorescence image (Figure 2–10A) and confocal reconstructed cross sections (Figure 2–10B) as well as stack sections (Figure 2–10C).

2.5 Data processing and statistical analyses

All numerical data were processed in GraphPad Prism version 4.03 for Windows (GraphPad Software) to generate graphical displays and analyse for statistical tests. Non-parametric tests were employed for the statistical analyses as indicated in each chapter. Differences were considered significant when \( p < 0.05 \), very significant when \( p < 0.01 \) ** and extremely significant when \( p < 0.001 \ ***.\)
Figure 2–9 Overlapped staining of target transcription factor with DAPI were confirmed to be nuclear localised with confocal microscopy sections

Ex vivo CD8⁺ central memory cells were stained with T-bet (green), Lef-1 (red), Eomes (pseudocoloured cyan) and DAPI (pseudocoloured grey) using the PF/GS/BSA protocol with sequential staining. All images were acquired on the Andor Revolution spinning disc confocal microscope system. A. Image acquired by wide field epifluorescence, analysed and presented by Cytosketch software. Single stains are shown for marked cells a, b, and c. B. Image acquired by confocal microscopy, analysed and presented by Imaris software. Cross sections of the 3 planes through the cell centres from the reconstructed 3-dimentional confocal images of cell a (top left, scale bar represents 1.5µm), cell b (top right, scale bar represents 2µm) and cell c (bottom left, scale bar represents 1.5µm). The flattened ends of the cells were the side of the cells adhered on the coverslips. C. Next page.
Figure 2–9 Overlapped staining of target transcription factor with DAPI were confirmed to be nuclear localised with confocal microscopy sections cont.

C. Confocal stack section images of the cells a, b, and c for every 0.23µm thick sections through the cells. Going across the page from left to right first then down the rows are the stack sections from the tip of the cells moving towards the end adhered to the coverslip. The scale bar represents 10µm. Due to the spinning disk set up for the confocal microscopy system, the images from confocal microscopy are horizontally inverted when compared to the wide field epifluorescence images.
Figure 2–10 Cytoplasmic and nuclear localisation of antigens were distinguishable by epifluorescence images

Ex vivo CD8+ central memory cells were stained with Tcf-1 (green), FoxO1 (red) and DAPI (pseudocoloured grey) using the PF/GS/BSA protocol. All images were acquired on the Andor Revolution spinning disc confocal microscope system. A. Image acquired by wide field epifluorescence, analysed and presented by Cytosketch software. Single stains are shown for marked cells a, and b. B. Image acquired by confocal microscopy, analysed and presented by Imaris software. Cross sections of the 3 planes through the cells from the reconstructed 3-dimentional confocal images of cell a (top panel) and cell b (bottom panel). The images on the left are those shown with Tcf-1, FoxO1 and DAPI staining. The images on the right are those shown with Tcf-1 and FoxO1 only without DAPI. The scale bars represent 1µm. The flattened ends of the cells were the side of the cells adhered on the coverslips. C. Next page.
Cytoplasmic and nuclear localisation of antigens were distinguishable by epifluorescence images cont.

C. Confocal stack section images of the cells a, and b for every 0.23µm thick sections through the cells. Top section shows Tcf-1 (green), Foxo1 (red) and DAPI (pseudocoloured grey) stains; bottom sections shows the same images with only FoxO1 staining. Going across the page from left to right first then down the rows are the stack sections from the tip of the cells moving towards the end adhered to the coverslip. The scale bars represent 10µm. Due to the spinning disk set up for the confocal microscopy system, the images from confocal microscopy are horizontally inverted when compared to the wide field epifluorescence images.
Chapter 3: Development of the IL-7 *in vitro* model –
Generation of rested human CD8$^+$ memory cells *in vitro*

3.1 Introduction

Immunological memory is the fundamental feature of the adaptive immune system, where successfully established memory cells provide rapid and vigorous protective responses against re-infection of the same or related pathogens. Memory cells are formed following naïve T cell activation, expansion into effector populations and contraction, where the integration of multiple receptor-mediated and cytokine-mediated signalling upon TCR stimulation dictates the quality and quantity of the resultant memory population (Mescher et al., 2006; Williams and Bevan, 2007). There is now considerable evidence that the third signals play an important role in the activation and regulation of CD8$^+$ T cell responses (Cox et al., 2011; Curtsinger and Mescher, 2010). However, data for the third signal regulations for human CD8$^+$ T cells are limited.

There is general consensus in support of more directed efforts into human immunological studies to improve translation of research findings into therapy (Davis, 2008), and limited investigations of programmed recall responses following the establishment of rested memory cells. Therefore we sought to establish an *in vitro* model for studies of human CD8$^+$ T cells. We sought to generate resting populations of CD8$^+$ memory cells from naïve cells *in vitro*, to enable us to assess the programming effects of priming signals in strictly controlled environments, as well as to enable studies of longitudinal progression of memory cell development.

Extrinsic growth factors, such as the members of the common gamma chain ($\gamma_c$) cytokine family, are essential to the survival, metabolism and growth of T cells (Rathmell, Vander Heiden, Harris, Frauwirth, and Thompson, 2000; Vander Heiden et al., 2001; Wieman, Wofford, and Rathmell, 2007). However, apart from T cell survival, $\gamma_c$ cytokines also direct T cell development, proliferation and differentiation, with each $\gamma_c$ cytokines harbouring differential potency towards each of these effects (Osborne and Abraham, 2010; Rochman et al., 2009). Understanding how to balance these effects with the use of $\gamma_c$ cytokines will enable the development of optimal conditions for survival and proliferation of human CD8$^+$ T cells *in vitro*.

In previous PhD research, our laboratory had established a protocol for expanding naïve CD4$^+$ T cells into a stable memory population *in vitro*, and also provided preliminary data on the *in vitro* culture of CD8$^+$ T cells with IL-7 (Brooks, 2007). In research for the current thesis, we aimed to confirm and optimise the expansion of CD8$^+$ T cells *in vitro* using IL-7. Our aim was to establish a trackable system to generate rested human memory CD8$^+$ T cells for the investigation of third signals and their programming effects. We tested a range of culture conditions and $\gamma_c$ cytokines for the support of CD8$^+$ T cell expansion, memory cell
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generation and maintenance *in vitro* for functional comparisons against IL-7. The optimal condition or cytokine would provide the best support for cell survival and population maintenance, and enable the best retention of cellular attributes associated with early differentiation. The establishment of an *in vitro* model will be useful for testing hypotheses about human CD8\(^+\) T cell differentiation, and regulatory factors that influence the generation of memory cells. Understanding the mechanisms for optimal generation and persistence of CD8\(^+\) memory T cells is expected to help us understand disease processes that involve CD8\(^+\) T cells, and to enable us to improve the current design of immunotherapies.
3.2 Methods

3.2.1 Cell culture media

All cells were cultured in complete culture medium consisting of RPMI1640 supplemented with penicillin, streptomycin and glutamine ("R0") with 5% AB human serum, denoted as RS5 (2.1.1). For the cell culture media experiment, three other media were also used: RF10 and OpTmizer™ CTS™ T cell expansion serum-free medium (GIBCO®, Invitrogen) and AIM-V® serum-free medium (GIBCO®, Invitrogen). RF10 denotes for R0 supplemented with 10% FBS. For the serum-free media, we supplemented OpTmizer™ with Pen/Strep and GlutaMAX™, whereas AIM-V® already includes glutamine, gentamicin and streptomycin.

3.2.2 Cells and cell culture

Naïve CD8^+ T cells were isolated from healthy PBMCs (2.1.3), incubated with 1 or 5ng/ml of IL-7 and washed prior to further experiments. For the naïve cell maintenance experiments, cells were either labelled or not labelled with CFSE and plated in equal concentrations for culturing with the following: no cytokines, IL-2, IL-4, IL-7, IL-15 or IL-21 (detailed in Table 2-1) at concentration from 0 to 50ng/ml for 14 days. For naïve cell expansion experiments, cells were stimulated for 48hr with a 1:1 ratio of anti-CD3/CD28 beads with IL-2 (10ng/ml), IL-7 (5 or 10ng/ml as indicated), IL-15 (10ng/ml) or no cytokines. Anti-CD3/CD28 beads were removed from culture at 48hr and cells replated for further expansion in the same cytokines. For the M1 cell maintenance experiments, cells expanded with IL-7 at least 20 days post-stimulation were washed from the original culture, either labelled or non-labelled with CFSE and plated in equal concentrations for further culturing with the following: no cytokines, IL-2, IL-4, IL-7 or IL-15 at concentrations from 0 to 50ng/ml for 7 days.

3.2.2.1 Lengthened duration of IL-2 during primary stimulation

Naïve CD8^+ T cells were stimulated with 1:1 ratio of anti-CD3/CD28 beads for 2 days and allowed to expand in either IL-2 (10ng/ml), IL-7 (10ng/ml) for 21 days or expand in IL-2 for 3, 5, 7, 10 or 15 days before the cultures were washed and continued with IL-7 supplementation for a total of 21 days.

3.2.3 Assessment of cell numbers

Cells were counted by the trypan blue dye exclusion method manually as previously described (2.2.1).

3.2.3.1 AICD assay – assessment of cell numbers by ATP quantification

Cells were plated in 96 well black assay plates with clear bottom. They were then stimulated with a 1:1 ratio of anti-CD3 antibodies (1µg/ml, Zymed) conjugated to CELLection™ beads as described in the
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Methods (2.2.2.2) for 24hr. The amount of ATP was quantified using the ATPlite™ assay kit for the assessment of cell numbers following the manufacturer's instructions (2.2.1.2).

3.2.4 Cell culture photography
Photographs of cells in culture were taken with a Leica DMI3000 B inverted microscope (2.2.3).

3.2.5 CFSE labelling
Cells were labelled with 0.125µM of CFSE at 1x10⁶/ml as previously described (2.2.4).

3.2.6 Assessment of cell deaths by flow cytometry
Cells were stained with DAPI and the percentage of live cell events was determined by the absence of DAPI together with live cell gating (2.3.1.1.1).

3.2.7 Cell surface receptor expression
Cell surface receptors were stained as described in the Methods chapter (2.3.1.1), and the following antibodies were employed: anti-CCR7-FITC (R&D or BD), anti-CD27-AF700 (BD or BL), anti-CD27-APC-Cy7, anti-CD28-PE-Cy5, anti-CD45RA-PE-TR, anti-CD45RO-PE-Cy7, anti-CD57-FITC, anti-CD62L-APC, anti-CD152-APC, anti-CD244-PE, anti-CD279-APC (as detailed in Table 2-3) and DAPI.

3.2.8 Expression of cytolytic molecules
This procedure is described previously (2.2.5.2), using anti-granzyme B-FITC and anti-perforin-APC antibodies (as detailed in Table 2-4).

3.2.9 Phosphorylated protein expression
Phosphorylated proteins were stained as described in the Methods chapter (2.3.1.3), using BD Phosflow™ anti-pAkt-AF647, anti-pErk-AF488 and anti-pSTAT-5-PerCP-Cy5.5 antibodies (as detailed in Table 2-6).

3.2.10 Cytokine secretion
Naïve cells were stimulated and supernatants were aspirated every 24hr for 120hr after stimulation. Secreted cytokines in supernatants were examined by CBA Human Soluble Protein Flex Set Assay as described in the Methods (2.2.5.3), using the cytokine capture beads specific for IL-2 and IFN-γ. Supernatants from the 24 and 48hr time points were diluted 1:10, those from 72hr were diluted 1:2 and the rest were not diluted.
3.3 Results

In order to advance our understanding of the regulation of memory CD8$^+$ T cell generation, we sought to confirm and/or further optimise a CD4$^+$ T cell in vitro model previously established in our laboratory for longitudinal studies of human CD8$^+$ T cells. In the work described in this chapter, we first investigated the potential use of serum-free media compared to serum-supplemented media for human CD8$^+$ T cell culture (3.3.1), and then the effects of different $\gamma_c$ cytokines to support the following: in vitro naïve cell survival (3.3.2), proliferation (3.3.3) and maintenance of antigen-experienced cells (3.3.4), and the secretion of cytokines by naïve cells stimulated in vitro with IL-7 (3.3.5) (Figure 3–1).

![Figure 3–1 Chapter 3 experimental overview](image)

A schematic diagram illustrating the general experimental outline for sections 3.3.2 to 3.3.5 of this chapter, and the time points of investigation for each set of experiments. D indicates days after stimulation. Other specific experiments are illustrated and described in individual sections. NB. The experiment described in Section 3.3.1 utilized a previously in vitro-generated population of memory cells to compare cell culture media. Since this followed a slightly different experimental scheme, it is not included in this diagram.
3.3.1 Human serum-supplemented medium best supports *in vitro* cell growth

Animal serum contains a wide range of proteins and biological agents, which provide constituents necessary to maintain cell survival and attachment, and to support proliferation for cell culturing. However, serum is an ill-defined product which shows batch-to-batch variation, and therefore introduces ambiguous factors into cell culture experiments (Gstraunthaler, 2003). The fetal bovine serum (FBS) commonly used for *in vitro* cultures is also a potential source of microbial contaminants, endotoxins and xenograftic components to human cells, and may not be optimal for human cell cultures. A number of chemically defined, serum-free media have been formulated for alternative solutions for FBS supplementation. OpTmizer™ and AIM-V® serum-free media were both developed for human lymphocytes. We sought to compare these two commercially available serum-free media against RPMI1640 medium supplemented with either 5% human serum (RS5) or 10% FBS (RF10), to determine their ability to support human CD8⁺ T cell survival and growth. If a serum-free medium was able to sustain primary cultures of human CD8⁺ T cells, elimination of sera from our cell culture model would potentially reduce experimental variability in our system.

We first investigated the ability of the four different media to support the survival overnight of an *in vitro*-generated population of human CD8⁺ antigen-experienced T cells. Cells cultured with AIM-V® serum-free media had the best survival, with an average of only 6% cell death. This was significant compared to the other three conditions (Figure 3–2A). We then stimulated the cells with anti-CD3/CD28 beads for 48hr to investigate the effects of the different media on cell proliferation. Either IL-2 or IL-7 was added to the cultures. To allow for variation in cell loss after the removal of anti-CD3/CD28 beads, we compared cell numbers six days post-stimulation in relation to cell counts following the removal of the beads. It was found that neither serum-free medium supported cell proliferation or survival following stimulation, RS5 provided the best cell growth and survival, and RF10 provided some cell growth but the fold change in cell numbers was at least four times less than that for RS5 (Figure 3–2B). Both IL-2 and IL-7 were tested to exclude potential differences brought about by different properties of the cytokines (Rochman et al., 2009). Although IL-2 overall enhanced proliferation in comparison to IL-7, an expected result for IL-2 as a T cell growth factor (D'Souza and Lefrancois, 2003), comparisons between the different media were similar across the two cytokines (Figure 3–2B). Cell death by day 4 post-stimulation was also evident from direct visualization of the cultures (Figure 3–2C). This suggests a requirement for serum in our *in vitro* cultures. Since human serum provides a better aid for cell expansion and survival, we subsequently used RS5 for our *in vitro* cell culture system.
3.3.2 Maintenance of naïve cells in vitro

Common gamma chain (γc) family cytokines have crucial roles in the development, survival, proliferation and differentiation of multiple haematopoietic cell lineages (Rochman et al., 2009). We sought to first investigate the most appropriate γc cytokine to maintain human naïve CD8⁺ T cells in vitro. Naïve CD8⁺ T cells, either labelled or non-labelled with CFSE, were cultured with titrating levels of IL-2, IL-4, IL-7, IL-15 or IL-21 for 14 days and assessed by flow cytometry for their effects on cell division, percentage of live cell
events and expression of differentiation markers. The ideal cytokine would support naïve cell survival with minimal cell divisions apart from slow homeostatic turnovers, and would maintain the naïve phenotype.

CFSE-labelled naïve cells showed that exogenous cytokine supplement was necessary to support their survival in vitro (Figure 3–3A). Of the five γc cytokines tested, IL-2, IL-4, IL-7 and IL-15 were able to support cell survival to various degrees, with some individual variations found between donors. IL-2 and IL-15 were both found to support cell survival, with a small population having extensively divided with low concentrations of both cytokines. A homeostasis-like proliferation occurred at a high concentration of 50ng/ml (Figure 3–3A, B), indicating that a small population of naïve cells had enhanced sensitivity to IL-2 and IL-15. The size of the population recruited into extensive cell division varied between donors, but for each individual appeared to be similar for IL-2 and IL-15 (Figure 3–3A, B). On the other hand, although the effects of IL-7 on naïve cell divisions also varied between donors, the small population of cells that was ultra-sensitive to IL-2 and IL-15 did not extensively divide in the presence of IL-7. For each donor, the gradual increase of IL-7 concentration caused minimal increment in both the number of cell divisions and the proportion of cells recruited into division (Figure 3–3A, C). Although IL-4 from a concentration of 1ng/ml was also able to support moderate naïve CD8+ T cell survival, it showed no additional effects with elevated concentrations. IL-21, however, was unable to maintain naïve cell survival at concentrations up to 50ng/ml (Figure 3–3A, D). A different concentration was required by each cytokine to reach its maximal survival effect. IL-4 and IL-7 both reached their optimal concentration at a low dose between 1 and 5ng/ml, with IL-7 supporting a higher percentage of live cell events when compared to IL-4. The optimal concentration for IL-15 was 5ng/ml and for IL-2, between 10 and 20ng/ml (Figure 3–3D).

The cytokines also had effects on the expression of phenotypic markers on the naïve cells. In all conditions, cells lost some expression of CCR7 and CD62L, and to a lesser extent, CD28 and CD27. IL-2 at all doses tested had the highest reduction of the expression of both the homing molecules and costimulatory receptors. While high IL-15 doses had similar effects, the effects of low IL-15 doses were less (Figure 3–3E). IL-7 doses from 1ng/ml were able to maintain the expression of CD28 and CD27, while high IL-7 concentrations maintained the expression of CCR7 and CD62L. IL-4 moderately maintained the differentiation marker expression, with no apparent dose effect (Figure 3–3E). This is and consistent with its lack of titration effect on naïve cell survival and induction of cell divisions.

In summary, IL-7 appeared to be the most suitable cytokine to maintain naïve CD8+ T cells in vitro, where 1–5ng/ml was chosen to sustain naïve population, providing optimal support for survival (Figure 3–3D, ▼) and the ability to maintain the expression of important surface molecules for functionality at these doses (Figure 3–3E). Although IL-4 showed similar attributes, it is a classical Th2 (T helper cell type 2) cytokine (Löhning, Richter, and Radbruch, 2002), which has potential to induce type 2 responses. IL-7 is also able to induce slow homeostatic proliferation (not seen with IL-4 supplementation), and may be important for the maintenance of the naïve cell populations. In contrast, IL-2 and IL-15 were found to induce extensive proliferation in a small subset of cells and reduce the expression of homing receptors and costimulatory
Figure 3–3 Naive CD8 T cells maintenance by γc cytokines

A-C. Naive CD8 T cells were labelled with CFSE, plated at 5x10^4/200µl and cultured for 14 days with indicated concentrations of cytokines for 3 healthy donors. CFSE dilutions were then assessed by flow cytometry. B and C show individual variations in the otherwise representative results from donor 1. The Y axes of the histograms were each scaled to individual maximum values. D. Percentage of live cell events for the CFSE-labelled cells from donor 1. E. The expression of indicated markers of naive CD8 T cells following 14 days in culture with the various cytokines. Phenotypic molecules were stained and expression assessed by flow cytometry. Data are representative of 2 donors.
 receptors, rendering these cytokines less ideal for naïve CD8$^+$ T cell maintenance. Although IL-15 was able to maintain phenotypic marker expression at lower doses, the survival signals were diminished. Therefore, IL-7 was employed for the maintenance of naïve cells in vitro prior to stimulation in subsequent experiments unless otherwise stated.

3.3.3 Naïve cell expansion in IL-2, IL-7 and IL-15

3.3.3.1 Kinetics of cell proliferation

Next we investigated the effects of different γc cytokines during naïve CD8$^+$ T cell expansion. We sought to compare IL-2, IL-7 and IL-15 since they were able to support naïve cell survival, whereas IL-4 was excluded from the investigation due to its potential to induce type 2 immune responses (Löhning et al., 2002). Isolated naïve CD8$^+$ T cells were first washed following prior incubation in IL-7, and then stimulated with anti-CD3/CD28 beads for 48hr with one of the following: no cytokines, IL-2, IL-7 or IL-15 at optimal concentrations (Figure 3–3D). Each cytokine was then continuously supplemented throughout the expansion and the expansion kinetics were assessed using cell numbers. Cells that had no cytokine supplementation died and did not proliferate, while cultures supplemented with IL-2, IL-7 or IL-15 successfully expanded (Figure 3–4). The expansion kinetics for these 3 cytokines were found to be similar for the first 6 days, but diverged from day 8. Cells grown with IL-2 had the highest proliferation rate from day 8. This continued steadily until day 20 and produced the largest population. IL-15 supplementation also led to a steady increase in cell numbers; however it was at a slower rate than IL-2 and plateaued from day 17. Naïve cell proliferation with IL-7 had the slowest kinetics, but a steady proliferation was also observed for the first 2 weeks. IL-7 treatment produced the smallest population following expansion (Figure 3–4).

**Figure 3–4 Expansion of naïve CD8$^+$ T cells**

Naïve CD8$^+$ T cells were plated at 2×10^5/200µl and stimulated with a 1:1 ratio of anti-CD3/CD28 beads with no cytokines (labelled as "0"), IL-2 (10ng/ml), IL-7 (5ng/ml) or IL-15 (10ng/ml). Beads were removed after 48hr, cells were counted and equal number of cells across each condition per donor were replated and allowed to continually expand in the same cytokines. Cell numbers were assessed by the trypan blue method on days 4, 6, 8, 11, 14, 17 and 20. Data are representative of 2 donors. A and B shows the same data set, B magnified the section of lower cell numbers of A up to the dotted line.
3.3.3.2 Phenotype and morphology of the in vitro-expanded cells

γc cytokines and cell expansions have been implicated in regulating CD8+ T cell differentiation and phenotype alteration (Rochman et al., 2009; Schlub et al., 2009; Wirth et al., 2010b). Since there were considerable differences between the γc cytokines in the magnitude of naïve cell expansion, we sought to investigate the phenotype of the in vitro-expanded cells 21 days after stimulation.

Cells expanded in IL-7 were found to maintain the highest expression of CCR7 and CD62L, while IL-2 and IL-15 treatment reduced their expression (Figure 3–5A). Although this loss of CCR7 and CD62L was not extensive at day 21, prolonged culture in IL-2 and IL-15 was observed to further reduce CCR7 and CD62L expression (data not shown). The IL-7-expanded cells were also found to retain the highest expression of CD28 and CD27. The loss of costimulatory receptor expression induced by IL-2 and IL-15 varied between donors. It was either similar for these two cytokines or more pronounced for IL-2 (Figure 3–5A and data not shown). Interestingly, we have found CD45RA to be expressed on expanded cells across all cytokine conditions, with an absence of CD45RO expression (Figure 3–5A) and low activation marker (CD69, CD38 and CD25) expressions (data not shown).

We also assessed the expression on the expanded cells for the following phenotypic markers associated with terminal differentiation and T cell exhaustion: CD279 (PD-1), CD152 (CTLA-4), CD244 (2B4) and CD57. The expression of the inhibitory receptors CD279, CD152 and CD244 was found to be absent on the cells expanded in all three cytokine conditions (Figure 3–5B). However, a small percentage of cells (<3%) were found to express CD57, with the expression being similar across the different conditions.

Direct visualization of the cells expanded in the three γc cytokines showed homogenous, healthy and rested cell morphology for the IL-7-expanded cells, consistent with their more homogenous molecular phenotype. Cells expanded in IL-2 and IL-15 were found to be more polarized with evident cell deaths (Figure 3–6).
Naïve CD8\(^+\) T cells were expanded as described in Figure 3–4. 21 days after naïve stimulation, cells were assessed for the expression of the indicated markers by flow cytometry. **A.** Markers in relation to differentiation. Data are representative of 3 donors from 2 independent experiments. **B.** Markers in association with terminal differentiation.
3.3.3.3 Cells expanded in IL-2 were prone to apoptosis upon restimulation, which is dependent on the duration of IL-2 exposure during primary stimulation but independent of IL-2 presence

Apart from its role in promoting proliferation and survival, IL-2 is also known for its unique role in activation-induced cell death (AICD). IL-2 is a sensitizer for apoptosis during lymphocyte expansion as a self-regulatory mechanism to limit T cell proliferation in an on-going immune response (Lenardo, 1991; Refaeli et al., 1998). We tested to see whether IL-2-expanded cells also had the same disadvantage upon restimulation in comparison to IL-7-expanded cells, and whether the continual presence of IL-2 or IL-7 during restimulation had an effect. IL-2- and IL-7-expanded cells were restimulated with anti-CD3 beads with or without respective cytokines for 24hr (Figure 3–7). ATP quantities were assessed as a relative measure of cell numbers following restimulation (the ATP assay was carried out by Dr. Anna Brooks using cells generated by the author).

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**Figure 3–7** Testing the sensitivity of IL-2- and IL-7-expanded cells to apoptosis upon restimulation
We found that a large portion of cells expanded in IL-2, but not those in IL-7, died upon restimulation, and the same results were found irrespective of the concurrent presence of cytokines (Figure 3–8). In contrast, cells expanded in IL-7 could withstand restimulation and the short term survival of cells following secondary stimulation was independent of IL-7 availability (Figure 3–8).

Figure 3–8 Susceptibility of IL-2-expanded cells to apoptosis, but not those expanded in IL-7
Naïve CD8\(^+\) T cells were expanded as described in Figure 3–4 with IL-2 or IL-7. 28 days after expansion, cells were washed and equal numbers were plated in black tissue culture plates with clear bottom in triplicates. They were stimulated with a 1:1 ratio of in-house labelled anti-CD3 beads with or without respective culture cytokines for 24hr. Relative cell numbers by quantification of ATP was assessed with the ATPlite assay kit and luminescence detected by Wallac 1450 Microbeta plus Liquid Scintillation Counter. Bars represent average of triplicates and error bars depict SEM. (This assay was carried out by Dr. A. E. S. Brooks.)
As the IL-2-induced sensitivity to apoptosis appeared to be independent of the presence of IL-2 during restimulation, we further tested whether the apoptosis sensitivity was dependent on the duration of IL-2 exposure during primary expansion. We cultured stimulated naïve CD8+ T cells in IL-2 for different lengths of time, which were then continued in IL-7 for a total of 21 days. Cell death upon secondary stimulation was assessed (Figure 3–9).

We found that CD8+ T cell populations expanded with longer durations of IL-2 exposure were correlated with a decreased proportion of live cells upon restimulation by both flow cytometry assessment (Figure 3–10A) and direct visualization of the cultures (Figure 3–10B).

Although the Fas (CD95) pathway of apoptosis has been widely implicated in AICD (Gorak-Stolinska, Truman, Kemeny, and Noble, 2001; Refaeli et al., 1998), Fas was observed to be minimally expressed across all populations of cells expanded with different durations of IL-2 exposure (data not shown). Intriguingly, inhibitors of granzyme B activity, perforin maturation and lytic granule formation have been found to reduce AICD in CD8+ T cells, indicating potential participation of cytolytic molecules in AICD (Gorak-Stolinska et al., 2001). At the same time, IL-2-driven effector differentiation has also been found to influence the expression of granzyme B and perforin (Kalia et al., 2010; Pipkin et al., 2010). Therefore, we also examined the effect of IL-2 exposure duration on the expression of granzyme B and perforin upon restimulation (Figure 3–9), and found a gradually enhanced expression of the cytolytic molecules with increased duration of IL-2 exposure, correlating with the diminishing survival of CTLs (Figure 3–10C) and indicating a potential contribution to the apoptotic sensitivity induced by IL-2.
Figure 3–10 Lengthened duration of IL-2 signalling during primary expansion is correlated with cell deaths and expression of cytolytic molecules upon restimulation

Naïve CD8⁺ T cells were stimulated and expanded in either IL-7 (10ng/ml), IL-2 (10ng/ml) or IL-2 (10ng/ml) for the indicated number of days in brackets before continued with IL-7 (10ng/ml) for a total of 21 days. Cells were then restimulated with 1:1 ratio of anti-CD3/CD28 beads for another 3 days in IL-7 for all conditions except “IL-2 only” condition, which was restimulated in IL-2. A. Percentage of live cell events determined by DAPI staining and live cell gating 3 days post-restimulation by flow cytometry. B. Photos of cell culture 3 days post-restimulation with anti-CD3/CD28 beads. Objective lens magnification 20x. Scale bars represent 50µm. C. Expression of granzyme B and perforin as a percentage of live singlet cells 3 days post-restimulation.

In summary, naïve cells expanded in IL-7 proliferated less than those expanded in IL-2 or IL-15. However, IL-7 exposure generated relatively healthier cultures with a more homogenous phenotype early in differentiation (CCR7⁺ CD62L⁺ CD28⁺ CD27⁺ CD45RA⁻ CD45RO⁻), as well as round and rested cell morphology, without any expression of the inhibitory receptors. IL-7-expanded cells were also able to withstand restimulation with minimal cell deaths. Therefore, cells expanded in IL-7 generated memory cells that were the least differentiated and had better capability to survive and take part in recall responses relative to the IL-2- and IL-15-expanded cells. Thus, we confirmed the use of IL-7 for the expansion of human naïve CD8⁺ T cells in vitro to generate memory populations. We denoted these cells as “M1”, for population of memory cells generated with IL-7 after one round of stimulation in vitro.
3.3.4 Maintenance of memory cells generated in vitro

Next, we investigated the effects of γc cytokines on the maintenance of the IL-7-expanded M1 cells in vitro. M1 cells expanded with IL-7, either labelled or non-labelled with CFSE, were cultured with titrated levels of IL-2, IL-4, IL-7 and IL-15 for 7 days, and assessed for survival, proliferation, cell morphology and expression of differentiation markers (Figure 3–11). The ideal cytokine would support memory cell homeostasis with minimal alterations to cellular attributes.

![Diagram](image.png)

**Figure 3–11 Testing the effects of γc cytokines on maintaining M1 populations generated with IL-7 expansion**

CFSE-labelled M1 cells showed that IL-7 at concentration of 1ng/ml or greater, and IL-15 from 10ng/ml, were able to maintain optimal cell survival, whereas IL-2 and IL-4 were unable to sustain cell survival at any concentrations up to 50ng/ml (Figure 3–12A-C). Consistent results for IL-7 and IL-15 were found in a separate assay of cell number assessment without CFSE-labelling. However, the CFSE non-labelled cells showed increased cell numbers at high doses of IL-2 while the population size was maintained in IL-4 (Figure 3–12D). This inconsistency for the maintenance of memory cells with IL-2 and IL-4 incubation may be due to a combination of CFSE-induced toxicity (Brooks, 2007) and the altered expression of IL-2Rα (CD25) by CFSE staining (Last'ovicka, Budinsky, Spisek, and Bartunkova, 2009). In contrast to the maintenance of resting naïve cells (Figure 3–3A-C), IL-7 signalling in M1 did not induce cell divisions up to a concentration of 50ng/ml, but IL-15 induced proliferation at a high dose (Figure 3–12A, B). Other γc family and related cytokines, IL-9, IL-21 and TSLP, were also tested, and were found unable to sustain M1 cell survival (data not shown).
Chapter 3

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C

![Graph showing percentage of live cell events](image)

D

![Graph showing cell numbers](image)

Figure 3–12 Maintenance of IL-7-expanded CD8⁺ M1 cells in culture with IL-2, IL-4, IL-7 or IL-15

CD8⁺ M1 cells were generated by expanding naïve cells as described in Figure 3–4 for 21 days in IL-7 (10ng/ml). Cells were then washed and labelled with CFSE and cultured with the indicated cytokines at titrating levels of concentration for 7 days. A, B. Dilutions of CFSE assessed by flow cytometry. B showed individual variation of response to IL-15 from the otherwise representative sample from donor 1 in A. Data are representative of 3 donors in 2 independent experiments. All histograms were plotted on the same Y axis scale. C. Percentage of live cell events for the corresponding CFSE-labelled cells from donor 1 by live cell gating in flow cytometry data. D. CD8⁺ M1 cells cultured in the same way without CFSE-labelling in indicated concentrations of cytokines in duplicate. Cell numbers were counted after 7 days of culture by the trypan blue method from a starting cell number of 3.92x10⁵ per duplicate. Data are representative of 3 donors in 2 independent experiments. E-G. Next page.
Development of the IL-7 in vitro model

E. Photos of cell culture after 6 days of incubation in the indicated concentrations of cytokine of cells from D. Objective magnification x20. Scale bars represent 50µm. F. Relative cell size and granularity assessed by FSC/SSC profiles with cells corresponding to donor 1 in A. G. Percentage of expression of the costimulatory receptors after 7 days of culture in the indicated concentrations of cytokines of cells from D. Data are representative of 3 donors from 2 independent experiments.

Figure 3–12 Maintenance of IL-7-expanded CD8⁺ M1 cells in culture with IL-2, IL-4, IL-7 or IL-15 cont.
By direct visualization of cell cultures, and flow cytometric assessment of the forward and side scatter (FSC/SSC) profiles, non-CFSE-labelled cells incubated in either IL-2 or IL-15 were found to be polarized with increased cell size and granularity, as well as cell clustering at a high dose (Figure 3–12E, F), which may potentially induce cell-to-cell interactions and signalling. However, maintenance of M1 populations in either IL-4 or IL-7 led to round resting cells with smaller cell sizes, but some cell deaths were found with IL-4 incubation even at a high dose of 50ng/ml (Figure 3–12E, F).

The expression of phenotypic markers was also investigated. We found the expression of CD28 and CD27 was decreased with higher concentrations of IL-2 and IL-15. This was not observed for IL-4 and IL-7 (Figure 3–12G). The percentage of CD28 and CD27 expression for M1 cells incubated in IL-4 was either slightly higher or the same as that with IL-7-incubated cells, which varied between donors (Figure 3–12G and data not shown). The expression of CCR7 and CD62L was also investigated. However, no consistent effects were observed across donors (data not shown), similar to an absence of γc cytokine effects on the expression of these receptors observed by others (Yang et al., 2013).

Overall, albeit an increase in the sample size may ease the identification of trends amongst individual variations, maintenance of M1 cells in IL-7 induced robust survival signals from relatively lower doses without alteration in rested cell morphology or induction of cell clustering. Therefore, we chose IL-7 to maintain M1 populations in vitro following naïve cell expansion.

### 3.3.4.1 Cytokine signalling in M1 cells

Interestingly, although IL-2, IL-7 and IL-15 all share common γ and β chains in their receptor complexes and activate STAT (signal transducers and activators of transcription)-5 as their main signalling protein (Rochman et al., 2009), these three cytokines were observed to induce differential effects on CD8\(^+\) T cells. Therefore, we sought to investigate whether there were quantitative differences in their abilities to phosphorylate the signalling molecules, Akt, STAT-5 and Erk. IL-7-expanded M1 cells were incubated with one of the following: IL-2, IL-7, IL-15 or no cytokines, and assessed for the expression of the phosphorylated signalling proteins. The differences in signalling molecule expression between the cytokine conditions were found to be relatively small. However, IL-7 was observed to induce the highest expression of both phosphorylated Akt (pAkt) and STAT-5 (pSTAT-5), while IL-15 induced marginally higher expression of phosphorylated Erk1/2 (pErk1/2) at both time points investigated (Figure 3–13). This may partially contribute to their differential effects.
3.3.5 Naïve cells expanded with IL-7 were able to secrete IL-2 and IFN-γ

Recently, IL-2 autocrine signalling during primary CD8⁺ T cell activation was found to be critical for optimal secondary expansions (Feau et al., 2011). Therefore, we checked to see whether naïve cells stimulated and expanded with IL-7 in vitro were able to secrete IL-2, and were thus capable of driving the critical autocrine signalling for functional memory cell development. The capability of in vitro-stimulated naïve cells to produce IFN-γ was also assessed to investigate effector function development during priming stimulation (Figure 3–14). Following stimulation, supernatants were collected from activated naïve CD8⁺ T cell cultures every 24hr for 5 days, in the presence of either no cytokines or IL-7, and the quantities of the secreted cytokines assessed.

![Figure 3–13 Phosphorylation of signalling molecules in IL-7-expanded CD8⁺ M1 cells by IL-2, IL-7 and IL-15](image)

CD8⁺ M1 cells were generated by stimulating naïve cells with anti-CD3/CD28 beads for 48 hours and allowed to expand for 21 days in IL-7 (10ng/ml). Cells were washed and resuspended into RS5 with either no cytokines, IL-2, IL-7 or IL-15 (10ng/ml each) for 3 days. Cytokine were added again prior to fixation and permeabilization for phosphorylated protein staining. pAkt was assessed after 2min of cytokine induction; pSTAT-5, 15min; pErk1/2, 3 and 10min. Relative expression values of each phosphorylated protein were plotted from fold changes of MFI between each cytokine condition and the no cytokine control.

![Figure 3–14 Investigation of IL-2 and IFN-γ secretion during naïve expansion accompanied by IL-7](image)
Naïve cells stimulated with either IL-7 or no cytokines were found to secrete both IL-2 and IFN-γ. Under both conditions, IL-2 was found in culture supernatants from 24hr after stimulation, peaked at 48hr and dropped to baseline by 72hr (Figure 3–15). The expression of the high affinity receptor of IL-2 (CD25) following activation (data not shown) along with the capability of receiving IL-2 signals (as observed in Figure 3–4) indicates that naïve CD8⁺ T cells stimulated in vitro were able to produce and potentially consume autocrine IL-2. This probable IL-2 autocrine signalling partially ascertain the quality of the generated memory cells. At the same time, IFN-γ was apparent in culture supernatants by 24hr following stimulation of naïve cells cultured in IL-7, peaked at 48hr and sustained in culture supernatant up to 120hr post-stimulation (Figure 3–15). Secretion of both IL-7 and IFN-γ was enhanced for cells cultured in IL-7 (Figure 3–15), with no dramatic differences in cell numbers up to day 4 post-stimulation (data not shown). This suggests that IL-7 encouraged the secretion of IL-2 and IFN-γ, which may partially explained by its effect on the enhancement of cell metabolism and survival signals (Rathmell, Farkash, Gao, and Thompson, 2001).

Figure 3–15 Naïve cells expanding in IL-7 secreted IL-2 and IFN-γ
Naïve CD8⁺ T cells were plated at 2x10⁵/200µl, stimulated and expanded in either no cytokines or IL-7 (5ng/ml). Supernatants were aspirated from cultures every 24hr post-stimulation for 5 days. Secreted IL-2 and IFN-γ in supernatants were detected using the BD CBA flex set assay.
3.4 Summary and discussion

In this chapter, we set out to establish an in vitro model and confirm the use of IL-7 for optimal in vitro culture for human CD8\(^+\) T cells. Following comparisons between cell culture media and \(\gamma_c\) cytokines, we have established a unique culture system to support long term maintenance and expansion of human CD8\(^+\) T cells in vitro. This culture system allows naïve CD8\(^+\) T cells to be stimulated, expanded, rested as memory populations and restimulated with the support of human serum-supplemented medium and IL-7. Each phase of the CD8\(^+\) T cell expansion was investigated with a number of \(\gamma_c\) cytokines and confirmed the optimal support by IL-7 for cell survival, homeostatic proliferation, maintenance of early differentiation attributes and rested cell morphologies in comparison to IL-2, IL-4 and IL-15. The IL-7-expanded cells were also found capable of undergoing secondary stimulations in vitro without prominent cell deaths, and to secrete IL-2 and IFN-\(\gamma\) during the primary expansion. This evidence indicates successful generation of functional and rested memory CD8\(^+\) T cells in vitro. The culture system could be utilized for longitudinal studies of human CD8\(^+\) T cell programming and differentiation.

From the comparison between two serum-supplemented media and two serum-free media, we found that although AIM-V\(^\circ\) serum-free medium maintained the best survival for human CD8\(^+\) T cells, both serum-free media were unable to support cell survival after stimulation; whereas human serum-supplemented-RPMI1640 medium provided superior support for cell proliferation. This is consistent with reports where AIM-V\(^\circ\) was found to be incapable of supporting cell expansion without serum (Sato et al., 2009), and autologous serum was supplemented into serum-free media to enhance survival and proliferation (Hickey et al., 2012; Sato et al., 2009). Since human serum-supplemented RPMI1640 was observed to provide optimal support for CD8\(^+\) T cell survival and proliferation, it was chosen for our culture system.

Following comparisons of \(\gamma_c\) cytokines, we have confirmed that IL-7 provides optimal support for human CD8\(^+\) T cell cultures across all phases of naïve cell expansion in comparison to IL-2, IL-4 and IL-15. IL-7 was observed to induce maximal population survival at the lowest concentration with a global homeostatic proliferation in the naïve populations that is important for population maintenance. This was not found with IL-2-, IL-4- and IL-15-supplemented cultures. IL-7 incubation also largely maintained the expression of homing and costimulatory receptors that are important for cellular functionality. The slow global proliferation observed with IL-7 is commensurate with its role in the maintenance of naïve T cell populations, and appeared similar to the lymphopenia-induced proliferation (LIP) found in vivo for both mice and humans with an overabundance of IL-7 (Sauce et al., 2012; Schluns, Kieper, Jameson, and Lefrancois, 2000). Cells undergoing LIP have also been observed to acquire memory phenotypic attributes (Goldrath, Bogatzki, and Bevan, 2000; Haluszczak et al., 2009), indicating that the loss of naïve phenotype expression incubated with \(\gamma_c\) cytokines is a natural process.

The IL-7-supported naïve CD8\(^+\) T cell expansion was observed to induce the smallest magnitude of proliferation in comparison to IL-2 and IL-15. This constrained cell proliferation is possibly advantageous in
terms of retaining proliferative potential, survival capability, ability to homeostatically proliferate and secretion of IL-2 following the generation of memory cells as previously observed (Klebanoff, Gattinoni, and Restifo, 2006). Indeed, IL-7-expanded cells were observed to retain the highest expression of the receptors associated with CD8+ T cell differentiation by the end of the 21-day expansion period. These receptors included CCR7, CD62L, CD28, CD27 and CD45RA, the expression of which was unexpectedly similar to naïve cells. A recent study has proposed a new memory T cell subset expressing predominantly naïve phenotypic markers (CCR7+ CD45RA+), with stem cell-like properties but enhanced memory cell functionalities compared to CM, EM and EMRA cells, which have been termed stem cell-like memory (SCM) cells (Gattinoni et al., 2011). The expression CD45RA and a lack of inhibitory receptor expression suggest that the IL-7-expanded cells generated in our study may be similar to SCM found in ex vivo human PBMCs (Gattinoni et al., 2011). In contrast, IL-2 and IL-15 induced greater expansions of naïve CD8+ T cells, with lower expression of differentiation markers and more polarized cell morphologies, indicating enhanced cellular metabolism and activities. At the same time, IL-2-expanded cells were found to be sensitive to apoptosis, making them less-than-ideal memory cells for further experimentation. Interestingly, cells expanded in each of the three cytokines showed a very small population of cells expressing CD57, a marker that is associated with replicative histories of CD8+ T cells (Brenchley et al., 2003). This implies some heterogeneity in the number of cell divisions undertaken by the naïve cells.

For the maintenance of M1 populations, IL-7 was observed to promote survival with an absence of cell divisions, and round cell morphologies with smaller cell sizes, indicating a stable resting state. At the same time, this IL-7-induced optimal M1 cell survival was found to be associated with the highest expression of phosphorylated STAT-5 and Akt when compared to IL-2 and IL-15. The effect of STAT-5 and its downstream activation of Akt are known to enhance effector and memory cell survival (Hand et al., 2010) by elevated levels of glucose uptake (Wofford, Wieman, Jacobs, Zhao, and Rathmell, 2008) and induction of Bcl-2 (Kelly et al., 2003). In contrast, IL-15 at high doses was found to induce proliferation, enlarged and polarized cell morphologies, cell clustering and an undesirable loss of costimulatory receptor expression, as well as the highest induction of Erk phosphorylation. Phosphorylated Erk is known to be responsible for the regulation of cell sizes and proliferation upon CD8+ T cell activation (D'Souza, Chang, Fischer, Li, and Hedrick, 2008), which is consistent with the observed effects in the current study of IL-15 on M1 populations. The effects of IL-7 and IL-15 on M1 populations are consistent with the current consensus for memory cell homeostasis regulations: IL-7 primarily supports cell viability via the induction of Bcl-2, and IL-15 induces basal homeostatic proliferation (Surh and Sprent, 2008). The data from the current study indicate enhanced cell metabolism and activities for M1 populations maintained in IL-15, as well as potentially unknown effects due to cell-cell contacts from IL-15-induced clustering. IL-7, however, supports optimal survival and cell resting. Owing to the inability of IL-2 and IL-4 to support the survival of IL-7-expanded cells under certain experimental conditions, we confirmed IL-7 to be the most suitable cytokine for memory cell maintenance.
Investigations for the establishment of the *in vitro* model also yielded interesting observations related to an absence of contraction *in vitro* following naïve cell expansion and mechanisms of sensitivity to apoptosis for IL-2-expanded cells. Interestingly, no contraction was apparent following the expansion of naïve CD8\(^+\) T cells *in vitro* irrespective of cytokine supplementation — although complete withdrawal of any of these cytokines caused a catastrophic collapse in the expanded populations. The mechanisms of programmed cell death upon resolution of an immune response are known to involve the pro-apoptotic protein Bim or the engagement of Fas or TNFR (Bouillet and O'Reilly, 2009; Hedrick, Chen, and Alves, 2010). Although enhanced IL-7 signalling or constitutive CD127 expression are incapable of rescuing effector cells from apoptosis upon contraction (Hand et al., 2007; Sun et al., 2006), the elevated levels of *in vitro* γc cytokines are likely to have upregulated the expression of anti-apoptotic proteins such as Bcl-2, Bcl-xL and Mcl-1 (Khaled and Durum, 2002; Plas, Rathmell, and Thompson, 2002), to restrain Bim from initiating CD8\(^+\) T cell apoptosis (Bouillet and O'Reilly, 2009; Khaled and Durum, 2002). At the same time, there is an absence of TNFR/DR ligands and Fas expression presented by APCs *in vitro*. These are required for the extrinsic pathway of CD8\(^+\) T cell apoptosis, and their absence results in a lack of contraction of effector cells *in vitro*. However, it is not clear whether the survival of the majority of the effector population has implications for the properties of the generated memory cells. This requires further investigation.

AICD implicated in the regulation of effector cell expansion is commonly observed following TCR restimulation of recently activated, cycling T cells in the presence of IL-2 (Refaeli et al., 1998; Schmitz et al., 2003; Zheng, Trageser, Willerford, and Lenardo, 1998). Although the sensitivity to apoptosis we have observed was also associated with IL-2 on cycling T cells, it did not require the presence of IL-2 during restimulation, and a prolonged period of time had elapsed since the previous TCR engagement. Therefore, the sensitivity to apoptosis for IL-2-expanded cells may differ from that observed shortly following primary T cell activation. Indeed, the expression of CD95 (Fas) and CD25 (IL-2Ra), molecules that have been implicated in AICD (Refaeli et al., 1998; Richter, Mollweide, Hanewinkel, Zobywalski, and Burdach, 2009), was not found to be elevated on the IL-2-expanded cells compared to the IL-7-expanded cells (data not shown). A different mechanism may thus be required to explain the sensitivity of the IL-2-expanded cells to apoptosis. We found that sensitivity to apoptosis was correlated with the duration of IL-2 exposure during primary stimulation, and the associated increase in granzyme B and perforin expression upon restimulation. As CTL cytolytic molecules have been previously found to contribute to AICD (Devadas et al., 2006; Gorak-Stolinska et al., 2001; Mateo et al., 2007), the IL-2-induced expression of cytolytic molecules provides a potential mechanism to explain the sensitivity of IL-2-expanded cells to apoptosis and a self-regulatory mechanism for augmented CTL effector functions. However, this does not exclude potential contributions from previously known mechanisms for IL-2-dependent apoptosis, such as the upregulation of Bim and the engagement of CD95 with CD95L (Brenner et al., 2008). Further mechanistic investigations are needed to establish the precise role of cytotoxic molecules in AICD following prolonged IL-2 exposure. It may be unlikely for T cells in an *in vivo* environment to be exposed to high levels of IL-2 for prolonged periods of time in the absence of antigen. Therefore, the form of AICD described above may potentially be restricted to *in vitro* environments.
Chapter 4: Measuring the effects of third signals during priming on human memory CD8\(^+\) T cells

4.1 Introduction

Following the establishment of an *in vitro* system for human naïve CD8\(^+\) T cells, we started to explore the effects of priming signals on the generation and programming of memory CD8\(^+\) T cells. A collection of *in vivo* and *in vitro* murine studies have provided evidence for the essential requirement of third signals for the activation of naïve CD8\(^+\) T cells. These signals are thought to promote optimal clonal expansion, development of effector functions, and the establishment of functional memory populations (Mescher et al., 2006). However, the specific effects of each cytokine as third signals and their “programming” effects on memory cells are so far inconclusive. Prior studies had focused mainly on IL-12 and IFN-α. However, confounding factors in the complex *in vivo* immune system and numerous variations in the *in vitro* experimental set-ups had made their differential effects difficult to distinguish.

For human CD8\(^+\) T cells, studies on the third signals are very limited. Two groups have investigated the effects of IL-12 and/or IFN-α during human CD8\(^+\) T cell stimulation, and have observed opposing IFN-α effects (Chowdhury et al., 2011; Hervas-Stubbs et al., 2012; Hervas-Stubbs et al., 2010; Ramos et al., 2009). While a number of other reports have studied the effects of IL-21 (Alves et al., 2005; Ansen et al., 2008; Kaka et al., 2009; Li, Bleakley, and Yee, 2005; Singh et al., 2011), these third signal cytokines have not been directly compared. The results of these studies are difficult to interpret due to many factors. These include the variation or combination of γc cytokines (IL-2, IL7 or IL-15) utilised in these experiments (Li et al., 2005; Ramos et al., 2009; Singh et al., 2011), and combinations of more than one priming cytokines (Kaka et al., 2009). Also, phenotypic and functional assessments were mostly done within 7 days following primary stimulation when the cells were still activated (Alves et al., 2005; Chowdhury et al., 2011; Hervas-Stubbs et al., 2010; Ramos et al., 2009), leading to discrepancies in the interpretation of data with proper recall responses often not investigated. Lastly, due to the lack of consensus in T cell biology nomenclature, the molecular definitions of T cell subset descriptions for both the starting populations and the phenotypic assessments of the generated cells varied between the investigations. This is important when the effects of priming cytokines differ between different cell subsets (Hervas-Stubbs et al., 2010).

Utilising the IL-7 model developed in the previous chapter, we were able to stimulate human naïve CD8\(^+\) T cells *in vitro* with strictly controlled signals, since IL-7 provided survival signals with minimal additional effects. Third signals could thus be tested in this controlled system. The stimulated CD8\(^+\) T cells were expanded to form memory populations, confirmed to be rested from activation, maintained *in vitro*, and withstood restimulation for functional recall assessments of stably formed memory cells. Therefore, we investigated the priming effects of a range of cytokines secreted by APCs and CD4\(^+\) T cells during naïve CD8\(^+\) T cell stimulation using the IL-7 *in vitro* model, and assessed their programming effects by
investigating the phenotypic, molecular and functional attributes of the resultant memory cells. Understanding the regulatory programming effects of the different third signal cytokines during CD8$^{+}$ stimulation on T cell differentiation, and memory and function generation will lead to improved immunization strategies and better construction of cells for adoptive immunotherapy.
4.2 Methods

4.2.1 Cells

4.2.1.1 Generation of memory cells, M1, in the presence of various cytokines during priming using the IL-7 model

Naïve CD8\(^+\) T cells were isolated from PBMC and stimulated to expand and generate memory populations, in different priming conditions as described in the Methods section (2.2.2). Briefly, naïve CD8\(^+\) T cells were incubated with IL-7 (5ng/ml) overnight following isolation from PBMC, and stimulated in the presence of the following priming or “programming” cytokines for 3 days: IFN-\(\alpha\), IL-2, IL-4, IL-7, IL-10, IL-12, IL-15, IL-18, IL-21, IL-23 or IL-27 (all at 10ng/ml; as detailed in Table 2-1). On day 3, IL-7 was added to cultures at 20ng/ml, and thereafter, at 5ng/ml, for a total of 21 days. All consequent cultures, restimulations and assays with the memory populations were supplemented with IL-7 (10ng/ml) only unless otherwise stated.

4.2.1.2 Reprogramming of the primed M1 populations

To investigate the plasticity of the programmed M1 populations, M1 cells primed by IL-4, IL-12, IL-21 or no cytokines during the primary stimulation were restimulated in the presence of IL-7 with one of the following: IL-4, IL-12, IL-21 or IL-7 alone (10ng/ml each).

4.2.2 Magnitude of cell expansion

Cells were counted 12 days post-stimulation by the trypan blue exclusion method with a Countess\textsuperscript{TM} Automated Cell Counter as described previously (2.2.1).

4.2.3 Cell surface receptor expression

Cell surface receptor staining was performed as described in the Methods section (2.3.1.1), and the following antibodies were employed: anti-CCR7-FITC (R&D or BD), anti-CD27-AF700 (BD or BL), anti-CD27-APC-Cy7, anti-CD28-PE-Cy5, anti-CD45RA-PE-TR, anti-CD62L-APC (as detailed in Table 2-3), and DAPI for live/dead discrimination (2.3.1.1.1).

Surface receptor expression data for cytokine primed M1 populations were analysed in two ways. First, each priming condition was compared to the no cytokine control using the Mann-Whitney test. Significant results are labelled with asterisks in the figures. The second approach was to investigate whether the receptor expression on each M1 population was above or below the population mean across all conditions. This method does not take individual variations into account, but provides a generalized view of the averaged expression relative to all the other conditions. This was done by compiling all data points for
each marker’s expression, constructing the 95% confidence interval (CI) of the mean (mean ± 2 x SEM) for the entire population, and then determining whether the average expression of each condition lay outside the 95% CI of the mean. This analysis was carried out for the double marker co-expression data.

4.2.4 Functional assays

4.2.4.1 Production of effector molecules

This procedure is described in the Methods chapter (2.2.5). The following antibodies were employed: anti-IFN-γ-AF647, anti-granzyme B-FITC and anti-perforin-APC (as detailed in Table 2-4).

4.2.4.2 Cytokine secretion

M1 cells were plated in flat-bottom 96 well tissue culture plates at 2x10^5/200µl and restimulated with IL-7 (10ng/ml). Supernatants were aspirated at 24 and 48hr after stimulation and examined by CBA Human Soluble Protein Flex Set Assay as described in the Methods (2.2.5.3), using cytokine capture beads specific for the following analytes: GM-CSF, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, MIP-1α and TNF.

Secreted cytokine data was also analysed by two approaches. The data was plotted by fold-changes relative to the donor-matched no cytokine condition. Any absence of cytokine secretions (0pg/ml) could not be plotted on the log scale, but these were included in the calculation of averages across donors. The donor-averaged cytokine secretion from each priming condition that was 2-, 4- or 16-fold more or less than the no cytokine condition was summarised qualitatively. However, to take into account the individual variations in each condition and also the deviation between sample data points, one-way ANOVA and Dunnnett’s post-test were employed to analyse differences for each donor in each secreted cytokine between all priming conditions and the cells primed without cytokines. These results were summarized where the effect of a specific condition on a specific secreted cytokine showed significant differences in 2 or 3 donors.

4.2.5 Transcription factor expression

Expression of transcription factors was investigated mainly by immunocytochemistry (ICC). For instances where flow cytometry was also utilized, the same clones of antibodies were employed.

4.2.5.1 Immunocytochemistry

The methanol-fixation, casein-block with FBS-supplemented antibody dilution buffer (M/C/FBS) protocol as detailed in the Methods (2.4) was employed for this chapter’s work. The following primary antibodies were employed: unconjugated anti-T-bet, anti-GATA-3, anti-Tcf-1, anti-Lef-1 (Table 2-10). Secondary antibodies
The effects of third signals during priming on memory CD8⁺ T cells

were goat anti-mouse IgG1-AF488, goat anti-rabbit IgG1-AF555 (Table 2-11) and DAPI for nuclear staining.

4.2.5.2 Intranuclear flow cytometry

To detect the expression of transcription factors by flow cytometry, dead cells were stained with LIVE/DEAD® Fixable Blue Dead Cell Stain where possible, and cells were fixed, permeabilized and stained as detailed in the Methods section (2.3.1.4). The following antibodies were employed: anti-T-bet-PE, anti-GATA-3-AF647, anti-IgG1-PE, anti-IgG1-AF647 (as detailed in Table 2-7 and Table 2-5).

4.2.5.3 Effect of anti-CD3 and anti-CD28 signalling on T-bet expression

Both naïve and antigen-experienced cells were investigated for T-bet expression under the influence of anti-CD3 and anti-CD28 signalling using in-house labelled beads. Naïve cells were first stimulated with titrated levels of anti-CD3 (0-1µg/ml, Zymed) and 0.1µg/ml of anti-CD28 (BD) both conjugated to CELLection™ beads as previously described (2.2.2.2). Cells were plated at 2x10⁵/200µl of RS5 in flat-bottom 96 well tissue culture plates and the anti-CD3 and anti-CD28 beads each administered at a 1:1 ratio to the cells in the presence of IL-7 (10ng/ml) and cultured for 2 days. 48hr post-stimulation, the beads were removed and the cells were re-plated and further cultured in IL-7 (10ng/ml). On day 4 post naïve stimulation, cells were intracellularly stained for T-bet and mouse IgG1 isotype control (4.2.5.2) and their expression detected by flow cytometry. The control for the naïve stimulation was naïve cells cultured in IL-7 (10ng/ml) only stimulated with 0µg/ml of anti-CD3 beads and 0.1µg/ml of anti-CD28 beads.

To examine the effect of anti-CD3 and anti-CD28 signalling upon restimulation, naïve cells stimulated with 0.01-1µg/ml of anti-CD3 beads were expanded for 14 days before restimulation in the presence of IL-7 (10ng/ml). These antigen-experienced cells were all plated at 3x 10⁵/200µl of RS5 in flat-bottom 96 well plates for restimulation. Cells generated with 1µg/ml of anti-CD3 were first used for restimulation with titrated levels of anti-CD3 on beads and anti-CD28 beads (0.1µg/ml), with cells at rest as control. The same cells were also restimulated with titrated levels of anti-CD28 on beads (0-1µg/ml) with or without anti-CD3 (1µg/ml). Then, M1 cells generated with each concentration of anti-CD3 beads (0.01-1µg/ml) were stimulated with titrated levels of anti-CD3 (0.01-1µg/ml) beads again with 0.1µg/ml of anti-CD28 beads. In all conditions, anti-CD3 and anti-CD28 coated beads were added at 1:1 ratio to the cells. Cells were assessed for T-bet expression 24hr post-restimulation.
Chapter 4

4.3 Results

Utilizing the IL-7 in vitro model, we investigated the programming effects of third signal cytokines on the quality of the in vitro-generated memory “M1” cells by assessing their proliferative capacity, recall effector functions and expression of transcription factors. Naïve CD8$^+$ T cells were stimulated in the presence of various priming cytokines for 72hr, and allowed to expand and rest in culture over a period of 21 days, in the presence of IL-7, to form stable memory populations. The magnitude of primary expansion was inspected at day 12 post-stimulation. The surface phenotypes of the generated M1 populations were then examined at day 21 and the M1 cells were restimulated to assess their effector functions and secreted cytokine profiles (Figure 4–1).

Figure 4–1 Chapter 4 experimental overview

A schematic diagram illustrating the general experimental outline for this chapter and the timing for each set of experiments. This chapter tests the effects of cytokine priming in the first 3 days of naïve cell stimulation using the IL-7 in vitro model, testing the cytokines secreted by APCs and CD4$^+$ T cells. The resultant cells are assessed by 4 sets of experiments (Sections 4.3.1 – 4.3.4). Section 4.3.5 examines the plasticity of programmed cells in response to additional cytokines added upon restimulation. Other specific experiments are described and illustrated in each individual section. D, days post naïve stimulation; TF, transcription factors.
4.3.1 Magnitude of primary expansion

The magnitude of initial T cell proliferation upon stimulation typically determines the size of the memory T cell population formed following the clearance of infection, and it is thus critical for subsequent immunity and protection (Hou, Hyland, Ryan, Portner, and Doherty, 1994; Murali-Krishna et al., 1998). Therefore, we investigated the magnitude of the initial expansion of naïve cells primed with a range of APC- or CD4+ T cell-secreted cytokines in the IL-7 in vitro model. A condition with no additional cytokines added to culture for the first three days of activation was included as a baseline reference control. The total cell numbers were examined at the peak of the primary expansion on day 12 following stimulation. This showed that the naïve cells expanded between 45 and 74-fold on average across the different priming conditions, which equated to at least 6-7 cell divisions (Figure 4–2). Cells primed in the presence of IL-12 appeared to be the most consistent in expanding to larger populations, but no significant differences were found for the magnitude of expansion across all conditions. We then observed that cells from all conditions could be sustained in culture for at least 3-4 weeks.


![Figure 4–2 Three-day cytokine priming does not significantly affect the magnitude of primary expansion](image)

Naïve CD8+ T cells were plated at 2x10^5/200µl and stimulated with a 1:1 ratio of anti-CD3/CD28 beads for 2 days in the presence of indicated cytokines for priming over the first 3 days of stimulation. IL-7 was provided from day 3 onwards to support expansion and survival. Cell numbers were assessed 12 days after stimulation by the trypan blue exclusion method. Each data point represents the average value of biological duplicate from one donor. Bars indicate mean ± SEM over 3 or 4 donors per condition from 2 independent experiments. The Mann-Whitney test was employed for the statistical comparisons.

4.3.2 Phenotypic expression

Next we sought to investigate the expression of differentiation-associated phenotypic markers on the M1 populations programmed by the various cytokine priming conditions. These markers define important memory cell characteristics including migratory properties and costimulatory signalling capabilities, and correlate with proliferative potentials and effector function capabilities. 21 days following naïve stimulation, the M1 populations were confirmed to be rested since they showed stable population size, low or absent CD25 expression and unpolarized cell morphology (data not shown). The expression of the phenotypic
markers was then assessed and the data analysed by comparisons to both the condition primed without cytokines and the population mean (detailed in 4.2.3).

Overall, the M1 populations generated with all priming conditions were found to largely maintain the expression of important phenotypic markers associated with early differentiation status, namely CCR7, CD62L, CD28 and CD27, and only modest differences were found between conditions. CCR7, CD27 and CD45RA were found to be expressed by high percentages of each M1 population with small variations between donors. For CD62L and CD28 expression, more inter-donor variations were observed (Figure 4–3).

There were no significant differences found between each priming condition and the no cytokine control for the expression of CCR7 and CD62L (Figure 4–3). However, the co-expression of these two lymphoid homing molecules was on average higher in cells primed in the presence of IL-4, IL-18 and IL-21 relative to the population mean, and lower in the populations primed in the presence of IL-12 (Figure 4–3, Table 4-1). The IL-4- and IL-21-primed cells also had the highest average expression of CD62L and CCR7, respectively (Figure 4–3). It is interesting to speculate that under the influence of IL-4 and IL-21, cytokines known to regulate B cell maturation and antibody production (Janeway, 2005; Spolski and Leonard, 2008), the need for CTL activities in the periphery reduces. This leads to the retention of the CTLs in the lymphoid organs through the expression of CCR7 and CD62L.

For the expression of costimulatory receptors, IL-4- and IL-12-primed cells appeared to have the highest expression of CD28. However, these results did not reach statistical significance when compared to the condition primed without cytokines (Figure 4–3). There was an overall high expression of CD27 across all conditions as expected, since CD27 expression tends to be lost only at the later stages of CD8+ T cell differentiation (Appay et al., 2002). Intriguingly, cells primed in the presence of IL-12 were found to have a significantly higher expression of CD27 when compared to the population primed without cytokines (Figure 4–3). For the co-expression of both CD27 and CD28, IL-4-, IL-12- and IL-21-primed memory cells showed higher co-expression than the population mean, while IFN-α-, IL-18- and IL-27-primed cells showed lower expression (Figure 4–3, Table 4-1).

CD45RA was found to be expressed, on average, in 80%-94% of the differentially primed M1 populations (Figure 4–3). The co-expression of CD45RA and CCR7 suggests the majority of the Ag-experienced populations had reverted or rested to a naive-like phenotype that is consistent with the recently reported stem cell-like memory (SCM) population (Gattinoni et al., 2011). There were no differences of CD45RA expression found between the differentially primed M1 populations. However, cells primed in the presence of IL-12 were found to have a significantly lower co-expression of CCR7 and CD45RA relative to the condition primed without cytokines (Figure 4–3). IL-21-primed cells had the highest expression of both CCR7 and CD45RA and IL-18-primed cells also had higher co-expression than the population mean (Figure 4–3, Table 4-1).
The effects of third signals during priming on memory CD8⁺ T cells

Figure 4–3 The expression of differentiation markers 21 days post-stimulation following cytokine priming

Naive CD8⁺ T cells were stimulated with a 1:1 ratio of anti-CD3/CD28 beads for 2 days in the presence of indicated cytokines for priming over the first 3 days of stimulation. IL-7 was provided from day 3 onwards to support expansion and survival. After 21 days of expansion, rested memory M1 populations were established and expression of indicated markers of memory differentiation were assessed by flow cytometry. Bars indicate average values of expression percentages across 3-4 donors in 2 independent experiments. Dotted lines are 95% CI of the population mean across all conditions (population mean ± 2x SEM). Significant differences between each cytokine priming condition and the cells primed without cytokines were compared using the Mann-Whitney test.

Figure 4–4 Distribution of differentiation subsets within each cytokine-primed M1 population

M1 populations generated as described in Figure 4–3; expression of CCR7 and CD45RA assessed on day 21 post naïve stimulation. Average percentages of each memory subset between 3-4 donors per condition are plotted.
Memory cell subsets could be specified in the M1 populations based on the heterogeneous phenotypic expression of CCR7 and CD45RA. The cells primed in the presence of IL-12 had the greatest loss of CCR7 and CD45RA expression across the priming conditions and encompassed, on average, 15.5% of cells expressing a CM-like phenotype, 4.4% of cells with an EM-like phenotype and 11.5% of cells with an EMRA-like phenotype in the M1 populations (Figure 4–4). These are the highest percentages of phenotypically CM and EMRA cells across all M1 populations, and indicated a more differentiated status. IL-4 and IFN-α priming also generated larger percentages of CM-like cells than other phenotypes, while IL-23 generated more EMRA-like cells. In contrast, IL-21 priming generated the lowest percentages of phenotypically CM, EM and EMRA cells, and produced the highest proportion of cells with a naïve- or SCM-like phenotype in the M1 populations across all the cytokine priming conditions (Figure 4–4).

In summary, the phenotypic expression of all the M1 populations generated with different priming cytokines were similar, with the majority of each population expressing a SCM-like phenotype without exhaustion and activation markers (data not shown). IL-12 was found to programme a significantly higher expression of CD27 and lower CCR7 and CD45RA co-expression relative to the condition primed without cytokines. In contrast, IL-4- and IL-21-primed M1 populations were found to have higher percentages of cells expressing both homing receptors and costimulatory molecules relative to the population mean, while IL-21 priming also programmed higher CD45RA expression (Figure 4–3, Table 4-1).

### Table 4-1 Expression of early differentiation markers above or below population average across all conditions

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Priming conditions</th>
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<tbody>
<tr>
<td></td>
<td>IFN-α</td>
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<tr>
<td>CCR7+ CD62L+</td>
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</tr>
<tr>
<td>CD28+ CD27+</td>
<td>↓</td>
</tr>
<tr>
<td>CCR7+ CD45RA+</td>
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Summarized data of double positive marker expression from Figure 4–3. Data from all priming conditions were first pooled to calculate the population mean and 95% confidence interval (CI) of the mean (dotted lines on Figure 4–3). Upward arrows (↑) indicate the average percentage of indicated marker expression is above the population 95% CI of the mean; downward arrows (↓) indicate those below the population 95% CI of the mean.

### 4.3.3 Effector functions

The effector functions of CD8+ T cells are critical for their ability to control infections and tumours. Such functions include inducing cytolytic events, and the secretion of cytokines for non-lytic effector activities and recruitment of immune cells (Dorner et al., 2002; Kaech et al., 2002b; Wherry and Ahmed, 2004). We restimulated rested M1 populations to determine the production of lytic molecules and the secretion of...
cytokines following various cytokine programming. By restimulating rested cells, we could avoid AICD, while correlating effector functions to the rested cell surface phenotypes without temporal alterations from cell activation, and simulate effector functions upon recall responses.

### 4.3.3.1 Expression of IFN-γ, granzyme B and perforin

Despite the small differences in cell surface phenotype between the M1 populations constructed under different priming conditions, polarizing differences in the production of IFN-γ were found upon restimulation. Whereas 28% of the M1 population primed without cytokines, on average, expressed IFN-γ following restimulation, IL-12 priming markedly increased this expression to an average of 83%, while IL-21 and IL-4 priming lowered the expression to an average 11% and 10%, respectively (Figure 4–5A). This significant IL-12 effect correlates with its reported inflammatory properties (Joshi et al., 2007). Surprisingly, despite IL-12 and IFN-α having been reported to have similar priming properties in murine models (Curtsinger and Mescher, 2010), IFN-α priming in our experiment produced a negligible effect on IFN-γ production. In contrast, IFN-γ production following IL-21 priming was found to be significantly reduced (Figure 4–5A). While IL-4 priming was also found to lower the expression of IFN-γ, albeit not significantly, both IL-21- and IL-4-primed populations correlated with their higher expression of early differentiation markers (Table 4-1). In the case of IL-4 priming, these data are consistent with type 2 responses to extracellular pathogen infections known to be initiated by IL-4. IFN-γ, however, is not crucial for the immune activity in this case and may inhibit type 2 responses (Apte et al., 2010; Lewkowich and HayGlass, 2002). The expression of IFN-γ for all other M1 populations was not significantly different to the condition primed without cytokines.

Although no statistically significant differences in granzyme B expression were found between each cytokine-primed M1 population and the cells primed without cytokines, IL-12 priming programmed the most consistent and highest expression of granzyme B (90%) in the M1 populations following restimulation (Figure 4–5B). This coincided with the ability of IL-12 to enhance IFN-γ production and is consistent with the more differentiated phenotype of M1 populations primed in the presence of IL-12 (Table 4-1). IL-18 and IL-27 priming, however, appeared to decrease the expression of granzyme B (65% and 47%, respectively) (Figure 4–5B), but did not correlate with other cellular attributes. For perforin expression, there were wide donor variations, and no statistically significant differences were found between priming conditions. However, the trend for perforin expression across the differentially primed M1 populations was found to roughly correlate with their granzyme B expression. Thus IL-12 priming programmed memory cells with the highest perforin expression, while IL-18 and IL-27 priming generated those with the lowest expression (Figure 4–5C). However, for each priming condition the perforin-expressing proportion of the M1 population was generally much lower than the proportion which expressed granzyme B (Figure 4–5B, C). Lastly, no expression of IFN-γ, granzyme B or perforin was found in the unstimulated controls. This indicates a rested status of the M1 populations with no persistent expression of lytic molecules, and thus suggests an absence of immediate cytolytic functions (Figure 4–5D-F).
Figure 4–5 The effect of 3 day cytokine priming on IFN-γ, granzyme B and perforin production upon restimulation

M1 cells were generated with the indicated priming cytokines as described in Figure 4–3. Biological duplicates of the M1 populations were plated at equal densities and either restimulated or not stimulated as controls. A. Percentage of cells positive for IFN-γ 6hr after restimulation. B, C. Percentage of cells positive for granzyme B (B) and perforin (C) 3 days post-restimulation. Each data point represents the average value from biological duplicate for one donor. Bars indicate mean ± SEM across 3-4 donors from 2 independent experiments. Each cytokine priming condition was compared to cells primed without cytokines using the Mann-Whitney test. D. The unstimulated controls from the IFN-γ assays described above (A). A representative of a technical repeat from one donor for each assay is shown. Normal black line, unstained control; filled grey area, isotype control; heavy black line, IFN-γ staining. E-F. Next page.
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Figure 4–5 The effect of 3 day cytokine priming on IFN-\(\gamma\), granzyme B and perforin production upon restimulation cont.

E-F. The unstimulated controls from the GzmB (E), Perforin (F) assays described above (B and C, respectively). A representative of a technical repeat from one donor for each assay is shown. Normal black line, unstained control; filled grey area, isotype control; heavy black line, GzmB, Perf staining.

4.3.3.2 Secreted cytokines

In the investigation of M1 population cytokine secretion profiles, we found that TCR and CD28 signals alone during primary stimulation were able to direct the secretion of a wide range of non-cytolytic effector cytokines and chemokines on restimulation. (Figure 4–6A). This range included CTL effector cytokines (IFN-\(\gamma\), TNF), the classical type 1 (IFN-\(\gamma\)) and type 2 (IL-4, IL-5, IL-13) cytokines (Löhning et al., 2002; Mosmann and Coffman, 1989), inflammatory cytokines (IL-6, TNF) (Akira, Hirano, Taga, and Kishimoto, 1990), chemokines that have been categorised with type 1 reactions (MIP-1\(\alpha\)) (Dorner et al., 2002), and regulatory or anti-inflammatory cytokines (IL-10) (Couper, Blount, and Riley, 2008). This observation suggested TCR-dependent, third signal-independent cytokine production, and a Tc0 (cytotoxic T cell type 0)-like phenotype (cells thought to be uncommitted to a specific set of cellular attributes characterised for immune reactions against different types of pathogens), marking these M1 cells polyfunctional as a
population. In vitro-generated CD8\(^+\) M1 cells were potent secretors of IFN-\(\gamma\), MIP-1\(\alpha\) and GM-CSF, with each of these cytokines present in a concentration greater than 1ng/ml in the culture supernatant.

To investigate the global shift in cytokine secretion profile caused by the priming conditions, we calculated the fold changes of the secreted cytokines for each priming condition relative to the baseline profile from the cells primed without cytokines (Figure 4–6B-H). Statistical comparisons between the priming conditions for each secreted cytokine were carried out as described in 4.2.4.2 and are summarised in Table 4-2. Overall, the secretions of IFN-\(\gamma\), TNF, IL-5 and IL-10 showed the greatest sensitivity to cytokine priming. Combining the results from the two methodologies of data comparison showed that these four secreted cytokines had either a donor-average of more than a 2-fold change or a statistically significant difference for 2 to 3 individuals in 4 or more priming conditions (Table 4-2).

IL-4-, IL-12- and IL-21-primed M1 populations showed the greatest change in the secreted cytokine profile upon secondary stimulation. IL-4 priming significantly induced the range of type 2 related cytokines, IL-4, IL-5, IL-13 and GM-CSF, with some evidence of moderate upregulations of IL-2, TNF, IL-10, and significant downregulation of IFN-\(\gamma\) (Figure 4–6C, Table 4-2). The downregulation of secreted IFN-\(\gamma\) at 48hr concurred with intracellular staining data for IFN-\(\gamma\) production 6hr post-restimulation (Figure 4–5A), indicating a continuation of reduced IFN-\(\gamma\) expression. IL-12 priming enhanced the secretion of all type 1 cytokines, IL-2, IFN-\(\gamma\), TNF, as well as MIP-1\(\alpha\) and IL-10, without significant changes to type 2 cytokines. Cells primed in the presence of IL-12 also induced the highest fold change in the secretion of IL-2, IFN-\(\gamma\) and MIP-1\(\alpha\) across all priming conditions (Figure 4–6D, Table 4-2). For IL-21 priming, quantitative changes in cytokine secretion were less than those found for IL-4 and IL-12 priming. However, there was a more global shift in the overall profile for the number of cytokines affected, with some increases observed for type 1 cytokines IL-2, TNF, and MIP-1\(\alpha\) accompanied by moderate downregulation of type 2 cytokines IL-4 and IL-5. IL-21 priming was also found to programme significantly reduced secretion of IL-6, as well as dramatic upregulation of IL-10 (Figure 4–6F, Table 4-2). Unexpectedly, IFN-\(\gamma\) secretion was found to be similar to the cells primed without cytokines at 48hr post-stimulation (Figure 4–6F). This indicates delayed production of IFN-\(\gamma\) following the reduced expression found at 6hr post-stimulation (Figure 4–5A).

Cells primed in the presence of IFN-\(\alpha\) showed a profile of marginally enhanced cytokine secretions for both type 1 and type 2 responses, including IFN-\(\gamma\), TNF, IL-5, IL-13 and IL-10, albeit not significantly (Figure 4–6B, Table 4-2). In contrast, IL-23, which shares the IL-12p40 chain with IL-12, modestly enhanced type 1 cytokines IFN-\(\gamma\), TNF and MIP-1\(\alpha\), without altering type 2 cytokines. IL-23 also induced IL-10 secretion, leading to a similar, albeit dampened, secretion profile that resulted from IL-12 priming (Figure 4–6G, Table 4-2). IL-18 and IL-27 priming caused minimal alterations to the cytokine secretion profile relative to cells primed without cytokines (Figure 4–6E, H, Table 4-2). This appears to coincide with an overall reduced activity of IL-18- and IL-27-primed populations, which also show diminished expression of lytic molecules (Figure 4–5B, C).
The effects of third signals during priming on memory CD8^+ T cells

Figure 4–6 Modulation and profile of secreted cytokines by cytokine priming
M1 cells were generated with the indicated priming cytokines as described in Figure 4–3, and restimulated with a 1:1 ratio of anti-CD3/CD28 beads in the presence of IL-7. Supernatants were aspirated from cultures at 24 and 48hr post-restimulation. The analytes indicated were detected using the BD CBA flex set assay. IL-2, IL-6 and IL-10 secretion were examined at 24hr; the rest of the analytes were examined at 48hr. The absolute concentrations of the secreted cytokines are shown for the M1 cells primed without cytokines during naïve stimulation (A); this is the baseline cytokine secretion profile. Fold changes against donor-matched baseline cytokine secretion were plotted for each cytokine priming condition (B-H). Each data point is the average value from the M1 biological duplicate per donor, with a total of 3 donors from 2 independent experiments. Bars depict mean values across the 3 donors. Zero cytokine secretions or those below detection point were not plotted, but included in the calculation for average values.
In summary, all M1 populations generated with various priming conditions adopted Tc0 phenotypes and were able to secrete pleiotropic cytokines. However, 3-day cytokine priming at the initiation of naïve CD8+ T cell activation programmed and skewed the cytokine secretion profiles with strong trends. IL-12, IL-4 and IL-21 priming induced the most prominent alterations in the global profiles, as well as statistically significant changes in the secretion of specific cytokines. The statistically significant changes for IL-12 priming included the upregulation of IL-2, IFN-γ, IL-6 and IL-10 secretion, while IL-4 priming augmented the secretions of GM-CSF, IL-4, IL5, IL-13 and downregulated IFN-γ secretion. IL-21 priming diminished IL-6 secretion and enhanced IL-10 secretion (Table 4-2, hollow arrows).

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<thead>
<tr>
<th>Secreted cytokines</th>
<th>Priming cytokines</th>
<th>IFN-α</th>
<th>IL-4</th>
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Summarized data from Figure 4–6. Single small arrows (↑) indicate > 2-fold change of cytokine secretion compared to baseline secretion; single solid arrows (é) indicate > 4-fold change; double solid arrows (éé) indicate > 16-fold change. Hollow arrows (ñ) indicate > 2 donors out of 3 showed statistically significant changes in cytokine secretion compared to baseline secretion per donor in one-way ANOVA analysis with Dunnett’s multiple comparison post-test.
4.3.4 Expression of transcription factors by cells primed in the presence of IL-4 or IL-12

The cytokine secretion profile of the IL-4- and IL-12-primed CD8+ T cells showed similar characteristics to CD4+ T cells polarized for type 2 (Th2) or type 1 (Th1) reactions, respectively, where master transcription factors GATA-3 and T-bet are known to direct the polarized responses (Murphy and Reiner, 2002). Therefore, we sought to investigate the expression of transcription factors in the M1 cells primed in the presence of IL-4 or IL-12 to see whether the skewed responses in CD8+ T cells are directed by the same master regulators. At the same time, we also assessed the expression of transcription factors Tcf-1 and Lef-1, which are associated with early differentiation (Rutishauser and Kaech, 2010). These factors are known to assist in the generation of functional CD8+ memory cells (Zhou and Xue, 2012). Following the generation of M1 populations by IL-4 and IL-12 priming, cells were rested and then restimulated in the presence of IL-7 only. The expression of transcription factors was then examined by immunocytochemistry (ICC) and flow cytometry. Only 2-dimensional fluorescence images are shown here, but co-staining of transcription factor with DAPI allowed confocal microscopy confirmation of nuclear localization (Figure 2–9).

4.3.4.1 T-bet

In rested M1 populations, no T-bet expression was detected by ICC (Figure 4–7A) and there was also minimal staining detected by flow cytometry (Figure 4–7B). Upon TCR stimulation, T-bet expression in both IL-4- or IL-12-primed M1 cells was found to peak at 24hr post-restimulation in almost all the cells in the population, suggesting an induction of T-bet expression by TCR signalling. During the following days, T-bet staining gradually reduced in both cell population fraction and staining intensity per cell. By day 4 post-restimulation, T-bet expression was almost completely ablated in both M1 populations. All expression was found in the nuclei, with consistent results by flow cytometric detection (Figure 4–7A, B). Comparison between the M1 populations primed in the presence of IL-4 and IL-12 showed negligible T-bet differences despite differing cytokine secretion profiles. To confirm this lack of cytokine priming effect for T-bet expression, M1 cells primed without cytokines were also investigated by flow cytometry for T-bet expression. They were found to follow the same pattern of expression kinetics with comparable T-bet quantities (Figure 4–7B). This generic expression of T-bet may contribute to the global secretion of CTL effector molecules across all priming conditions tested. Other regulatory mechanisms must therefore be responsible for enhanced type 1 responses in the M1 cells primed in the presence of IL-12.
Chapter 4

Figure 4–7 Expression of T-bet in M1 populations primed in the presence of IL-4 or IL-12 upon restimulation

M1 cells generated with IL-4 and IL-12 priming were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time point, cells were all extracted at the same time and stained for T-bet by both immunocytochemistry (A) and flow cytometry (B) in separate experiments. A. Cells were co-stained with DAPI (pseudocoloured grey) and T-bet (green). Left panels show overlays of DAPI and T-bet; right panels show T-bet only. Objective magnification x100. B. Fold difference of median fluorescence intensity (MFI) between T-bet and isotype control. Control cells primed without cytokines were also included for flow cytometry staining. Data are representative of 2 donors.
4.3.4.1.1 Expression of T-bet is dependent on anti-CD3 signal strength and independent of anti-CD28 signals

To confirm the induction of T-bet by TCR stimulation and investigate the contribution of anti-CD3 and anti-CD28 signals to T-bet expression, we used in-house labelled beads with titrated levels of anti-CD3 and anti-CD28 antibodies to stimulate either naïve T cells or antigen-experienced cells in four experimental setups. In each experiment, the T-bet expression was examined by flow cytometry (Figure 4–8A-D, and detailed in 4.2.5.3).

**Figure 4–8 Section 4.3.4.1.1 experimental outlines**

A schematic diagram illustrating the experimental set-up to investigate the induction of T-bet expression by CD3- and CD28-mediated signalling on naïve and antigen-experienced cells. **A-C.** Experimental outlines for the results in figures A-C, respectively, in Figure 4–9. **D.** days post-stimulation; **Ag.** antigen. **D.** Next page.
Figure 4–8 Section 4.3.4.1.1 experimental outlines cont.
A schematic diagram illustrating the experimental setup for the results in figures D-F, respectively, in Figure 4–9. D, days post-stimulation; Ag, antigen.

Stimulation of naïve CD8⁺ T cells with titrated levels of anti-CD3 beads and a uniform concentration of anti-CD28 beads (Figure 4–8A) resulted in a gradual increase of T-bet expression with elevated levels of anti-CD3 antibody concentration, suggesting an association between TCR signalling and T-bet expression upon primary stimulation (Figure 4–9A). The absence of differential T-bet expression between resting cells and cells stimulated with anti-CD28 signals only (0µg/ml anti-CD3) indicated that T-bet expression is either independent of CD28 signalling or requires anti-CD3 co-signalling. Next, we tested to see whether the same phenomenon applied to antigen-experienced cells (Figure 4–8B), and indeed, a similar pattern of graded T-bet expression was also observed upon restimulation of cells with titrated anti-CD3 beads (Figure 4–9B). The declining percentages of T-bet⁺ cells were also correlated with a diminishing MFI of the T-bet⁺ population (data not shown). This suggests the reduction of the T-bet⁺ population percentages was not merely a consequence of an increasing proportion of cells not receiving TCR stimulation due to the lowered anti-CD3 concentration on beads. Rather, it was a genuine reduction of T-bet expression on a per cell basis. We then tested T-bet expression when antigen-experienced cells were stimulated with titrated anti-CD28 beads, either with or without anti-CD3 beads at a constant concentration (Figure 4–8C). This showed that anti-CD28 beads do not alter T-bet expression (Figure 4–9C). Together, these results indicated that T-bet expression is correlated to CD3- but not CD28-mediated signalling in both naïve and antigen-experienced cells. There was some expression of T-bet observed in the unstimulated antigen-experienced cells (Figure 4–9B, C), in contrast to the previous data (Figure 4–7).
The effects of third signals during priming on memory CD8+ T cells

Naive CD8+ T cells were stimulated with titrated levels of anti-CD3 (0-1µg/ml) on beads at a 1:1 ratio, together with a 1:1 ratio of anti-CD28 beads (0.1µg/ml) for 2 days in IL-7, whereas control cells were rested in IL-7 for 14 days. A. T-bet and isotype control were stained 4 days post naive cell stimulation. B, C. Antigen-experienced cells generated in (A) with 1µg/ml of anti-CD3 were restimulated in IL-7 with (B) titrated levels of anti-CD3 on beads and anti-CD28 beads (0.1µg/ml) both at 1:1 ratios or (C) titrated levels of anti-CD28 on beads with or without anti-CD3 (1µg/ml). T-bet was stained 24hr post-restimulation. D-F. Cells stimulated with anti-CD3 (0.01-1µg/ml) during primary stimulation were restimulated again with titrated levels of anti-CD3 (0.01-1µg/ml) on beads with anti-CD28 beads (0.1µg/ml) in IL-7 for 24hr, and assessed for cell size and granularity by FSC (D) and SSC (E), as well as T-bet expression (F). MFI, median fluorescence intensity; Unstim., unstimulated control; FSC, forward scatter; SSC, side scatter.

Figure 4–9 Expression of T-bet modulated by CD3-mediated signalling
This was possibly due to the early restimulation time point (day 14 post-primary stimulation rather than day 21) for the M1 cells in Figure 4–7. The observation suggests the cells were not fully rested at 14 days post-primary stimulation and maintained T-bet expression at this time point.

It was then of interest to determine whether antigen-experienced cells have T-bet expression memory of their primary antigen stimulus upon secondary stimulation. Naïve cells stimulated in the same anti-CD3 titrations as those in Figure 4–9A were expanded for 14 days, and cells from each condition were restimulated again with titrated levels of anti-CD3 plus a uniform concentration of anti-CD28 beads (Figure 4–8D). Cell size and granularity (cell density) were found to be largely dependent on the anti-CD3 concentration from the secondary stimulations, and independent of the primary stimulations as indicated by the FSC and SSC profiles (Figure 4–9D, E). This finding is consistent with studies showing that enhanced metabolic activities are necessary for T cell activation (Pearce, 2010; Rathmell, Elstrom, Cinalli, and Thompson, 2003). However, there was a sharp change in the FSC profile for cells initially stimulated by anti-CD3 concentrations of 0.1µg/ml and 0.03µg/ml, and then restimulated with 0.1µg/ml of anti-CD3. This may indicate the presence of some form of threshold for the accumulation of TCR signals. Assessment of T-bet expression showed that antigen-experienced CD8+ cells did retain some form of TCR stimulation memory from their primary activation. Although the highest anti-CD3 concentration during the secondary stimulation induced high expression of T-bet across all cell populations, at each anti-CD3 concentration lower than 1µg/ml during secondary challenge, we found a graded expression of T-bet which correlated with the concentrations of anti-CD3 during both the primary and secondary stimulations (Figure 4–9F).

### 4.3.4.2 GATA-3

ICC staining showed no expression of GATA-3 in M1 populations at rest – a similar result to that seen for T-bet. In the IL-4-primed population, GATA-3 expression was upregulated in a subset of cells upon secondary stimulation. The expression peaked at 24hr post-restimulation, but slightly declined in intensity at 48hr with approximately the same proportion of expression. Expression then reduced to an almost indistinguishable level over the following 2 days. All staining was located in the nuclei (Figure 4–10A). This experiment shows that the IL-4-primed population is heterogeneous despite the gross phenotypic homogeneity suggested by differentiation markers (Figure 4–3). It also suggests that only a subset of cells may be responsible for the dramatically enhanced secretion of IL-4, IL-5 and IL-13, as well as the reduced IFN-γ secretion (Figure 4–6, Table 4-2). No GATA-3 expression can be observed by ICC in the IL-12-primed cells (Figure 4–10A). Detection of GATA-3 by flow cytometry, however, showed minimal levels of expression in both cells primed in the presence of IL-12 and no cytokines (Figure 4–10B). This basal level of expression was below the detection limit for ICC (at a similar threshold as that observed for T-bet, Figure 4–7, ≈5 fold change in MFI). This result is consistent with murine CD4+ T cell studies of GATA-3 expression in Th1 and neutral conditions (Zhu, Cote-Sierra, Guo, and Paul, 2003), and may explain the global, albeit low, type 2 cytokine secretions. In IL-4-primed cells, there was some enhancement of GATA-3 expression detected by flow cytometry over the 4 days following restimulation (Figure 4–10B).
The effects of third signals during priming on memory CD8+ T cells

Figure 4–10 Expression of GATA-3 in M1 cells primed in the presence of IL-4 or IL-12 upon restimulation

M1 cells generated with IL-4 and IL-12 priming were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time point, cells were all extracted at the same time and stained for GATA-3 by both immunocytochemistry (A) and flow cytometry (B) in separate experiments. A. Cells were co-stained for nucleus with DAPI (pseudocoloured grey) and GATA-3 (green). Left panels show overlays of DAPI and GATA-3; right panels show GATA-3 only. Objective magnification x100. B. Fold difference of median fluorescence intensity (MFI) between GATA-3 and isotype control. Control cells primed without cytokines were also included for flow cytometry staining. Data are representative of 2 donors.
However, changes in MFI were not substantial. Nevertheless, the difference in GATA-3 expression between cells primed in the presence of IL-4 and IL-12 by ICC staining is distinctive. This observation suggests an IL-4 priming programming effect in naïve CD8+ T cells so that they “remember” and induce GATA-3 expression on secondary stimulation.

It was intriguing to find that the most M1 populations primed in the presence of IL-4 or IL-12 express T-bet, while the M1 population primed in the presence of IL-4 specifically upregulate GATA-3 expression upon secondary stimulation. Other researchers have found that T-bet and GATA-3 are inversely regulated in CD4+ T cells under Th1 and Th2 conditions (Zhu and Paul, 2010), and T-bet has been found to inhibit GATA-3 in CD4+ T cells (Usui et al., 2006). We therefore examined the flow cytometry data shown in Figure 4–7B and Figure 4–10B to check whether the expression of GATA-3 found by ICC in the IL-4-primed M1 population was co-expressed with T-bet in the same cells. We found that GATA-3 and T-bet were indeed co-expressed for the majority of cells in all three M1 populations investigated, with the co-expression being more prominent, with enhanced GATA-3 expression, in the IL-4-primed population (Figure 4–11). This suggests that the transcription factor regulatory network differs in CD4+ and CD8+ T cells.

![Figure 4–11 Co-expression of GATA-3 and T-bet](image)

M1 cells generated by IL-4, IL-12 priming and no cytokine exposure were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time point, cells were all extracted at the same time and stained for GATA-3 and T-bet by flow cytometry. Co-expression of GATA-3 and T-bet are shown here from data in Figure 4–7B and Figure 4–10B. Gates are drawn from the isotype controls at each time point. Data are representative of 2 donors.
4.3.4.3 Tcf-1 and Lef-1

Both Tcf-1 and Lef-1 were found to be expressed in resting M1 populations. Expression reduced upon TCR stimulation over the first 48hr and was restored during day 3 and 4 post-restimulation. All expression was located in the nuclei (Figure 4–12, Figure 4–13). The kinetics of expression were similar between the IL-4- and IL-12-primed cells. However, the overall intensity of staining appeared to be dimmer for those

Figure 4–12 Expression of Tcf-1 in M1 populations primed in the presence of IL-4 or IL-12 upon restimulation
M1 cells generated with IL-4 and IL-12 priming were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time points cells were all extracted at the same time and stained for Tcf-1 (green) and nucleus with DAPI (pseudocoloured grey) by immunocytochemistry. Left panels show overlays of DAPI and Tcf-1; right panels show Tcf-1 only. Objective magnification x100. Data are representative of 2 donors.
primed in IL-12 relative to IL-4 (Figure 4–12, Figure 4–13). This suggests an overall early differentiation status for our generated M1 populations, and a correlation of Tcf-1 and Lef-1 expression with phenotypic differentiation expression, where cells primed in the presence of IL-12 appeared to be more differentiated with lower CCR7 expression (Figure 4–3, Figure 4–4).

**Figure 4–13 Expression of Lef-1 in M1 populations primed in the presence of IL-4 or IL-12 upon restimulation**

M1 cells generated with IL-4 and IL-12 priming were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time points cells were all extracted at the same time and stained for Lef-1 (red) and nucleus with DAPI (pseudocoloured grey) by immunocytochemistry. Left panels show overlays of DAPI and Lef-1; right panels show Lef-1 only. Objective magnification x100. Data are representative of 2 donors.
The similarity in expression patterns between Tcf-1 and Lef-1 prompted us to investigate the co-expression pattern of the two T cell factor/lymphoid enhancer binding factor family transcription factors. Double staining revealed concurrent expression of Tcf-1 and Lef-1, especially at rest and on day 4 post-restimulation. On day 3 post-restimulation, the expression pattern was more heterogeneous and appeared to be in the restoration process following downregulation by TCR stimulation (Figure 4–14).

**Figure 4–14 Co-expression of Tcf-1 and Lef-1**

M1 cells were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time points cells were all extracted at the same time and stained for Lef-1 (red), Tcf-1 (green) and nucleus with DAPI (pseudocoloured grey) by immunocytochemistry. Left panel shows overlay, middle panel shows Lef-1 only and right panel shows Tcf-1 only. Objective magnification x100. Data shown is from IL-4-primed cells.
Further co-stainings of Lef-1 with T-bet (Figure 4–15) and GATA-3 (Figure 4–16) showed a general trend of inverse expression kinetics between Lef-1 and the effector function transcription factors. The segregation of expression is more distinct between T-bet and Lef-1, with only a few cells showing co-expression. However, there appears to be no inverse correlation between the staining intensities of Lef-1 and T-bet at the single cell level (Figure 4–15).

**Figure 4–15 Inverse correlation of kinetics of expression between Lef-1 and T-bet**

M1 cells primed in the presence of IL-12 were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time points cells were all extracted at the same time and stained for Lef-1 (red), T-bet (green) and nucleus with DAPI (pseudocoloured grey) by immunocytochemistry. Left panel shows overlay, middle panel shows Lef-1 only and right panel shows T-bet only. Objective magnification x100.
Although GATA-3 is also induced upon anti-CD3/CD28 bead stimulation, and has an overall inverse relationship with Lef-1 for the kinetics of expression following restimulation, a higher proportion of cells co-stained with faint intensities for GATA-3 and Lef-1 (Figure 4–16), relative to T-bet and Lef-1.

**Figure 4–16 Inverse correlation of kinetics of expression between Lef-1 and GATA-3**

M1 cells primed in the presence of IL-4 were restimulated in IL-7. Multiple sets of cells were restimulated consecutively every 24hr over 4 days. At each indicated time points cells were all extracted at the same time and stained for Lef-1 (red), GATA-3 (green) and nucleus with DAPI (pseudocoloured grey) by immunocytochemistry. Left panel shows overlay, middle panel shows Lef-1 only and right panel shows GATA-3 only. Objective magnification x100.
4.3.5 Plasticity of programmed cells

Following the successful programming of human CD8⁺ T cells, we sought to investigate whether the programmed M1 populations have plasticity potential with regard to their cytokine secretion profiles. M1 populations generated with IL-4, IL-12 and IL-21 priming, or no cytokines, during naïve stimulation were utilised for this experiment since they have the most distinctive cytokine secretion profiles. Each of these four M1 populations was restimulated in IL-7 alone, or in IL-7 together with IL-4, IL-12 or IL-21. Culture supernatants were then examined for secreted cytokines at either 24hr or 48hr post-secondary stimulation. Data are presented as fold change of each secondary reprogramming condition against the control where the cells were restimulated in IL-7 only.

Overall, we found that programmed M1 cells were able to be reprogrammed by cytokine exposure upon secondary stimulation, with regard to their cytokine secretion profile. An interaction between the effects of cytokine programming from the primary and secondary challenges was apparent for most secreted cytokines. These included IL-2, IFN-γ, IL-6, IL-5, IL-13 and IL-10 (Figure 4–17). This suggests that memory CD8⁺ T cells are able to adjust imprinted functional attributes according to the signals received during antigen re-encounter, while still retaining third signal effects acquired during previous stimulations. The secretion of IL-2 and IFN-γ provided good examples of the cytokine programming interaction between the primary and secondary stimulations. Although secretion of both these cytokines was enhanced by IL-12 priming during primary TCR stimulation, the effect of IL-12 during restimulation was dependent on naïve cell priming conditions. IL-12 reprogramming increased the secretion of IL-2 and IFN-γ for IL-4- or IL-21-primed M1 populations. The effect was similar to that observed during naïve cell priming. However, IL-12 re-exposure of IL-12-primed cells maintained or decreased the secretion of IL-2 and IFN-γ, respectively, while negligible IL-12 effects were found in the M1 population primed without cytokines (Figure 4–17, Table 4-2).

Some secreted cytokines appeared to be more dependent on either the priming conditions during the primary stimulation or the reprogramming conditions upon the secondary stimulation. We have found the change in secretion of TNF, MIP-1α and GM-CSF upon restimulation to be similar across each reprogramming condition regardless of the primary priming condition. This indicates a more important role for cytokine exposure during secondary stimulation for the secretion of these cytokines. In particular, IL-12 exposure during restimulation enhanced the secretion of all 3 of these cytokines, while IL-4 exposure uniformly downregulated the secretion of MIP-1α (Figure 4–17). In contrast, although IL-4 secretion cannot be measured for cultures reprogrammed in IL-4, reprogramming with either IL-12 or IL-21 altered IL-4 secretion upon secondary restimulation in a similar fashion to that seen for the primary priming conditions (Figure 4–17).
The effects of third signals during priming on memory CD8+ T cells

Figure 4–17 Plasticity of cytokine secretion capabilities after differential cytokine exposures during primary and secondary stimulations

M1 cells were generated with the indicated primary priming cytokines as described in Figure 4–3, and restimulated in the presence of IL-7 alone or IL-7 together with IL-4, IL-12 or IL-21. Supernatants were aspirated from cultures at 24 and 48hr post-restimulation and the analytes indicated were detected using the BD CBA flex set assay. IL-2, IL-4, IL-6 and IL-10 secretion were examined at 24hr, and the rest of the analytes were examined at 48hr. Fold changes of each reprogrammed condition against no reprogramming (IL-7 only condition) during secondary stimulation were plotted and grouped for each primary priming condition. Error bars are SEM of duplicate data. Dotted lines indicate 2-fold change. Zero cytokine secretions or those below the detection limit were not plotted but were included in calculations of mean and SEM values. n/a, not applicable; <DL, secretion from both duplicate were less than detection limit.
As described above, the effects of cytokine reprogramming upon secondary stimulation were not necessarily the same as those resulting from naïve cell priming. However, overall outcome for many secreted cytokines was partially dependent on cytokine priming conditions during both stimulations. Using the most significant cytokine secretion results from naïve cell priming (4.3.3.2), we summarised statistically significant results from individual donors (hollow arrows in Table 4-2) which had at least a 2-fold change in donor-averaged data relative to the control primed without cytokines (solid arrows in Table 4-2). We then examined the changes in these secreted cytokines during secondary stimulation by the same programming cytokines (Table 4-3). We found IL-4 exposure during secondary stimulation to further enhance the secretion of IL-5, paralleling its effect during naïve cell priming. IL-12 programming was found to augment secretion of MIP-1α and IL-10, and IL-10 secretion was augmented by IL-21 programming (Table 4-3). This indicates the potential of immune response enhancement by using identical third signals for both primary and secondary stimulations (e.g. IL-5 and MIP-α secretion). A continual increase in IL-10 secretion resulting from either IL-12 or IL-21 programming may self-regulate overt responses. For the secretion of GM-CSF and IL-13 induced by IL-4 priming, and the secretion of IL-6 diminished by IL-21 priming, changes in secretion amounts were negligible upon secondary stimulation (Table 4-3). This implies that the response was not increased by further cytokine exposure.

This capability of the M1 populations to alter the skewed cytokine secretion profiles upon secondary TCR stimulation suggests their ability to appropriately adjust immune response outcomes according to both the third signals received during primary stimulation as well as the contexts of the newly encountered antigen.

**Table 4-3 Comparison of cytokine secretion modulation by reprogramming cytokines against significant changes during primary stimulation**

<table>
<thead>
<tr>
<th>Priming / Reprogramming cytokines</th>
<th>Priming cytokines</th>
<th>Reprogramming cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secreted cytokines</strong></td>
<td><strong>Primary</strong></td>
<td><strong>Secondary</strong></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>IL-4</td>
<td>↑</td>
<td>n/a</td>
</tr>
<tr>
<td>IL-5</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>IL-13</td>
<td>↑</td>
<td>-</td>
</tr>
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</table>

Significant modulation of cytokine secretions during primary stimulation, defined by statistically significant changes (hollow arrows in Table 4-2) together with a minimal of 2-fold change in donor-averaged data (solid arrows in Table 4-2), are listed in the “Primary” column and compared against cytokine secretion modulations during secondary stimulation. Arrows in the “Secondary” column indicate a minimal of 2-fold change by reprogramming cytokine compared against no reprogramming condition across all 4 primary priming conditions. “-” indicates the changes by the reprogramming cytokine are less than 2-fold change. n/a, not applicable; P.D., reprogramming modulation is primary priming condition dependent.
4.4 Summary and discussion

Utilising the IL-7 in vitro model, we were able to investigate specific programming effects of cytokine exposure during naïve CD8+ T cell stimulation on the quality of the resultant memory cells. We observed distinctively programmed cytokine secretion profiles and transcription factor expression in the M1 populations produced by three-day cytokine exposure which will be summarized here and discussed more in depth in the General discussion chapter.

Following the investigation of a range of cytokines as third signals, we found the most prominent effects following exposure to IL-12, IL-4 or IL-21. In these cases, three days of cytokine priming were able to skew the functional profiles of the memory cells 21 days following stimulation. Priming naïve CD8+ T cells in the presence of IL-12 skewed cytokine secretion towards a type 1 profile, with enhanced secretion of IL-2, IFN-γ and MIP-1α being observed. IL-12 exposure also induced elevated levels of granzyme B expression. IL-4 priming was found to induce the secretion of type 2 cytokines including IL-4, IL-5, IL-13 and GM-CSF, while IL-21 priming reduced the secretion of IL-6, delayed the production of IFN-γ and enhanced the secretion of IL-10.

Alterations in phenotypic expression of important differentiation markers on the rested memory cells were very modest following three-day cytokine exposure. High percentages of each cytokine-primed M1 population expressed CCR7, CD62L, CD28, CD27 and CD45RA and thus possessed a phenotype similar to the SCM population. On closer examination, we observed that IL-12 exposure moderately lowered CCR7 and CD45RA expression and slightly enhanced CD27 expression, whereas IL-4 and IL-21 exposure generated M1 populations with moderately elevated expression levels of CCR7, CD62L as well as CD28 and CD27. The alterations in the expression of these markers may affect the capability of these cells to traverse lymphoid organs and circulate through peripheral tissues (Bromley et al., 2005; Kansas, 1996; Schuster et al., 2009). There may also be effects on their ability to provide costimulatory signals for survival and cell cycle progression upon the next stimulation (Appleman et al., 2002) via IL-2 production, Bcl-xL expression (Boise et al., 1995) and glucose uptake and cellular metabolism (Frauwirth et al., 2002).

The investigation of transcription factor expression found that rested M1 populations did not have constitutive prominent expression of effector master transcription factors T-bet or GATA-3. Both IL-12- and IL-4-primed M1 populations, as well as M1 cells primed without cytokines, were observed to express T-bet at similar levels upon secondary stimulation, and the expression of T-bet appeared to be dependent on the strengths of TCR signals. The populations primed in the presence of IL-4 were found to specifically upregulate expression of GATA-3 upon activation. GATA-3 was co-expressed with T-bet, providing evidence for the programming of human CD8+ T cells through brief exposure to IL-4 during naïve cell activation. Both T-bet and GATA-3 expression were found to gradually diminish 3 or 4 days following restimulation. In contrast, Tcf-1 and Lef-1 were found to be expressed in rested M1 populations. Expression was reduced upon secondary stimulation with re-expression observed by day 3 or day 4 post-
restimulation. At the same time, Tcf-1 and Lef-1 were found to have higher expression in M1 populations primed in the presence of IL-4 relative to those primed in the presence of IL-12. This pattern of expression was inversely related to T-bet and/or GATA-3 expression. This is the first time protein transcription factor expression has been investigated in cytokine-primed human CD8\(^+\) T cells.

The effects of three-day cytokine exposure with IFN-\(\alpha\), IL-18, IL-23 and IL-27 were moderate or insignificant. This was unexpected for IFN-\(\alpha\), as it is known to share redundant roles in the murine system with IL-12 as a third signal to induce proliferative and cytotoxic effects for CD8\(^+\) T cells (Curtsinger et al., 2005; Kolumam et al., 2005).

Cytokine exposures showed no significant effects on the magnitude of naïve CD8\(^+\) T cell expansion \textit{in vitro}. This could be due to either the presence of exogenous IL-7 in our culture system or potential masking by high stimulatory signals from anti-CD3/CD28 beads. In murine CD8\(^+\) T cells, proliferation induced by high concentrations of peptide-MHC complex on microspheres gave similar results either with or without IL-12 (Curtsinger et al., 2003b). Also, a recent study that showed significant differences in human CD8\(^+\) T cell expansion with or without IFN-\(\alpha\) used a much lower beads-to-cells ratio (1:10) than the ratio used for our studies (Hervas-Stubbs et al., 2012). The only previous work to investigate the effect of third signal cytokines on human CD8\(^+\) T cell proliferation examined IFN-\(\alpha\) (Hervas-Stubbs et al., 2012; Hervas-Stubbs et al., 2010) and no other cytokines. Thus, further investigation of third signal effects is warranted.

Re-exposure of M1 populations to third signal cytokines upon secondary stimulation showed their plasticity potential, with skewed cytokine secretion profiles being observed in some cases. Many cytokine secretion profiles showed an interactive effect between the primary and secondary stimulations. Affected cytokines included IL-2, IFN-\(\gamma\), IL-5, IL-6, IL-13 and IL-10. The secretion of TNF, MIP-1\(\alpha\) and GM-CSF, however, appeared to be more dependent on cytokine exposure during secondary stimulation.
Chapter 5: The effects of exposure to combined IL-12 and IL-21 during priming of human memory CD8$^+$ T cells

5.1 Introduction

Following the optimisation of the IL-7 in vitro model and the investigation of different cytokines as third signals for the generation of human CD8$^+$ memory cells, we found that priming naïve CD8$^+$ T cells with IL-12 for three days programmed memory cells with enhanced capability to secrete IL-2, IFN-γ, TNF and MIP-1α, the type 1 response cytokines, indicating their superior effector functions (Hinrichs et al., 2009; Wilde et al., 2012). However, the phenotype of the M1 populations primed in the presence of IL-12 had lost CCR7 and CD45RA co-expression and appeared to be further differentiated. In contrast, IL-21 priming during naïve CD8$^+$ T cell stimulation was found to programme retention of CCR7, CD28 and CD27 expression in the rested memory populations. Expression of CD28 and CD27 indicates that appropriate costimulatory signals from the next antigen encounter can be delivered, enables the augmentation of TCR signals, and prevents the induction of anergy or deletion (Bandyopadhyay, Soto-Nieves, and Macian, 2007; Viola and Lanzavecchia, 1996). At the same time, CCR7 expression permits lymphocyte trafficking to secondary lymphoid organs (Bromley et al., 2005). It also enhances movement velocity and cell positioning within the secondary lymphoid organs, and increases T-APC contacts for T cells to receive survival and functional signals (Kehrl, Hwang, and Park, 2009; Mortier et al., 2009; Schuster et al., 2009). However, the M1 populations primed in the presence of IL-21 exhibited less functional capacity, showing a delayed IFN-γ production capability upon secondary stimulation. Therefore, we sought to determine the possibility of synergising the desirable programming effects of IL-12 and IL-21 to achieve superior effector functions and less differentiated phenotypes. Accordingly, the two cytokines were combined for naïve CD8$^+$ T cell priming. We also investigated whether extended IL-21 exposure could augment its more subtle effects. Adjustment of priming conditions to generate optimal memory T cells may lead to better future control of viral infections or tumours.
Chapter 5

5.2 Methods

5.2.1 Cells
Naïve CD8⁺ T cells were isolated from PBMC and stimulated to expand and generate memory populations, M1, in different priming conditions as previously described (2.2.2). Briefly, naïve CD8⁺ T cells were incubated with IL-7 (5ng/ml) overnight and stimulated in the presence of priming cytokines, IL-12 (10ng/ml) and/or IL-21 (10ng/ml), for either 3 or 14 days. IL-7 was added to cultures again on day 3 (20ng/ml) and thereafter (5ng/ml) to support the survival and maintenance of the expanding cells, and the resulting memory populations. All subsequent cultures, restimulations and assays with the memory populations were supplemented with IL-7 (10ng/ml) only.

5.2.2 Division kinetics and magnitude of expansion size

5.2.2.1 Division kinetics
Cells were labelled with 0.125µM of CFSE at 1x10⁶/ml as previously described (2.2.4) and plated at equal concentrations. They were then either stimulated with proliferation kinetics being assessed on day 4, or given a titrated dose of IL-7 (0-50ng/ml) for 7 days to examine the homeostatic proliferation.

5.2.2.2 Cell counting
Cells were counted by the trypan blue exclusion method either manually or with the Countess™ Automated Cell Counter as described in the Methods section (2.2.1).

5.2.3 Cell surface receptor expression
Cell surface receptor staining was performed as described previously (2.3.1.1), and the following antibodies were employed: anti-CCR7-FITC (R&D or BD), anti-CD25-APC-Cy7, anti-CD27-AF700 (BD or BL), anti-CD27-APC-Cy7, anti-CD28-PE-Cy5, anti-CD45RA-PE-TR, anti-CD62L-APC, anti-CD127-PE (as detailed in Table 2-3), and DAPI for live/dead discrimination (2.3.1.1.1).

5.2.4 Functional assays

5.2.4.1 Effector molecules
Procedures were as described in the Methods chapter (2.2.5). The following antibodies were employed: anti-IFN-γ-AF647, anti-granzyme B-FITC and anti-perforin-APC (as detailed in Table 2-4).
5.2.4.2 Cytokine secretion

M1 cells were plated in flat-bottom 96 well tissue culture plates at $2 \times 10^5 / 200 \mu l$ and stimulated with IL-7 (10ng/ml). Supernatants were aspirated at 24 and 48hr after stimulation and examined by CBA Human Soluble Protein Flex Set Assay as described in the Methods section (2.2.5.3). Cytokine capture beads specific for the following analytes were used: IL-2, IL-6 and IL-10, which were sampled at 24hr post-stimulation, and IFN-γ, MIP-1α and TNF, which were sampled at 48hr post-stimulation.
5.3 Results

To investigate the effects of combined cytokine priming, naïve CD8\(^+\) cells were stimulated with both IL-12 and IL-21. IL-12 was supplemented for 3 days and IL-21 for either 3 or 14 days. Cell proliferation kinetics were investigated at several different time points: upon primary and secondary stimulations, during naïve expansion, and during homeostatic proliferation of the rested M1 populations. M1 cell surface phenotype, effector functions and secreted cytokines were also characterised (Figure 5–1, Figure 5–12). These were compared with naïve CD8\(^+\) T cells primed with either IL-12 or IL-21 alone, or no cytokines. For parts of this chapter, Chapter 4 data from single cytokine priming or condition primed without cytokines are presented again for comparative purposes.

5.3.1 Three-day priming of naïve CD8\(^+\) T cells with IL-12 and IL-21

![Experimental overview for Chapter 5, Section 5.3.1](image)

A schematic diagram illustrating the general experimental outline for the first section of this chapter (Section 5.3.1). Naïve CD8\(^+\) T cells were primed with both IL-12 and IL-21 for three days and expanded with IL-7 to generate M1 populations. The generated cells and cells during expansion were assessed by 3 sets of experiments. The time points of investigation and corresponding result figures for each experiment set are shown.

5.3.1.1 Cell proliferation

The ability to rapidly expand and establish a large population of effector and memory cells is desirable for CTL activity and immune protection (Murali-Krishna et al., 1998). We sought to determine whether
stimulating naïve CD8⁺ T cells with combined IL-12 and IL-21 priming enhances cell proliferation upon activation for both primary (5.3.1.1.1, 5.3.1.1.2) and secondary stimulations (5.3.1.1.4), or augments self-renewal capabilities when memory populations have been established (5.3.1.1.3).

5.3.1.1.1 Proliferation kinetics upon stimulation of naïve cells

We first evaluated the initial TCR-triggered proliferation kinetics of naïve CD8⁺ T cells primed with IL-12 and IL-21. We labelled naïve CD8⁺ T cells with CFSE and stimulated the cells with or without both IL-12 and IL-21 for the first 3 days. On day 4, it was found that the maximum number of cell divisions was the same for both conditions. However, cells primed with IL-12 and IL-21 had a higher proportion of cells that underwent five divisions compared to the condition primed without cytokines, as well as fewer cells that divided 1, 2 or 3 times (Figure 5–2). This indicated that IL-12 and IL-21 priming moderately enhanced initial TCR-triggered proliferation in naïve CD8⁺ T cells.

![Figure 5–2 Proliferation of naïve CD8⁺ T cells stimulated with IL-12 and IL-21 priming](image)

Naïve CD8⁺ T cells were labelled with CFSE and plated at 1.5x10⁵/200µl in 96 well flat bottom plates. Cells were stimulated with or without the presence of IL-12 and IL-21 (10ng/ml each) for 3 days, in duplicate. Beads were removed at 48hr and cells were re-plated at 5x10⁵/200µl for further expansion. IL-7 (5ng/ml) was given from day 3 onwards to support survival and proliferation. Cells were harvested on day 4 and CFSE dilutions assessed by flow cytometry. Red lines are CFSE-labelled samples stimulated with or without priming cytokines; blue lines are CFSE-labelled cells not stimulated but cultured in the presence of IL-7; black lines are unstained cells. Grey dotted lines indicate the number of divisions by CFSE dilution.

However, since we used IL-7 to support cell viability in vitro, the enhanced initial proliferation of stimulated naïve cells could be due to a synergistic effect between IL-7 and the IL-12/IL-21 priming combination.

5.3.1.1.1.1 Synergistic effect between IL-7 and the IL-12/IL-21 priming combination

We tested the synergy hypothesis by investigating whether titrated IL-7 had an effect on the proliferative kinetics. We stimulated naïve CD8⁺ T cells with and without IL-12 and IL-21 priming, and supplemented cultures with increasing concentrations of IL-7 from day 3 post-stimulation. The cell numbers were then
determined 6 days after the first IL-7 addition. We found elevated cell numbers in both populations primed in the presence of IL-12/IL-21 or no cytokines with increasing IL-7 concentrations, as well as augmented cell numbers in the IL-12 and IL-21-primed populations for every IL-7 concentration tested (Figure 5–3). These data indicate that both IL-7 concentration and IL-12/IL-21 priming have an influence on TCR-driven proliferation (P<0.0001, P=0.0112, respectively). Furthermore, we found that differences in cell numbers between the populations primed in the presence of IL-12/IL-21 and no cytokines with increasing IL-7 concentrations as well as augmented cell numbers in the IL-12 and IL-21-primed populations for every IL-7 concentration tested (Figure 5–3). This suggests that there is indeed a synergistic effect between IL-7 and the IL-12/IL-21 priming combination on the proliferation of naïve CD8+ T cells (for the interactive effect between the two cell number variables, P=0.0028).

5.3.1.1.2 IL-7Rα expression is enhanced by IL-12/IL-21 priming on stimulated naïve cells

Having found a synergistic effect between combined IL-12/IL-21 priming and IL-7 on the proliferation of activated naïve CD8+ T cells, we investigated whether the interactive effect resulted from IL-12/IL-21 priming regulation of IL-7Rα (CD127) expression. Four days after stimulation, we found the expression of IL-7Rα in the primed population to be dramatically increased compared to the population primed without cytokines (Figure 5–4). This is a surprising result since recent IL-7 signalling and TCR stimulation are both known to downregulate IL-7Rα transcription and protein expression (Mazzucchelli and Durum, 2007; Park et al., 2004; Wirth, Harty, and Badovinac, 2010a). Enhanced expression of IL-7Rα and IL-7 signalling may enhance the expression of Bcl-2, promoting survival, the maintenance of glycolytic flux for metabolism, and the capacity to undergo mitogenesis (Jacobs, Michalek, and Rathmell, 2010; Kittipatarin and Khaled, 2007). This would contribute to augmented proliferation of IL-12/IL-21-primed cells. The expression of IL-7Rα adopted a unimodal distribution in the IL-12/IL-21-primed population (Figure 5–4), suggesting that elevated IL-7 signalling and enhanced proliferation of IL-12/IL-21-primed cells may be driven by the entire population rather than only a fraction of it.
The effects of exposure to combined IL-12 and IL-21 during priming

Figure 5–4 Naïve CD8⁺ T cells stimulated with IL-12 and IL-21 priming have higher expression of IL-7Rα (CD127)

Naïve CD8⁺ T cells were stimulated with or without the presence of IL-12 and IL-21 (10ng/ml each) for 3 days, in duplicate. IL-7 (5ng/ml) was given from day 3 onwards. Cells were harvested on day 4 to assess CD127 expression. Data are representative of 3 donors.

5.3.1.1.3 Enhanced proliferation kinetics by IL-12/IL-21 priming occurs prior to IL-7 addition

To further ascertain the effect of IL-12/IL-21 priming on the kinetics of TCR-triggered proliferation, we tested to see whether proliferation was enhanced prior to the addition of IL-7. CFSE-labelled naïve CD8⁺ T cells were stimulated, and harvested 2 days post-stimulation. Cells from both populations primed in the presence of IL-12/IL-21 and no cytokines were found to have had entered their first division 2 days following activation, but the IL-12/IL-21-primed population possessed a larger fraction of cells that had undergone division (Figure 5–5). This provided evidence for a direct IL-12/IL-21 priming effect on the kinetics of TCR-triggered naïve cell proliferation. This was additional to augmentation of IL-7Rα expression on activated cells, and the synergistic effect with IL-7 which enhanced initial naïve cell proliferation.

5.3.1.1.2 Magnitude of expansion 12 days post-stimulation

Next, we investigated whether the enhanced initial proliferation kinetics following IL-12/IL-21 priming led to an augmented magnitude of expansion. This was also compared to IL-12 and IL-21 priming alone to seek individual contributions by the two combined cytokines. We assessed cell numbers 12 days post-stimulation, and found that IL-12 and IL-21 priming either together or alone minimally increased cell numbers compared to cells primed without cytokines (Figure 5–6A). The difference did not reach statistical significance (P=0.4657). Examining donor-matched data points failed to reveal any consistent trends in cell population sizes across priming conditions (Figure 5–6B). This suggests that there are no differences between priming conditions for the magnitude of expansion at day 12, and that, by day 12, cells primed without cytokines had achieved similar cell expansion to primed cells, despite the slower initial proliferation.
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Figure 5–5 IL-12 and IL-21 priming enhanced the proliferation kinetics as early as 2 days following TCR stimulation on naïve CD8+ T cells

Naïve CD8+ T cells were labelled with CFSE and plated at 1.5x10⁵/200µl in 96 well flat bottom plates. Cells were stimulated with or without IL-12 and IL-21 (10ng/ml each) for 3 days, in duplicate. Beads were removed at 48hr and harvested for analysis by flow cytometry.

Figure 5–6 Effect of IL-12 and IL-21 priming on the magnitude of expansion

Naïve CD8+ T cells were stimulated with or without the presence of IL-12 and/or IL-21 (10ng/ml each) for 3 days in duplicates of 2x10⁵ cells/200µl in 96 well flat bottom plates. IL-7 was given from day 3 onwards. Cell numbers were assessed 12 days after stimulation by the trypan blue dye exclusion method. Each data point depicts the average value of biological duplicates per donor, with a total of 4 donors from 2 independent experiments. A, Data symbols grouped by priming conditions (solid symbols), bars indicate mean ± SEM of the 4 donors. Data were analysed by Friedman test, P value stated. B. Same data set, data symbols grouped by donors (open symbols).

5.3.1.3 Homeostatic proliferation of M1 cells generated with combined IL-12 and IL-21 priming

We investigated the homeostatic proliferation ability of M1 cells generated with combined IL-12 and IL-21 priming. Rested M1 populations generated either with or without IL-12/IL-21 priming were labelled with CFSE, and cultured with titrated doses of IL-7 for 7 days to examine their rate of homeostatic proliferation. We found that both populations failed to survive without IL-7, and that a minimum IL-7 concentration of 5ng/ml was required for cell division. The maximum effective dose of IL-7 was found to differ between the memory populations primed in the presence of IL-12/IL-21 and no cytokines. Homeostatic cell divisions
reached maximum levels in an IL-7 concentration range of 5-10 ng/ml for the cells primed without cytokines, while the IL-12/IL-21-primed population continued to recruit cells into division with IL-7 concentrations up to 50 ng/ml (Figure 5–7). This suggests that IL-7 sensitivity is enhanced for the IL-12/IL-21-primed M1 population.

**Figure 5–7** M1 populations generated with combined IL-12 and IL-21 priming undergo heightened homeostatic proliferation and have enhanced sensitivity for IL-7

M1 cells were generated by stimulating naïve CD8^+^ T cells with or without combined IL-12 and IL-21 priming during the first 3 days of stimulation. IL-7 was given from day 3 onwards to allow further expansion and rest over 3 weeks. 20 days after primary stimulation, the M1 populations were labelled with CFSE, plated at equal densities in duplicates in 96 well flat bottom plates and cultured with the indicated IL-7 doses for 7 days. The scale of each histogram was adjusted to its maximum count. Data are representative of 2 donors.

**5.3.1.4 Proliferation kinetics upon secondary stimulation**

Rapid activation and CTL responses by memory T cells enable an accelerated immune response for secondary antigen encounters (Rogers, Dubey, and Swain, 2000). Therefore, we investigated whether combined IL-12 and IL-21 priming had programmed the M1 populations to show enhanced proliferation kinetics upon secondary stimulation. The M1 populations primed in the presence of IL-12/IL-21 or no cytokines were labelled with CFSE and restimulated in the presence of IL-7 only. Four days after restimulation, the IL-12/IL-21-primed M1 populations were found to have divided more than the M1 cells primed without cytokines (Figure 5–8). Combined IL-12 and IL-21-priming may therefore have effected
enhanced proliferation kinetics and a consequent capacity for accelerated response to TCR stimulations (Figure 5–7).

![Figure 5–8 M1 cells generated with IL-12 and IL-21 priming proliferate faster upon secondary stimulation]

M1 cells were generated as described in Figure 5–7. 20 days after stimulation, the M1 populations were labelled with CFSE, plated at equal densities in duplicate, and restimulated with IL-7 (10ng/ml) only. Cells were harvested on day 4 to assess CFSE dilution. Red lines are stimulated and CFSE-labelled cells; blue lines are CFSE-labelled cells not stimulated but cultured in IL-7; black lines are unstained cells. The dotted line indicates the CFSE dilution peak after 5 cell divisions. Data are representative of 3 donors from 2 independent experiments.

5.3.1.2 M1 effector functions

Given the enhanced proliferation kinetics for the IL-12/IL-21-primed M1 populations in response to TCR stimulations, and to IL-7 for homeostatic proliferation, we next investigated the effector capabilities of these cells. The M1 populations programmed with or without IL-12 and/or IL-21 priming were restimulated to assess their production of effector molecules and cytokine secretions.

5.3.1.2.1 IL-21 partially inhibited IL-12 induced IFN-γ production but maintained granzyme B expression

Compared to the polar alterations of IFN-γ production induced by either IL-12 or IL-21 priming (Chapter 4, Figure 4–5A), we found the combined priming effect of IL-12 and IL-21 programmed M1 populations to express an intermediate level of IFN-γ. The percentage of IFN-γ+ cells assessed 6hr post-restimulation was reduced following addition of IL-21 to IL-12 priming. However, total IFN-γ expression appeared to be greater than that observed for the condition primed without cytokines (Figure 5–9A). The median fluorescence intensity (MFI) of the IFN-γ+ population for each priming condition was found to correlate with the percentage of IFN-γ+ cells in each population (data not shown). This indicated that priming naïve CD8+ T cells with IL-12/IL-21 had an effect not only on the percentage of cells expressing IFN-γ, but also on the amount of IFN-γ production per cell.

For the production of lytic molecules, M1 populations generated with IL-12/IL-21 priming were able to express granzyme B at the same consistent and heightened levels as cells primed in the presence of IL-12
The effects of exposure to combined IL-12 and IL-21 during priming

(Figure 5–9B). In contrast, the expression of perforin was found to have greater inter-individual variability compared to IFN-γ or granzyme B expression (SEM range for IFN-γ, 2.7 – 7.6; GzmB, 0.7 – 7.1; Perf, 7.8 – 13.11). This is consistent with observations described in Chapter 4 (Figure 4–5C). No differences in perforin production were found between different priming conditions, and no trends were observed across donors (Figure 5–9C).

Figure 5–9 Production of IFN-γ, granzyme B and perforin by M1 cells primed with IL-12 and IL-21
M1 cells were generated with the indicated priming conditions, in duplicate, as described in Figure 5–7. Each duplicate M1 population was plated at equal densities and restimulated in the presence of IL-7 (10ng/ml) only. IFN-γ (A) was examined after 6hr of restimulation. Granzyme B (B) and perforin (C) were examined after 3 days of stimulation. Percentages of live, singlet cells positive for each effector molecule were plotted. Each data point is the averaged value of the biological duplicates of the M1 populations per donor, with a total of 4 donors from 2 independent experiments. Graphs in the upper panel have data grouped by priming conditions (solid symbols). Bars indicate mean values ± SEM across the 4 donors. Data were analysed by Friedman tests and Dunn’s post-tests, and P values from the Friedman tests are shown. Graphs in the bottom panel show the same data set, but the data symbols were grouped by donors (open symbols).
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5.3.1.2.2 **IL-12/IL-21 priming generated M1 populations with a distinct profile of cytokine secretions**

Following secondary stimulation of the M1 populations, IL-2 secretion by the IL-12/IL-21-primed M1 cells did not differ from that seen in either no cytokine or singularly-primed conditions (P=0.2066). Secretion of IFN-γ and TNF differed significantly across the 4 priming conditions tested (P=0.033, P=0.033, respectively). At 48hr post-stimulation, concentrations of IFN-γ and TNF secreted by IL-12/IL-21-primed M1 populations were found to be similar to those secreted by M1 cells primed in the presence of IL-12 (Figure 5–10). At 6hr post-stimulation, however, IFN-γ expression was reduced for the IL-12/IL-21-primed M1 populations relative to populations primed in the presence of IL-12 (Figure 5–9A). This indicated a delay in IFN-γ production following the addition of IL-21.

IL-6 secreted by IL-12/IL-21-primed cells was found to be consistently reduced compared to that secreted by cells primed without cytokines, and similar to that secreted by IL-21-primed cells (Figure 5–10). Interestingly, the IL-10 secretion trend across the 4 priming conditions appeared to be inversely correlated to the IL-6 secretion trend. Thus, IL-12/IL-21-primed cells showed the highest secretion of IL-10. For two donors out of the three examined, the effect of IL-12 priming and IL-21 priming appeared to be additive for the production of IL-10, with IL-10 secretion increased relative to amounts secreted by either IL-12 or IL-21 singularly-primed cells. Of all the cytokines examined, IL-10 also had the greatest fold changes in secretion relative to the condition primed without cytokines. This observation suggests that regulation of IL-10 production regulation is very sensitive to *in vitro* priming conditions. Lastly, MIP-1α (CCL3) secretion did not differ significantly between the 4 priming conditions (Figure 5–10).

In summary, the effect of IL-12/IL-21 priming on cytokine secretion was found to be specific for each cytokine, generating a distinct profile of cytokine secretions. M1 populations programmed with IL-12/IL-21 priming were able to secrete enhanced levels of IFN-γ and TNF compared to the condition primed without cytokines. However, IFN-γ and TNF levels were similar to those observed for M1 cells primed in the presence of IL-12. While no differences were found for either IL-2 or MIP-1α production, IL-6 secretion was found to be reduced relative to that observed for both no cytokine and IL-12 priming conditions. It was similar to that observed for IL-21-primed cells. For the secretion of IL-10, IL-12/IL-21 priming appeared to have additive effects, resulting in elevated levels of IL-10 production compared to priming by either IL-12 or IL-21 alone.
The effects of exposure to combined IL-12 and IL-21 during priming

Figure 5–10 Cytokine secreted upon secondary stimulation by M1 cells primed with IL-12 and IL-21

M1 cells were generated with the indicated priming conditions, in duplicate as described in Figure 5–7. Each duplicate M1 population was plated at 2x10^5/200µl and restimulated in the presence of IL-7 (10ng/ml) only. Supernatants were aspirated from cultures at 24 and 48hr. The analytes indicated were detected using the CBA flex set assay at either 24hr (for IL-2, IL-6 and IL-10) or 48hr (for IFN-γ, TNF and MIP-1α). Absolute concentrations of each analyte (top rows of each panel, solid symbols, grouped by priming conditions) and fold changes relative to the no cytokine control (bottom rows of each panel, open symbols, symbols grouped by individual donors) were plotted. Each data point is the averaged value from the biological M1 duplicates per donor, with a total of 3 donors from 2 independent experiments. In the upper row graphs of each panel, bars indicate mean values of absolute concentrations. Solid lines in absolute concentration graphs indicate maximum CBA assay standard range, any data above were extrapolated. Dotted lines in fold-change graphs indicate no change to the no cytokine control. Absolute concentration data were analysed by Friedman tests and Dunn’s post-tests. P values are stated from the results of the Friedman tests; no significant results were found from Dunn’s post-tests.
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5.3.1.3 The IL-12/IL-21-primed M1 populations had similar expression of phenotypic markers to M1 cells primed in the presence of IL-12, with reduced expression of IL-2Ra

Next, we examined the expression of cell surface differentiation markers on the M1 populations primed in the presence of IL-12/IL-21. For all the markers examined (CCR7, CD62L, CD28, CD27 and CD45RA), we found the overall expression of the differentiation markers by IL-12/IL-21-primed M1 populations to be very similar to that expressed by M1 cells primed in the presence of IL-12. For some of these markers (CD62L, CD28 and CD27), expression was also similar to that observed for cells primed in the presence of IL-21. Co-expression of CCR7 and CD45RA appeared to be slightly increased relative to IL-12-primed cells, indicating reduced potential for IL-12-induced differentiation (Figure 5–11A).

The expression of high affinity chains for the IL-7 and IL-2 receptors was also investigated. These chains are mediators of survival and proliferation signals, while the expression of IL-2Ra (CD25) is also an indicator of cell activation. No differences between the 4 priming conditions were found for the expression of IL-7Ra (CD127) (P=0.2066) However, significant differences were observed for the expression of IL-2Ra (P=0.033). The IL-12/IL-21-primed M1 cells appeared to show reduced IL-2Ra expression compared to those primed with IL-12 alone (Figure 5–11B), suggesting a more rested cell status.
The effects of exposure to combined IL-12 and IL-21 during priming

M1 cells were generated with the indicated priming conditions, in duplicate as described in Figure 5–7. Cell surface phenotype was examined 21 days after naive stimulation for the expression of differentiation markers (A), plotted as percentages of positive expression from live singlet populations, and cytokine receptors (B), plotted as median of fluorescence intensity (MFI). Data were collected from 3 donors in 2 independent experiments. For 2 donors, the expression was examined for biological duplicates. Each data point represents the averaged values of the duplicates, where applicable, per one donor. Bars indicate the mean values across donors. Data were analysed by Friedman tests and Dunn’s post-tests. P values are stated from the results of the Friedman tests and asterisks between conditions indicate significant results from Dunn’s post-test.

Figure 5–11 The combined effect of IL-12 and IL-21 priming on the M1 expression of the differentiation markers and cytokine receptors
5.3.2 Priming naïve CD8\(^+\) T cells with three days of IL-12 and an extended duration of IL-21

The incorporation of IL-12/IL-21 priming during the primary stimulation of naïve CD8\(^+\) T cells programmed M1 populations with combined characteristics of those primed with either IL-12 or IL-21 alone. The addition of IL-21 to IL-12 priming moderately relieved differentiation effected by IL-12 priming. IFN-γ production was delayed and the expression of CCR7 and CD45RA increased moderately, while the ability to produce lytic molecules and to secrete IL-2 and TNF was maintained. Therefore we sought to investigate whether an extended duration of IL-21 exposure during the primary expansion would produce memory populations with less differentiated phenotype. We stimulated naïve CD8\(^+\) T cells with IL-12 priming for three days, in combination with IL-21 exposure for either 3 or 14 days. The resulting M1 populations were assessed for their effector functions, phenotype and expansion kinetics during the primary and secondary expansions (Figure 5–12).

Figure 5–12 Experimental overview for Chapter 5, Section 5.3.2

A schematic diagram illustrating the general experimental outline for the second section of this chapter (Section 5.3.2). Naïve CD8\(^+\) T cells were stimulated with IL-12 priming for 3 days and IL-21 exposure for 3 or 14 days and then expanded with IL-7 to generate M1 populations. The resultant cells and cells during expansion were assessed by 3 sets of experiments. Time points and corresponding result figures for each experiment set are shown.
5.3.2.1 M1 effector functions

5.3.2.1.1 The production of IFN-γ and the lytic molecules was maintained with extended duration of IL-21 exposure

The combination of IL-21 priming together with IL-12 resulted in delayed production of IFN-γ (Figure 5–9A, Figure 5–10). Interestingly, we found that 14 days of IL-21 exposure did not alter the percentage of cells expressing IFN-γ (Figure 5–13A), but moderately increased the fluorescence intensity levels (MFI) of the IFN-γ⁺ population, indicating a minor enhancement of IFN-γ production per cell (Figure 5–13B). The expression of the lytic molecules granzyme B and perforin was also investigated. It was found that the extended exposure of IL-21 did not affect either the percentage of cells expressing these lytic molecules or their MFI (Figure 5–13A, B).

![Graph A](image1.png)
![Graph B](image2.png)

Figure 5–13 The effect of extended IL-21 exposure on the production of effector molecules

Naïve CD8⁺ T cells were stimulated in the presence IL-12 for 3 days and IL-21 for 3 or 14 days, in duplicate. IL-7 was given from day 3 onwards for a total of 21 days. The biological duplicates of the M1 populations were plated at equal densities and restimulated with IL-7 (10ng/ml) only. Intracellular IFN-γ was examined after 6hr of stimulation. Granzyme B and perforin were examined after 3 days of restimulation. Percentages of live, singlet cells positive for each effector molecule were plotted (A), as well as the median fluorescence intensity, MFI, of each positive population (B). Each data point represents the averaged value of the biological duplicates per donor. Bars indicate mean values across donors. Data were analysed by Mann-Whitney tests. ns, no significant difference; n/a, non-applicable (P value could not be calculated due to an absence of numerical difference for 2 out of 3 donors between the 2 conditions).
5.3.2.1.2 Extended duration of IL-21 exposure modulated the secretion of TNF and IL-6

For M1 populations generated following extended IL-21 exposure, no statistically significant differences were found for secretion of the type 1 cytokines, IL-2, IFN-γ and TNF. However, the secretion of TNF appeared to be moderately enhanced relative to that seen after 3-day IL-21 priming. For IL-6 and IL-10 secretion, there appeared to be a reduction of IL-6 with extended IL-21 exposure, but no difference in IL-10 secretion. This contrasted with the observed augmentation of IL-10 secretion after IL-12 and IL-21 singular cytokine priming as well as their combined priming (Figure 5–14).

Figure 5–14 The effect of extended IL-21 exposure on the secretion of cytokines

M1 populations were generated with 3 days of IL-12 priming in combination with 3 or 14 days of IL-21 exposure as described in Figure 5–13. The biological duplicates of the M1 populations were plated at 2x10^5/200µl and restimulated with IL-7 (10ng/ml) only. Supernatants were aspirated from cultures following stimulation and the concentrations of cytokines assessed using the BD CBA flex set assay at either 24hr (IL-2, IL-6, IL-10) or 48hr (IFN-γ, TNF). The absolute concentrations of the secreted cytokines were plotted. Each data point represents the mean value of the biological duplicates per donor. Bars indicate mean values across donors. Solid lines indicate maximum CBA assay standards, any data above the line were extrapolated. Data were analysed by Mann-Whitney tests. ns, no significant difference.
5.3.2.2 Extended IL-21 exposure moderately enhanced CD28 expression

Expression of phenotypic markers previously found to be altered by IL-21 (Figure 5–11) (Spolski and Leonard, 2008) was examined for rested M1 populations programmed with either 3 or 14 days of IL-21 exposure. None of the 4 receptors tested showed statistically significant differences in expression between 3- or 14-day exposure. CD28 expression, however, showed a trend to increased expression following 14-day IL-21 exposure (Figure 5–15). This suggests that extended IL-21 exposure may lead to preservation of memory cells in a less differentiated state, in which the expression of important costimulatory receptors is retained for future CD8+ T cell stimulation and function.

Figure 5–15 The effect of extended IL-21 exposure on the expression of differentiation markers on M1 cells

M1 cells were generated with IL-12 priming for 3 days and IL-21 exposure for 3 or 14 days. IL-7 was given from day 3 onwards and the cells were allowed to expand and rest. Cell surface phenotypes were examined 21 days after naive stimulation for the expression of differentiation markers, plotted as percentages of positive expression. Data were collected from 3 donors. Bars indicate the averaged values across donors. Data were analysed by Mann-Whitney tests. ns, no significant difference.

5.3.2.3 Proliferation kinetics during the primary and secondary expansions

Despite the enhanced proliferation kinetics of cells primed with IL-12/IL-21 immediately following TCR stimulation, this effect did not translate into a significant difference in the magnitude of expansion (5.3.1.1). We sought to investigate whether extended IL-21 exposure affected cell proliferation on a gross scale by determining cell numbers over the duration of the primary and secondary expansions. No significant differences between cells subjected to extended IL-21 exposure and controls were found in proliferation kinetics or overall magnitude of expansion during primary expansion (Figure 5–16A). For secondary expansions, the overall magnitude of proliferation was greatly reduced relative to that observed for primary expansions. The initial proliferation kinetics upon secondary expansion appeared similar between the 2 conditions, but the M1 population generated with extended exposure of IL-21 showed slightly longer periods of continual proliferation. However, neither M1 population could accumulate from 7-10 days post-
stimulation, and both gradually diminished over the next two weeks (Figure 5–16B). This unexpected result indicates that M1 populations are unable to expand, or accumulate, upon restimulation, in contrast to their successful primary expansion and polyfunctional memory cell formation.

**Figure 5–16 The effect of extended IL-21 exposure on the proliferation kinetics during the primary and secondary expansions**

**A.** Naive CD8\(^+\) T cells were stimulated in the presence IL-12 for 3 days and IL-21 for 3 or 14 days, in duplicate, with starting cell numbers of 2x10\(^5\)/200µl. IL-7 was given from day 3 onwards and the cells were allowed to expand for a total of 21 days to generate rested M1 populations. The cell numbers were assessed on days 3, 6, 9, 12, 15 and 20 post-stimulation by the trypan blue dye exclusion method. **B.** The biological duplicates of the M1 populations were plated at 4x10\(^5\)/200µl and restimulated with IL-7 only. Cell numbers were assessed on days 3, 5, 7, 10, 15 and 20 post-restimulation. Each data point depicts mean values of the biological duplicates ± SEM. Data are representative of 3 donors.
5.4 Summary and discussion

In this chapter, we aimed to determine whether combined priming by IL-12/IL-21 could programme CD8+ memory cells with the desirable attributes induced by IL-12 or IL-21 priming alone, i.e. superior effector functions and less differentiated phenotypes. We found that addition of IL-21 exposure to IL-12 priming delayed IFN-γ production, but its overall quantity maintained by 48hr post-stimulation. The combined regimen also maintained expression of lytic molecules and the secretion of IL-2, TNF and MIP-1α. Extended IL-21 exposure was able to enhance CD28 expression, indicating that the combination of IL-12 and IL-21 priming could indeed synergise their desirable programming effects. At the same time, we found that the addition of IL-21 to IL-12 priming reduced the programmed secretion of IL-6, while increasing the production of IL-10, with reduced CD25 expression, as well as enhanced proliferation kinetics at the initiation of TCR stimulations and augmented homeostatic proliferation. However, the enhanced initial proliferation kinetics upon naïve cell stimulation did not yield significant alterations to the overall magnitude of expansion.

The increased initial division kinetics by IL-12/IL-21 priming are consistent with previous observations of IL-12’s ability to promote expansions of antigen-specific cells upon murine infections (Curtsinger et al., 2003a; Lisiero, Soto, Liau, and Prins, 2011; Xiao et al., 2009). They are also consistent with that IL-21 can augment TCR-driven proliferation in both murine and human CD8+ T cells in the presence of γc cytokines (Ansen et al., 2008; Li et al., 2005; Zeng et al., 2005). We provided evidence for a potential mechanism of the synergistic activities between IL-21 and IL-7, whereby IL-12/IL-21 priming was observed to upregulate IL-7Rα expression and IL-7 signalling despite recent TCR engagement and the presence of exogenous IL-7. This contributes to enhanced proliferation and is likely due to IL-7’s ability to not only enhance cell survival and proliferation (Mackall, Fry, and Gress, 2011), but also induce cellular metabolism processes by elevating the levels of glucose uptake (Jacobs et al., 2010; Kittipatarin and Khaled, 2007). The synergistic effect of IL-21 and IL-7 on naïve CD8+ T cells has also been observed to increase antigen responsiveness via decreased CD5 expression, which negatively modulates TCR signalling (Gagnon et al., 2010). This evidence indicates that the combined priming of IL-12/IL-21 is able to overcome potentially masked influences of priming cytokines on TCR-triggered proliferation kinetics (see previous chapter). At 12 days post-stimulation, however, the magnitude of expansion with IL-12/IL-21 priming was not significantly different from that observed after priming with either IL-12 or IL-21 alone. This suggests that IL-12 plus IL-21-primed populations had faster, but shorter, durations of active proliferation. Alternatively, the similar magnitude of expansions across priming conditions may be due to maximum survival signals induced by the continuous presence of IL-7.

The enhanced homeostatic proliferation found in rested IL-12 and IL-21-primed M1 cells indicates an enhanced capability to maintain memory populations, as well as prolonged sensitivity towards continuous IL-7 signalling. This is in contrast to M1 populations primed without cytokines, which maintained survival and cell metabolism in the presence of IL-7, but did not show significant alterations in homeostatic cell

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cycling in response to increasing concentrations of IL-7. This indicates a reduced capacity to persist as a population. In contrast to the enhanced initial proliferation kinetics upon TCR signalling with IL-12/IL-21 exposure, where an augmented expression of IL-7Rα contributed to their synergistic effect, the expression of IL-7Rα on the rested M1 populations primed in the presence of IL-12/IL-21 was not significantly different to the cells primed without cytokines, despite the enhanced homeostatic proliferation with higher doses of IL-7. This may potentially suggest a faster cycling of IL-7Rα expression in the rested, IL-12/IL-21-primed M1 populations or enhanced IL-7 signalling via the common β and γ chains of the IL-7 receptor complex, and requires further confirmation.

Unexpectedly, IL-12/IL-21-primed M1 populations were found incapable of full expansion and accumulation upon secondary stimulation despite the expression of an early differentiation phenotype and the capability to exert multi-effector functions. Although IL-7 has been found to support the survival of memory CD8+ T cells (Goldrath et al., 2002; Schluns et al., 2000), it has been observed that prolonged cultures of human CD4+ T cells in IL-7 eventually cease STAT-5 signalling and cell cycling (Swainson, Verhoeyen, Cosset, and Taylor, 2006), indicating a potential need for alternative in vitro survival signals. IL-15 appears to be a potential candidate in this case, where memory CD8+ T cells are known to require IL-15 for in vivo survival and persistence in murine models (Becker et al., 2002; Kennedy et al., 2000). Another possible explanation for the inability to proliferate upon secondary challenge may be a deficiency of essential signals during naïve cell activation. It has been observed that in the absence of IL-2R signalling during primary stimulation, CD8+ T cells generated memory cells that did not accumulate after re-challenge (Mitchell et al., 2010; Williams et al., 2006). Subsequently Feau et al. (2011) found that the IL-2 signal requirement originated from CD8+ T cell autocrine signalling, and that helper T cell CD40L-dependent interaction with APCs was also essential to generate fully functional memory cells. Therefore, there is a possibility that our in vitro-generated M1 cells had reduced IL-2R signalling due to the lack of CD4 help and/or restricted inflammatory signals during the primary challenge. We sought to investigate these possibilities next for the accumulation of effector cells upon secondary stimulation.
Chapter 6: Further development of the \textit{in vitro} model – the addition of IL-2 and IL-15

6.1 Introduction

In the previous chapters, we found that although optimised third signal cytokine priming enhanced the cellular attributes of the memory cells, the M1 populations generated with IL-12 and IL-21 priming were unable to sustain secondary proliferation. A number of potential factors may contribute to this inability of M1 populations to accumulate upon restimulation. One possibility is an insufficiency of autocrine IL-2 signals during the primary expansion with the absence of CD4$^+$ help and APCs. Therefore, we sought to supplement naïve expansions with a short duration of IL-2 exposure to determine whether additional IL-2 signals could rescue the incompetency of the M1 populations to expand upon secondary stimulation. Because strong IL-2 signals are known to drive CD8$^+$ T cell differentiation (Kalia et al., 2010; Pipkin et al., 2010), we also compared cells generated with or without a short duration of IL-2 supplementation in the IL-7 model with naïve cells expanded with long-term IL-2. This is a common protocol for the expansion of antigen-specific cells in current ACT regimes. Another possible reason for the inability of M1 populations to expand upon secondary stimulation is an inadequate provision of survival signals. IL-15 is a $\gamma_c$ cytokine best characterised for its role in the maintenance of CD8$^+$ memory populations. It has also been found to contribute to all phases of CD8$^+$ T cell biology, including development, activation, proliferation and cytotoxicity (Castillo and Schluns, 2012; Verbist and Klonowski, 2012). The survival and self-renewal capabilities of CD8$^+$ memory cells are critically dependent on the availability of IL-15 (Becker et al., 2002; Kennedy et al., 2000), as discussed in the Introduction. Therefore, given the known importance of IL-15 to CD8$^+$ memory cells, we sought to also determine whether addition of recombinant IL-15 during the secondary stimulation could rescue the non-accumulating secondary effectors.

Investigations of functional capabilities and anti-tumour efficacies of differential CD8$^+$ memory cell subsets have indicated an association of superior CTL functionalities with cells in earlier differentiation status, either in the order N (naïve) $>$ CM $>$ EM, or SCM $>$ CM $>$ EM. It has been proposed that the higher proliferative potential, longer telomere lengths and better survival capabilities of the less differentiated cells contribute to the engraftment and persistence of the adoptively transferred cells, and thus correlate to efficacy in ACT (Berger et al., 2008; Heslop et al., 2010; Restifo et al., 2012; Rosenberg et al., 2011; Wang et al., 2011). Since we appeared to have generated human memory CD8$^+$ T cell populations \textit{in vitro} with advantageous attributes for T cell transfers in ACTs, we sought to compare the expression of transcription factors of M1 populations to \textit{ex vivo} CD8$^+$ memory cell subsets. The aim was to acquire an indication of differentiation status of our \textit{in vitro}-generated memory cells in relation to \textit{in vivo} generated memory cells. A similarity between transcription factor expression patterns between M1 populations and naïve or SCM cells suggests desirable traits for ACT.
6.2 Methods

6.2.1 Cells

6.2.1.1 Generation of M1 populations with cytokine priming and exogenous IL-2
Naïve CD8\(^+\) T cells were isolated from PBMC by MACS separation, as described in the Methods chapter (2.1.3), and expanded to generate M1 memory populations in vitro with different priming conditions, with or without exogenous IL-2 supplementation. After isolation from PBMC, naïve CD8\(^+\) T cells were first incubated with IL-7 (5ng/ml) overnight, then stimulated in the presence of priming cytokines. Three cytokine conditions were investigated in this chapter. Two conditions utilised the IL-7 model system with 3-day IL-12 (10ng/ml) and 14-day IL-21 (10ng/ml) priming with or without the addition of IL-2 (10ng/ml) for the first 7 days following stimulation. These two conditions both had IL-7 added to the cultures from day 3 onwards and were labelled as “IL-21” and “IL-21 + IL-2” conditions, respectively. The third condition examined was an IL-2 (10ng/ml)-based expansion with only IL-12 (10ng/ml) as a priming cytokine for 3 days, which was labelled as “IL-2 only” condition (Figure 6–1A). Cells were allowed to expand and rest for at least 21 days to permit the formation of M1 populations prior to functional assays.

6.2.1.2 Generation of M2 populations with IL-7 or IL-15
M1 populations generated from “IL-21” and “IL-21 + IL-2” priming conditions were restimulated to generate M2 populations. Cells were stimulated again and the cells were then allowed to further proliferate and rest for at least 24 days. Three cytokine conditions were investigated to support the expansion and survival of the M2 populations. M1 cells were either restimulated and expanded in IL-7 (10ng/ml) alone, IL-15 (10ng/ml) alone or given IL-7 (10ng/ml) for the first 7 days of restimulation then IL-15 (10ng/ml) for the rest of the period (labelled as “IL-7/15” condition) (Figure 6–1B).

6.2.1.3 Ex vivo memory subsets
Total CD8\(^+\) T cells were isolated from PBMC by MACS separation and sorted into memory subsets by fluorescence-activated cell sorting (FACS) as described in the Methods chapter (2.3.3). Using the gating strategy in Figure 6–2 and post-sort validation in Figure 6–3, single, live cells were sorted into four memory subsets on FACSAria II based on expression of CCR7 and CD45RA, after exclusion of CD4- and CD56-expressing cells as detailed in Table 6-1. Cells were mounted onto coverslips, fixed immediately following sorting, and assessed for transcription factor expression by immunocytochemistry.
Further development of the in vitro model: Addition of IL-2 & IL-15

Figure 6–1 Cytokine priming and experimental culture conditions for the generation of M1 and M2 populations

Cytokine supplementation for both priming and survival support are shown for the experimental conditions investigated to generate M1 (A) and M2 (B) populations in this chapter. A. Dark blue boxes denote the 3 condition labels for the generation of M1. Each big arrow is the base cytokine to support survival of naïve cells from stimulation, expansion to rest and formation of memory M1 populations. Small arrows indicate the cytokines supplemented for priming and the exogenous addition of IL-2. Numbers in bracket denote the number of days the cytokines were given to the cultures from the point of stimulation. B. M1 cells generated from the IL-7-based expansion conditions were each restimulated under 3 conditions to produce the M2 populations, labelled as “IL-7”, “IL-7/15” and “IL-15” conditions. The IL-7 in the “IL-7/15” condition was given for the first 7 days post-restimulation.

Table 6-1 Sorting criteria by phenotypic marker expression for ex vivo CD8^+ memory subsets

<table>
<thead>
<tr>
<th>Markers</th>
<th>Naive (N)</th>
<th>Central Memory (CM)</th>
<th>Effector Memory (EM)</th>
<th>EM with CD45RA (EMRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD56</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCR7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD45RA</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Total CD8^+ T cells were isolated from PBMC by MACS separation, stained for CD4, CD56, CCR7 and CD45RA expression and sorted into four memory subsets: naïve, central memory, effector memory and effector memory with CD45RA expression.
Figure 6–2 Gating strategy for ex vivo CD8⁺ memory cell subset sort

Magnetically enriched ex vivo CD8⁺ T cells were first gated by cell size and doublets. CD4⁺ and CD56⁺ cells were excluded then gated for the four memory cell subsets by CCR7 and CD45RA expression to sort on BD FACSria II with BD FACSDiva Software. N, naïve cells (CCR7⁺ CD45RA⁺); CM, central memory cells (CCR7⁺ CD45RA⁻); EM, effector memory cells (CCR7⁻ CD45RA⁻); EMRA, effector memory cells with CD45RA expression (CCR7⁻ CD45RA⁺).

Figure 6–3 Ex vivo CD8⁺ T cell memory subsets before and after sorting

CD8⁺ T cells were isolated from PBMC by MACS separation and stained for CCR7, CD45RA, CD4 and CD56 expression prior to and following cell sorting. The data from the sorted subsets are overlayed in the bottom panel. FACS plots are representative of 4 donors.
6.2.2 Cell proliferation

6.2.2.1 Proliferation kinetics by assessment of cell numbers

Cells were counted by the trypan blue exclusion method either manually or with Countess™ Automated Cell Counter as described in the Methods chapter (2.2.1).

6.2.3 Cell surface receptor expression

Cell surface receptor staining was performed as described previously (2.3.1.1), and the following antibodies were employed: anti-CCR7-FITC (R&D or BD), anti-CD25-APC-Cy7, anti-CD27-AF700 (BD or BL), anti-CD28-PE-Cy5, anti-CD45RA-PE-TR, anti-CD45RO-PE-Cy7, anti-CD56-PE-Cy7, anti-CD57-FITC, anti-CD62L-APC, anti-CD71-PE, anti-CD98-FITC, anti-CD152-APC, anti-CD244-PE, anti-CD279-APC, anti-CD45RA-PE-TR, anti-CD45RO-PE-Cy7, anti-CD56-PE-Cy7, anti-CD57-FITC, anti-Glut1-APC (as detailed in Table 2-3) and DAPI for live/dead discrimination (2.3.1.1.1).

6.2.4 Functional assays

All restimulations of M1 populations for functional assays were performed in biological duplicates in IL-7 (10ng/ml) for the “IL-21” and “IL-21 + IL2” conditions, or IL-2 (10ng/ml) for the “IL-2 only” condition unless otherwise stated.

6.2.4.1 Effector molecules

Processes were as described in the Methods chapter (2.2.5), using the following antibodies: anti-IFN-γ-AF647, anti-granzyme B-FITC and anti-perforin-APC (as detailed in Table 2-4).

6.2.4.2 Cytokine secretion

Cells were plated in flat-bottom 96 well tissue culture plates at 2x10^5/200µl and stimulated. Supernatants were aspirated at 24 and 48hr after stimulation and examined by CBA Human Soluble Protein Flex Set Assay as described in the Methods chapter (2.2.5.3). Cytokine capture beads specific for the following analytes were used: IL-2, IL-6, IL-10, IFN-γ and TNF.

6.2.4.3 Susceptibility to apoptosis

Cells were plated at equal cell numbers per well in U-bottom 96 well tissue culture plates and either stimulated or rested for 24hr. Cells were stained with DAPI and analysed by flow cytometry. Live cell events were determined by DAPI staining and live cell gating from flow cytometric data.
6.2.5 Expression of transcription factors by immunocytochemistry

Two protocols were employed for immunocytochemistry in this chapter, one with methanol-fixation, casein-block and FBS-supplemented antibody dilution buffer (labelled as “M/C/FBS” protocol), and the other with paraformaldehyde-fixation, goat-serum-block and BSA (bovine serum albumin)-supplemented antibody dilution buffer (labelled as “PF/GS/BSA” protocol). Both were performed with sequential staining as described in the Methods chapter (2.4). For the assessment of T-bet, Lef-1 and Eomes expression, either protocol was used for different experiments. For the staining of naïve cells 5 days post-stimulation (Figure 6–13) and rested M1 populations (Figure 6–14), M/C/FBS protocol was employed; for the staining of M1 cells 1 day post-restimulation (Figure 6–15), M2 populations (Figure 6–18) and ex vivo memory subsets (Figure 6–20), PF/GS/BSA protocol was employed. Staining expression for these antigens was validated to be the same across the two protocols (Figure 2–7). All staining for FoxO1 and Tcf-1 was performed with the PF/GS/BSA protocol. The following primary antibodies were employed: unconjugated anti-T-bet, anti-Lef1, anti-Eomes, anti-FoxO1, anti-Tcf-1 and AF647-conjugated anti-Eomes for sequential staining (details of the antibodies are delineated in Table 2-10). The following secondary antibodies were employed: goat anti-mouse IgG1-AF488, goat anti-rabbit IgG1-AF555 (as detailed in Table 2-11) and DAPI for nuclear staining.
6.2.5.1 Heterogeneity of IL-2-generated M1 cells – expression of transcription factors in phenotypically different subsets of IL-2-generated M1 population

M1 cells generated with the “IL-2 only” condition (6.2.1.1, Figure 6–1) were stained with either anti-CCR7-FITC (BD), anti-CD27-FITC or anti-CD28-PE in RPMI1640 with no Phenol red and 5% human serum. Single, live cells were then sorted on FACSaria II cell sorter based on positive or negative expression of CCR7, CD27 or CD28 separately (Figure 6–4). Cells were then adhered onto coverslips for fixation and stained with anti-Eomes by the PF/GS/BSA immunocytochemistry protocol described above.

![Pre-sort and Post-sort histograms](image)

Figure 6–4 M1 cells generated under the “IL-2 only” condition sorted by CCR7, CD28 or CD27 expression

M1 cells generated in the “IL-2 only” condition (as described in Figure 6–1) were stained for the expression of CCR7, CD28 and CD27 prior to and following cell sorting 24 days post-naive stimulation. For the post-sort data, the filled grey histograms represent unstained cells, the blue lines represent the negative expression subsets and the red lines represent the positive expression subsets for each marker.

6.2.5.2 Expression of transcription factors in M1 cells generated with IL-7, IL-2 or IL-15 during naïve expansion

M1 cells were generated by stimulation and expansion of naïve cells with IL-7, IL-2 or IL-15 alone (10ng/ml each) as described in Chapter 3 (3.2.2). On day 24 following naïve stimulation, rested M1 cells were adhered onto coverslips for fixation and stained for Lef-1, FoxO1 and Tcf-1 expression by the PF/GS/BSA immunocytochemistry protocol described above.
6.3 Results

6.3.1 Supplementation of IL-2 enhanced primary expansions but had negative effects on secondary expansions

We investigated whether a limited duration of additional IL-2 supplementation during the initiation of the primary expansion of naïve CD8\(^+\) T cells could rescue the failure of M1 populations to expand upon secondary challenge. Naïve CD8\(^+\) T cells were stimulated with the optimised condition of 3 days of IL-12 exposure and 14 days of IL-21 exposure with the IL-7 \textit{in vitro} model with and without 7 days of exogenous IL-2 (labelled as "IL-21" and "IL-21 + IL-2" conditions) and compared against an IL-2-based expansion with a 3-day exposure to IL-12 (labelled as "IL-2 only" condition) (Figure 6–1A). The proliferation of the primary and secondary expansions was tracked by assessing the cell numbers during both expansions.

During the primary stimulation, naïve CD8\(^+\) T cells across the 3 culture conditions proliferated with similar kinetics up to 9 days following TCR activation, in a manner similar to that reported in Chapter 3 (Figure 3–4). From day 6 onwards, those cells expanded without exogenous IL-2 proliferated at a steady, albeit slow, rate for the rest of the expansion. The presence of exogenous IL-2 induced a sharp increase in proliferation kinetics 9 days following stimulation. For the cells exposed to IL-21 and a limited duration of IL-2, the sharply enhanced proliferation kinetics continued until day 12, when the expansion kinetics gradually reduced to a plateau between day 15 and day 20 after stimulation. This procedure generated a population of M1 cells about 2 fold greater than those expanded without exogenous IL-2. For those cells expanded in the "IL-2 only" condition, the proliferation kinetics remained at a rapid steady rate of increment from day 9 onwards for the rest of the expansion, generating the largest population of M1 cells (Figure 6–5A). This suggests that the duration of IL-2 exposure during the primary expansion correlates to the final magnitude of expansion.

During the secondary challenge, the expansion of both cell populations grown in the IL-7 model with or without exogenous IL-2 peaked on day 7 post-restimulation and declined thereafter. The addition of IL-2 for a limited duration during the primary stimulation modestly reduced the overall magnitude of the secondary expansion. The M1 populations expanded in the IL-2-based cultures were found to reduce in size upon restimulation. Although some proliferation was observed following the initial decline, the cell numbers quickly plateaued and started to diminish again from day 15 post-restimulation (Figure 6–5B). These data suggest that although additional IL-2 signals can augment the magnitude of the primary expansion, they sensitized cells to apoptosis following restimulation. IL-2 is not the limiting factor for the inability of the M1 populations to accumulate upon secondary stimulation.
Further development of the in vitro model: Addition of IL-2 & IL-15

6.3.2 Addition of IL-15 supported the secondary expansions

Next, we sought to test whether IL-15 could support secondary expansion of the M1 populations. Due to the ability of IL-15 to induce loss of differentiation marker expression as previously observed (Figure 3–12G), we investigated the support of secondary expansions by IL-15 with 2 durations of exposure in comparison to an IL-7 only expansion. M1 cells generated from the “IL-21” and “IL-21 + IL-2” primary expansion conditions were employed for the secondary stimulations, where cells were restimulated with either IL-7 or IL-15 alone for the duration of the secondary expansion (labelled as “IL-7” and “IL-15” conditions), or IL-7 for the first 7 days post-restimulation followed by IL-15 for the rest of the expansion (labelled as “IL-7/15” condition) (Figure 6–1B). Cell numbers were tracked for the duration of the expansions.

We found the addition of IL-15 from day 7 post-restimulation rescued the effector cells for accumulation during the secondary expansion and elevated the magnitude of proliferation. The supplementation of IL-15 from the point of restimulation augmented expansion kinetics from day 3 or day 5 following stimulation in comparison to those stimulated with IL-7, and plateaued around day 15 post-restimulation. Then cell numbers modest declined towards the end of the expansion. Some donor variations were observed for the proliferation kinetics of the IL-15-supplemented conditions, but the IL-7 only condition consistently generated the largest population of cells, and the IL-7/15 condition also enhanced the magnitude of proliferation.

Figure 6–5 IL-2 augments primary expansions but reduces secondary expansions of naïve CD8+ T cells

A. Naïve CD8+ T cells were stimulated in duplicate with a 1:1 ratio of anti-CD3/CD28 beads for 2 days, in the presence of IL-12 for 3 days, and either with IL-21 for 14 days (“IL-21”) or IL-21 for 14 days and IL-2 for 7 days (“IL-21 + IL-2”). Both conditions received IL-7 from day 3 onwards. These conditions were compared to naïve cells stimulated in the presence of IL-12 for 3 days with IL-2 only (“IL-2 only”). Cell numbers were assessed on days 3, 6, 9, 12, 15 and 20 post-stimulation by the trypan blue exclusion method. B. M1 populations generated by the indicated primary priming conditions were stimulated again with anti-CD3/CD28 beads in 1:1 ratio for 2 days in either IL-7 for the “IL-21” and “IL-21 + IL-2” conditions, or IL-2 for the “IL-2 only” condition. Cell numbers were assessed on days 3, 5, 7, 10, 15 and 20 post-restimulation by the trypan blue exclusion method. Data depicts mean ± SEM of biological duplicates and is representative of 3 donors.
expansion above those expanded with only IL-7 (Figure 6–6). There was no consensus in the proliferative kinetics for the “IL-21” and “IL21 + IL-2” M1 populations upon secondary stimulation between the two donors examined. However, the trend of secondary proliferations for the three IL-7 and/or IL-15 conditions was consistent. Accumulation of cells upon secondary expansion with IL-15 supplementation suggests that the M1 populations were functional and had intrinsic capability to proliferate given adequate availability of survival signals. Cell survival following the secondary stimulation enables further assessments of memory cell qualities resulting from additional signals during primary as well as secondary expansions. We have denoted the rested cells generated following secondary expansions “M2” populations, for memory cells generated after two rounds of in vitro stimulation.

![Figure 6–6 IL-15 augments secondary expansions and allow the accumulation of M2 populations](image)

**Figure 6–6 IL-15 augments secondary expansions and allow the accumulation of M2 populations**

M1 populations generated by either primary conditions of “IL-21” (IL-12, 3 days; IL-21, 14 days; and IL-7 from day 3 onwards) or “IL-21 + IL-2” (IL-12, 3 days; IL-21, 14 days; IL-2, 7 days; and IL-7 from day 3 onwards) were restimulated with anti-CD3/CD28 beads in 1:1 ratio for 2 days in IL-7, IL-15, or IL-7 for the first 7 days then IL-15 from day 7 onwards (IL-7/15). Cell numbers were assessed on days 3, 5, 7, 10, 15 and 20 post-restimulation by the trypan blue exclusion method. 2 donors are shown.

### 6.3.3 Cellular attributes of M1 cells generated with IL-2 supplementation

The survival and proliferation of M1 populations upon secondary stimulation in the presence of IL-15 affirmed the functionality of these M1 cells. However, it was unclear how short term IL-2 supplementation had affected the cellular attributes of the M1 populations. Therefore, we assessed the functional properties of the populations generated with and without the 7-day exposure to IL-2 in the IL-7 in vitro model with IL-12 and IL-21 priming, and compared these to an IL-2-based expansion with IL-12 priming only (Figure 6–7).
Further development of the in vitro model: Addition of IL-2 & IL-15

6.3.3.1 Dynamic expression of phenotypic markers during primary expansion

We tracked the dynamic expression of several groups of phenotypic markers during primary expansions of the naïve CD8+ T cells expanded in the “IL-21”, “IL-21 + IL-2” and “IL-2 only” conditions. Our aim was to examine alterations in phenotypic marker expression over time to identify differences between different conditions.

For the expression of differentiation-associated markers, the “IL-2 only” condition induced the most substantial alterations. For this condition, the expression of CD62L, CD28 and CD27 was significantly reduced after 3–7 days post-stimulation relative to IL-7-based expansions. Expression of these markers in the IL-7-based conditions was largely maintained throughout the expansion, either with or without the short supplementation of IL-2 (Figure 6–8A). For CD27, some donor variations were observed. Expression for cells cultured in the “IL-2 only” condition either modestly inclined (data as shown) or plateaued (data not shown) following the minimum expression reached between day 5 to day 7 following stimulation, resulting in a final expression that was always lower than those observed for the IL-7 conditions. The expression of CCR7 fluctuated for the first 7 days following stimulation, but by day 7 reduction of expression was similar.
for the “IL-21 + IL-2” and “IL-2 only” conditions. However, those cells expanded with a short exposure of IL-2 recovered the CCR7 expression faster than “IL-2 only” cells. The fraction of cells expressing a naïve or SCM-like phenotype (CCR7⁺ CD45RA⁺) had similar alteration kinetics to CCR7 expression following a complete loss of co-expression at day 7 post-stimulation (Figure 6–8A).

The MFI of CD45RA largely reflected the percentage of cells with CCR7 and CD45RA co-expression, which reached a minimum at day 7 post-stimulation, and then recovered during the later phases of the expansions. A moderate increase of CD45RA expression across all conditions at day 3 post-stimulation suggested that CD45RA expression is temporarily affected by TCR stimulation. However, re-expression of CD45RA towards the end of the expansion was different across the three conditions. The M1 cells generated with no exogenous IL-2 had the highest re-expression of CD45RA on a per cell basis, followed by those generated with the “IL-21 + IL-2” condition, while the “IL-2 only” condition had the lowest expression (Figure 6–8B). From day 3 onwards, the MFI of CD45RO expression displayed an inverse pattern to CD45RA expression, with an increase to maximum expression on day 7, followed by a gradual decline to no expression by the end of the expansion (Figure 6–8B). The expression of CD25 (IL-2Rα) was largely similar across the different expansion conditions with a maximum on day 5 post-stimulation, and a decline to no expression between days 7 and 14 (Figure 6–8B).

The expression of the exhaustion-associated markers CD279 (PD-1) and CD152 (CTLA-4) was both observed to upregulate immediately following TCR activation. Their expression peaked at day 3 post-stimulation and downregulated to baseline levels by day 7 post-stimulation (Figure 6–8C). Minor fluctuations were observed from day 7 till the end of the expansion for these regulatory receptors, but the expression during this period was not significant (data not shown).

The expression of mTOR activity-associated markers also showed no particular differences across expansion conditions. Expression kinetics for CD71 (transferrin receptor) and CD98 (amino acid transporter) were superimposable across the three conditions (Figure 6–8D). Although some differences were observed for the expression of Glut1 (glucose transporter 1) between the different conditions, there were no consistency across donors other than the gross fluctuation patterns over time (data not shown).

Overall, the addition of IL-2 to the IL-12/IL-21-primed IL-7 expansion for the first 7 days of stimulation had minimal effects on phenotypic expression for a wide range of markers across the duration of naïve stimulation. The only exception was the expression of CCR7, where the recovery rate of CCR7 expression on “IL-21 + IL-2” cells was much slower than the rate for those cultured in the “IL-2” condition. At the end of the expansion period, however, they showed similar CCR7⁺ population fractions. In contrast, cells expanded in the IL-2-based culture showed significantly reduced expression for a number of differentiation markers. However, they showed similar expression kinetics to cells expanded in the IL-7-based conditions for markers associated with activation, mTOR activities and exhaustion.
Further development of the in vitro model: Addition of IL-2 & IL-15

Figure 6–8 Modulation of phenotypic molecules during primary expansion
Naïve CD8\(^+\) T cells were stimulated as described in Figure 6–5 with the indicated priming conditions. On days 3, 5, 7, 14 and 21 post-stimulation the cells were examined for the indicated markers by flow cytometry. **A.** Markers associated with differentiation plotted as percentage of cells positive for expression. **B.** Markers associated with differentiation and activation plotted by the median fluorescence intensities (MFI). **C.** Inhibitory receptors associated with exhaustion plotted as percentage of cells positive for expression. **D.** Markers associated with mTOR activity plotted by the MFI. Data shown are representative of 3 donors.
6.3.3.1 Heterogeneity of the IL-2-generated M1 population

The loss of expression of differentiation-associated markers by substantial proportions of the population expanded with the “IL-2 only” condition suggests potential generation of heterogeneous cell subsets. Therefore, we sought to determine whether the differences in phenotypic expression in the IL-2-expanded population were associated with transcription factor expression. We sorted the “IL-2 only” M1 population based on their expression of CCR7, CD28 or CD27, and stained for transcription factors Eomesodermin (Eomes) and T-bet by immunocytochemistry. We found that Eomes was expressed only in the CCR7⁻ populations but not in the CCR7⁺ populations, while no differences in Eomes expression were observed between the CD28⁺ and CD28⁻ populations. For the CD27⁺ and CD27⁻ subsets, Eomes expression was much more prominent in the CD27⁻ population for one donor, but showed minimal expression in both CD27⁺⁻ populations for the second donor (Figure 6–9). As for T-bet, there was either minimal or no expression found in all sorted cell subsets (data not shown). This indicates that prolonged culture with IL-2 creates heterogeneous populations in vitro, with distinct phenotypes and Eomes expression patterns that are associated with the loss of CCR7 expression and possibly CD27 expression.
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M1 cells generated in the “IL-2 only” condition (as described in Figure 6–5) were sorted on day 24 post-naïve stimulation by expression of CCR7, CD28 or CD27 and fixed on coverslips for immunocytochemistry from 2 donors. Cells were stained for DAPI (pseudocoloured grey) and Eomes (pseudocoloured cyan). Overlays and single colour stain for Eomes are shown. Objective magnification x40.

Figure 6–9 Heterogeneous expression of Eomes in the IL-2-generated M1 population is associated with CCR7 and CD27 expression
6.3.3.2 Effector functions

Next, we investigated whether IL-2 addition into the primary expansion of naïve CD8+ T cells had an effect on the development of effector functions upon secondary stimulation. M1 populations generated from the three conditions, “IL-21”, “IL-21 + IL-2” and “IL-2 only”, were restimulated and examined for their expression of IFN-γ, granzyme B, perforin and secretion of selected cytokines.

6.3.3.2.1 Expression of IFN-γ and lytic molecules, granzyme B and perforin

The supplementation of exogenous IL-2 for the first 7 days of primary expansion did not appear to have significant effects on the expression of IFN-γ, granzyme B or perforin upon secondary stimulation. Differences were observed only when the cells were expanded with prolonged exposure to IL-2. In this case, M1 population fractions capable of granzyme B or perforin expression were reduced, but expression of granzyme B on a per cell basis was increased (Figure 6–10).

![Figure 6–10 Effect of exogenous IL-2 during primary expansion on the expression of IFN-γ, granzyme B and perforin](image)

M1 populations generated by the indicated conditions, as described in Figure 6–5, with biological duplicates were restimulated in the presence of either IL-7 for the “IL-21” and “IL-21 + IL2” conditions or IL-2 for the “IL-2 only” condition. IFN-γ expression was examined after 6hr of restimulation. Granzyme B and perforin expression was examined after 3 days of restimulation. Percentages of live cells expressing each effector molecule and the median fluorescence signal (MFI) of each positive population were plotted. Each data point is the average value of the biological duplicates per donor. Bars indicate mean values across the 3 donors. Data were analysed by Friedman test and Dunn’s multiple comparison post-test. ns, not significant.
6.3.3.2.2 Secretion of cytokines

The addition of IL-2 for the first 7 days of naïve expansion did not have any significant effects on the secretion of the following cytokines: IL-2, IFN-γ, TNF, IL-6 and IL-10. However, cells expanded in the “IL-2 only” condition had significant higher IL-6 secretion and moderately lower TNF and IL-10 secretion compared to the “IL-21” condition (Figure 6–11A, B). The higher IL-6 and lower IL-10 secretions are likely to be due to the absence of IL-21, which was earlier found to downregulate IL-6 and enhance IL-10 (Chapters 4 (Figure 4–6) and 5 (Figure 5–10)). As IL-2 secretion could not be assessed for the M1 cells generated in the “IL-2 only” condition, we separately investigated the capability of the “IL-2 only” M1 population to secrete IL-2 by restimulation with IL-7. We found that “IL-2 only” M1 cells were capable of IL-2 secretion at similar levels as those generated in IL-7-based conditions (Figure 6–11C).

Figure 6–11 Effect of exogenous IL-2 during primary expansion on cytokine secretion upon secondary stimulation

A, B. M1 populations generated by the indicated conditions, as described in Figure 6–5, with biological duplicates were stimulated again at 2x10^6/200µl. Supernatants were aspirated from cultures at 24 and 48hr. The analytes indicated were detected using the BD CBA flex set assay at either 24 (A) or 48 (B) hr. Absolute concentrations of each analyte were plotted. Each data point is the average value from the M1 biological duplicates per donor. Bars indicate mean values across donors. Data was analysed by the Friedman test and Dunn’s multiple comparison post-test, except for IL-2, which was analysed by the Wilcoxon signed rank test. C. Cells grown in the “IL-2 only” condition were restimulated in the same manner in the presence of IL-7. Supernatants were collected and analysed for IL-2 secretion as above at 24hr. Data was from one donor. Solid line indicates maximum concentration for standard curve. ns, not significant; na, not applicable due to the supplementation of exogenous IL-2 in culture.
6.3.3.3 Susceptibility to apoptosis

Next, we investigated sensitivity to apoptosis upon restimulation for the M1 populations generated with the three expansion conditions. This was done by assessing the percentage of live cells by flow cytometry following a 24hr restimulation. We found that the addition of IL-2 had some effects on cell survival. The percentages of live cell events in the rested “IL-21 + IL-2” M1 populations were moderately reduced, while the live cell events in both “IL-21 + IL-2” and “IL-2 only” conditions also diminished upon restimulation, indicating their reduced capability for survival and enhanced susceptibility to apoptosis (Figure 6–12).

6.3.3.4 Expression of transcription factors

We then investigated the expression of a group of transcription factors known to be associated with CD8+ T cell effector function, differentiation and quiescence. Immunocytochemistry was used to assess naïve CD8+ cells expanded in the three expansion conditions. Three time points were assessed: 5 days post-naïve stimulation, rested M1 cells, and 1 day post-restimulation of M1 cells.

6.3.3.4.1 Transcription factor expression on day 5 following naïve stimulation

5 days following the primary stimulation of naïve CD8+ T cells, we found that the quiescence or differentiation arrest-associated transcription factors, Lef-1, FoxO1 and Tcf-1, appeared to be similarly expressed across the three expansion conditions. However, some differences were detected for the expression of the differentiation-associated transcription factors, T-bet and Eomes (Figure 6–13Ai, B, C). T-bet expression was observed to be higher for cells stimulated in the “IL-2 only” condition compared to cells expanded in the IL-7-based conditions (Figure 6–13Ai). Eomes expression detected by the conjugated (Figure 6–13Ai) and unconjugated (Figure 6–13B) versions of the same antibody with different immunocytochemistry protocols both showed that cells stimulated in the “IL-2 only” condition also
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Figure 6–13 Transcription factor expression 5 days following naïve stimulation

Naïve CD8+ T cells were stimulated in the conditions indicated (as described in Figure 6–5), extracted from culture 5 days following stimulation and fixed on coverslips for immunocytochemistry. A.i. Cells were stained with DAPI (pseudocoloured grey), Lef-1 (red), T-bet (green) and Eomes (AF647-conjugated, pseudocoloured cyan). Overlays and single colour stains for Lef-1, T-bet and Eomes are shown, in order from left to right. A.ii. Same images from (A.i) cut out and enlarged to show co-expression (Eomes pseudocoloured blue). B/C. (Next page) Cells stained with DAPI, FoxO1 (red) and either (B) Eomes (green, purified version) or (C) Tcf-1 (green). Overlays, single stains are shown for each condition, one cut out and enlargement are shown for each stain. Objective magnification x40.
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**B**

IL-21

IL-21 + IL-2

IL-2 only

**C**

IL-21

IL-21 + IL-2

IL-2 only

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expressed the highest levels of Eomes, while the “IL-21 + IL-2” cells in expressed slightly more Eomes than “IL-21” cells. Interestingly, the assessment time point fell within the period where exogenous IL-2 was added into the “IL-21 + IL-2” condition. This suggests that IL-21 priming may contribute to the reduction of T-bet and Eomes expression induced by IL-2.

While most transcription factor expression was found in the cell nuclei by an overlap with DAPI staining, FoxO1 was the only investigated transcription factor expressed in the cell cytoplasm only. This nuclear exclusion of FoxO1 was observed for all three populations of cells stimulated with different conditions.

From transcription factors co-staining, we found that differentiation arrest- or quiescence-associated transcription factors can be co-expressed with differentiation-associated transcription factors. Lef-1 was found to co-express in the nuclei with both T-bet and Eomes (Figure 6–13Aii), while FoxO1 and Eomes were expressed in multiple combinatory patterns (Figure 6–13B). Both FoxO1 and Eomes can be singularly expressed (Figure 6–13B, marker a), co-expressed in the nuclei (marker b), or present with FoxO1 expressed in the cytoplasm and Eomes expressed in the nuclei (marker c). FoxO1 and Tcf-1 were also found to co-express regardless of the FoxO1 expression location (Figure 6–13C). T-bet and Eomes could be either co-expressed or singularly expressed (Figure 6–13Aii). These heterogeneous patterns of co-expression suggest that these transcription factors do not directly influence the expression of one another.

6.3.3.4.2 Transcription factor expression in rested M1 cells

For rested M1 populations, we found Lef-1 to be uniformly expressed across the 3 expansion conditions, while the expression of T-bet and Eomes reduced to a few cells in “IL-21 + IL2” and “IL-2 only” populations, leaving the 3 conditions with similar expression (Figure 6–14A). In contrast, the expression of Tcf-1 in rested M1 cells was observed to be reduced for the “IL-21 + IL-2” condition, with a greater reduction for the “IL-2 only” condition. The expression of FoxO1 had mostly become cytoplasmic expression across all conditions, and showed reduced intensity for the “IL-2 only” condition (Figure 6–14B).

6.3.3.4.3 Transcription factor expression on day 1 following restimulation of M1 cells

Upon restimulation of the M1 populations, all expressed Lef-1 and FoxO1, but not Tcf-1. For the expression of Eomes and T-bet, there were relatively more cells expressing Eomes, but similar number of cells expressing T-bet, in the “IL-2 only” condition compared to the IL-7-based conditions (Figure 6–15).
Figure 6–14 Transcription factor expression in rested M1 cells

Naïve CD8\(^+\) T cells were stimulated in the conditions indicated (as described in Figure 6–5), allowed to expand and rest before fixation for immunocytochemistry. **A.** Cells were stained with DAPI (pseudocoloured grey), Lef-1 (red), T-bet (green) and Eomes (pseudocoloured cyan). Overlays and single colour stains for Lef-1, T-bet and Eomes are shown, in order from left to right. **B.** Cells stained with DAPI, Tcf-1 (green) and FoxO1 (red). Overlays and single colour stains for Tcf-1 and FoxO1 are shown, in order. FoxO1 single coloured images were also cut out and enlarged from each condition (fourth column). Data are representative of 2 donors, 1 donor repeated in 2 independent experiments. Objective magnification x40.
Further development of the in vitro model: Addition of IL-2 & IL-15

Figure 6–15 Transcription factor expression 1 day post-restimulation of M1 cells

M1 cells generated in conditions indicated (as described in Figure 6–5) were restimulated with anti-CD3/CD28 beads for 24hr either with IL-7 for the IL-21 inclusive conditions or IL-2 for the "IL-2 only" condition. At 24hr post-restimulation, the beads were removed and cells fixed on coverslips for immunocytochemistry. **A.** Cells were stained with DAPI (pseudocoloured grey), Lef-1 (red), T-bet (green) and Eomes (pseudocoloured cyan). **B.** Cells were stained with DAPI, FoxO1 (red) and Tcf-1 (green). Overlays of either all antigens or each transcription factor with DAPI are shown. Eomes is also shown as a single colour stain. Data are representative of 2 donors, 1 donor repeated in 2 independent experiments. Objective magnification x40.
6.3.4 Cellular attributes of M2 cells with the addition of IL-15 during secondary expansion

We have found that IL-15 enables the accumulation of cells during secondary expansion of M1 populations. However, we have also shown in Chapter 3 that culturing CD8⁺ T cells with IL-15 induces loss of key receptors associated with an undifferentiated phenotype. Thus, we generated M2 populations with the supplementation of IL-15 either for the entire course of the secondary expansion (labelled as “IL-15” condition) or from day 7 onwards post-restimulation (labelled as “IL-7/15” condition). These were compared with an IL-7-based restimulation (labelled as “IL-7” condition) (Figure 6–1B) to see whether there were differences in the expression of phenotypic markers and transcription factors in the rested M2 populations (Figure 6–16).

Figure 6–16 Experimental overview for Chapter 6, Section 6.3.4

A schematic diagram illustrating the experimental outline for Section 6.3.4, where M1 cells generated with the “IL-21” or “IL-21 + IL-2” conditions were restimulated under 3 cytokine supplementation regimes, “IL-7”, “IL-7/15” or “IL-15”, as described in Figure 6–1B, for the generation of M2 populations. The generated cells were assessed by 2 sets of experiments. The time point of investigation is shown. TF, transcription factor.
6.3.4.1 Surface expression of phenotypic molecules on rested M2 cells

We found the addition of IL-15 induced loss of CCR7 and CD28 expression in rested M2 populations (Figure 6–17A). CCR7 loss was the greatest in the “IL-15” condition and intermediate for the “IL-7/15” condition, suggesting the percentage of CCR7 loss related to the duration of IL-15 exposure, while expression of the other lymphoid homing molecule, CD62L, appeared to be unaffected by IL-15. The “IL-7/15” and IL-15 only conditions also induced the loss of CD28 expression to a similar extent; whereas expression of the other costimulatory receptor, CD27, was maintained, and no differences were observed across the three conditions. The percentage of the M2 populations expressing a naïve or SCM-like phenotype (CCR7+ CD45RA+) decreased with lengthened duration of IL-15 in culture, similar to the expression pattern of CCR7 between the three conditions. The majority of the cell population that was not co-expressing CCR7 and CD45RA was found to be CCR7+ CD45RA+, as shown with the inverse correlation of these two phenotypes with these three culture conditions. The expression of differentiation-associated molecules by the rested M2 populations appeared to be more prominently affected by the secondary expansion conditions, and no differences were observed between cells generated by the different primary expansion conditions (Figure 6–17A).

For expression of exhaustion-associated markers, there were marginal upregulations of CD279 (PD-1), CD244 (2B4) and CD57 by the supplementation of IL-15 during secondary expansion (Figure 6–17B). However, these upregulations were donor specific, not dependent on primary expansion conditions and the proportion of the population expressing them tended to be very small. Interestingly, a marker usually found on NK cells, CD56, was found to be significantly upregulated on cells exposed to IL-15, regardless of the duration of exposure (Figure 6–17B).
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Figure 6–17 Modulation of phenotypic molecule expression by IL-15 during secondary expansion
M1 populations generated by either the “IL-21” or “IL-21 + IL-2” conditions (as described in Figure 6–5) were restimulated in IL-7, IL-15 or IL-7 for the first 7 days then IL-15 from day 7 onwards (IL-7/15). Expression of indicated markers was assessed 24 days post-restimulation. A. Differentiation associated phenotypic markers. B. Exhaustion associated phenotypic markers and CD56. The percentage of expression was plotted for 2 donors.

6.3.4.2 Expression of transcription factors in M2 populations
Expression of the 5 transcription factors investigated previously in the M1 populations (6.3.3.4) was examined in the rested M2 populations generated in “IL-7/15” and IL-15 only conditions. We were unable to examine the M2 population generated in the IL-7 only condition due to low number of cells that survived till the end of the expansion. For the expression of quiescence- or differentiation arrest-associated
transcription factors, Lef-1 was found to be expressed in almost all cells in the populations examined (Figure 6–18A), similar to the previous time points investigated (Figure 6–13, Figure 6–14, Figure 6–15). In contrast, the expression of Tcf-1 was found to be abrogated in all the M2 populations examined (Figure 6–18B). The proportion of cells expressing FoxO1 appeared to have moderately reduced, especially for those that were expanded in the “IL-15” condition for the secondary expansion or in the “IL-21 + IL-2” condition during the primary expansion. For the differentiation-associated transcription factors, T-bet expression was not found in the rested M2 populations, and while some Eomes expression was observed, the proportion of the cell populations with positive Eomes expression remained relatively low. Eomes expression was expressed in the fewest cells in the population primarily expanded under “IL-21” condition and then under the “IL-7/15” condition following secondary stimulation (Figure 6–18A).

Figure 6–18 Transcription factor expression in M2 cells
M1 cells generated with the “IL-21” or “IL-21 + IL-2” conditions (as described in Figure 6–5) were restimulated with either IL-7 for the first 7 days then IL-15 (IL-7/15) or IL-15 alone (as described in Figure 6–6). Cells were allowed to expand and rest. 29 days following restimulation, cells were extracted and fixed on coverslips for immunocytochemistry. A. Cells were stained with DAPI (pseudocoloured grey), Lef-1 (red), T-bet (green) and Eomes (pseudocoloured cyan). Overlays and single colour stains for Lef-1, T-bet and Eomes are shown, in order from left to right. Data are representative of 2 donors. Objective magnification x40. B. Next page.
6.3.4.2.1 The reduction of Tcf-1 expression is associated with IL-7 and IL-2 signalling, but the uniform expression of Lef-1 and FoxO1 is not due to IL-7 support during primary expansion

It was interesting to have found a relatively uniform expression (or lack of expression) of Lef-1, FoxO1 and Tcf-1 across all the primary and secondary expansion conditions for most of the time points examined. Therefore, we sought to investigate whether IL-7 signals received during the priming expansion had a role in the expression of these quiescence-associated transcription factors. We stimulated and expanded naïve CD8+ T cells in IL-7, IL-2 or IL-15 alone, as performed in Chapter 3, and stained for Lef-1, FoxO1 and Tcf-1 expression by immunocytochemistry when the cells had rested from the primary expansion. We found both Lef-1 and FoxO1 were still uniformly expressed across the three M1 populations expanded in different cytokines, and FoxO1 expression was found mostly in the cytoplasm across the different conditions. However, uniform Tcf-1 expression was only found in the IL-15-expanded M1 population and greatly reduced in the IL-7- and IL-2-expanded M1 cells (Figure 6–19). This suggests that IL-7 and IL-2 signalling may have had a role in the reduction of Tcf-1 expression in the M2 populations, whereas the uniform expression of Lef-1 and FoxO1 was not due to constant IL-7 signalling in vitro.
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Naïve CD8⁺ T cells were stimulated with IL-7 (10ng/ml), IL-2 (10ng/ml) or IL-15 (10ng/ml). Cells were allowed to expand and rest with continuous supplementation of the respective cytokines. On day 24 post-stimulation, cells were extracted and fixed on coverslips for immunocytochemistry. Cells were stained with DAPI (pseudocoloured grey) and either Lef-1 (red) or FoxO1 (red) with Tcf-1 (green). For each transcription factor stained, an overlay with DAPI and single colour stains are shown. Enlarged cut outs for FoxO1 are also included (bottom panel, 4th column). Objective magnification x40.
6.3.5 Comparison to ex vivo memory subsets by the expression of transcription factors

We have so far established that expanding naïve CD8⁺ T cells in vitro in an IL-7-based system with short term IL-12 exposure and long term IL-21 exposure generated functional memory cells with many enhanced cellular attributes. The addition of IL-2 for the first week of primary stimulation appeared to enhance the primary expansion with no major undesirable effects, and these in vitro-generated memory cells were able to undergo secondary expansion with the supplementation of IL-15. However, how these cells compare to the in vivo-generated memory cells are unknown. Therefore, we sought to investigate the expression of transcription factors of the ex vivo CD8⁺ memory cell subsets in comparison to the in vitro-generated M1 populations as an indication for the differentiation status of the M1 cells. Isolated ex vivo CD8⁺ T cells were sorted by fluorescence activated cell sorting (FACS) by their expression of CCR7 and CD45RA into naïve (N; CCR7⁺ CD45RA⁺), central memory (CM; CCR7⁺ CD45RA⁻), effector memory (EM; CCR7⁻ CD45RA⁻) and effector memory with CD45RA expression (EMRA; CCR7⁻ CD45RA⁺) populations, and fixed immediately to examine their transcription factor expression by immunocytochemistry.

We found the expression of Lef-1, FoxO1 and Tcf-1 gradually decreased with the more differentiated phenotypes of ex vivo CD8⁺ T cells. Lef-1 was expressed in all cells in the naïve population, a proportion of cells in the CM subset and a minimal number of cells in both the EM and the EMRA populations (Figure 6–20A). The expression pattern of Tcf-1 across the memory subsets was similar to that found with the expression of Lef-1, where Tcf-1 expression was found in all cells in the naïve population, a proportion of cells in the CM subset and no expression in the EM and EMRA populations (Figure 6–20B). FoxO1 appeared to be expressed in all ex vivo memory subsets. However, the intensity of staining was the brightest in the naïve cells and very dim in the EM and EMRA populations. Based on the expression of these quiescence-associated transcription factors, the M1 populations generated in the IL-7-based cultures were the most similar to the ex vivo naïve cells, with the majority of the cell populations expressing Lef-1 and FoxO1. Tcf-1 expression in the M1 cells generated with the “IL-21” condition was also similar to those found in the naïve cells, although the “IL-21 + IL-2” condition generated M1 populations were more similar to the ex vivo CM subset with reduced Tcf-1 expression (Figure 6–14). Interestingly, the expression of Lef-1 and FoxO1 by the M2 cells closely resembled those found in the CM subset, but the abrogated expression of Tcf-1 was more similar to those found in the EM and EMRA populations (Figure 6–18), perhaps due to the long durations of exposure to IL-7 and/or IL-2 (Figure 6–19).

For the expression of the differentiation-associated transcription factors, T-bet was rarely expressed in any of the ex vivo memory cell populations, while the expression of Eomes was increased with the more differentiated phenotypes (Figure 6–20A). The expression of Eomes was found to be variable between donors; some showed a much more dramatic increase in the expression of Eomes in the ex vivo EM and EMRA populations than others (Figure 6–20C). The expression of Eomes found in both the M1 and M2 populations was relatively low, similar to the expression found in the naïve or CM cells. Overall, both the
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rested M1 and M2 populations had a transcription factor expression pattern most similar to the ex vivo naïve or CM cells.

Figure 6–20 Transcription factor expression in ex vivo CD8+ memory subsets
CD8+ T cells were isolated from PBMC by MACS separation, sorted into memory subsets (N=Naïve, CCR7+ CD45RA+; CM=central memory, CCR7 CD45RA+; EM=effector memory, CCR7 CD45RA+; EMRA=effector memory with CD45RA expression, CCR7 CD45RA+) and fixed on coverslips for immunocytochemistry. A. Cells were stained with DAPI (pseudocoloured grey), Lef-1 (red), T-bet (green) and Eomes (pseudocoloured cyan). Overlays and single colour stains for Lef-1, T-bet and Eomes are shown, in order from left to right. Data are representative of 3 donors from 2 independent experiments. Objective magnification x40. B/C. Next page.
Figure 6–20 Transcription factor expression in ex vivo CD8+ memory subsets cont.

**B.** Cell stained with DAPI (pseudocoloured grey), FoxO1 (red) and Tcf-1 (green). Overlays and single colour stains for FoxO1 and Tcf-1 are shown, in order from left to right. **C.** Eomes staining with DAPI from a different donor. Data are representative of 3 donors from 2 independent experiments. Objective magnification x40.
6.4 Summary and discussion

In this chapter, we aimed to rescue the incompetency of M1 populations to accumulate upon secondary stimulation, and found that while additional IL-2 signals during the primary expansion did not enable their survival, supplementation of IL-15 during the secondary expansion was critical. We then compared the transcription factor profile of M1 populations with ex vivo CD8+ memory cell subsets, and found that M1 populations had similar transcription factor expression to ex vivo naïve or CM cells, confirming their early differentiation status at the level of gene transcription regulations.

6.4.1 Addition of exogenous IL-2 for the generation of M1 cells

Following the investigation of the effects of additional exogenous IL-2 for the first 7 days of primary expansion in the IL-7 in vitro model with IL-12/IL-21 priming, it was found that supplementation of IL-2 was able to enhance the magnitude of primary expansion as expected, but that additional IL-2 signals did not enable the accumulation of cells upon secondary stimulation, and moderately reduced the magnitude of secondary expansions. This suggests that IL-2 was not the limiting factor for the inability of the M1 populations to expand upon secondary stimulation. However, interesting observations were made for the effects of IL-2 on these cells.

Memory cells generated with IL-2-based expansions were observed to have reduced expression of CCR7, CD62L, CD28 and CD27, enhanced susceptibility to apoptosis, and altered production of effector molecules and secretion of cytokines, as well as reduced expression of the transcription factors Tcf-1, FoxO1 and moderately enhanced expression of T-bet and Eomes. Unlike IL-2-based expansions, 7-day IL-2 supplementation to M1 cells generated with the IL-7-based expansion had negligible effects on the surface phenotype, effector molecule expression or cytokine secretion capabilities, despite its distinctive effect on the enhancement of the primary expansion. Short-term IL-2 exposure for cells expanded in the IL-7 model with IL-12 and IL-21 priming induced moderate reduction of cell survival following restimulation, and reduced expression of Tcf-1 in rested M1 populations. Expression of other transcription factors, including Lef-1, FoxO1, T-bet and Eomes, was found to be similar with or without short-term IL-2 exposure in the IL-7 model. Finding such minimal alterations following the addition of IL-2 into the IL-7-based expansion was unexpected. It indicates that short-term IL-2 exposure can be used to increase the magnitude of proliferation for the primary expansion of naïve CD8+ T cells with limited effects on cellular attributes. These results suggest that prolonged IL-2 exposure is necessary to drive effector development and terminal differentiation of CD8+ T cells, and/or the IL-7-based in vitro culture together with IL-21 priming minimised differentiation as previously implicated (Albrecht et al., 2011; Hinrichs et al., 2008). This capability of IL-21 to restrain IL-2-induced differentiation may play a role in chronic infections, by sustaining CTL functionality (Elsaesser, Sauer, and Brooks, 2009; Frohlich et al., 2009; Yi, Du, and Zajac, 2009). It suggests that IL-2 signalling routes for cellular proliferation and sensitivity to apoptosis may be different.
from those that induce differentiation and effector functions, where specific downstream effects could be selectively inhibited.

The extensive alterations in proliferation kinetics, surface phenotypes, effector functions, apoptosis upon restimulation and expression of transcription factors in the “IL-2 only” condition were consistent with previous studies. IL-2 has long been identified to be a potent T cell growth factor (Miyazaki et al., 1995) and has been found to sustain T cell expansions (D'Souza and Lefrancois, 2003). Indeed, we have shown that naïve expansion in IL-2 with IL-12 priming augmented the proliferation of naïve CD8+ T cells, leading to linear expansion kinetics that lasted for at least 20 days post-stimulation. Interestingly, it appeared that the presence of IL-2 did not alter proliferation kinetics for the first 6 days following naïve cell activation. During this period, the intrinsic programme of cell cycling activities following strong TCR stimulation may be the dominant driving force. However, the magnitude of the secondary expansions of M1 populations generated with exposure to IL-2 was found to be reduced. This may be due to IL-2-induced susceptibility to apoptosis and reduced survival capabilities following restimulation as discussed previously in Chapter 3.

The loss of receptors associated with an early differentiation phenotype that regulates the capability for lymphoid organ homing and costimulatory signalling suggests that IL-2-generated memory cells are directed to travel to peripheral tissues with reduced capability to re-mount immune responses. IL-2 expansions also generated heterogeneous cell subsets, where the loss of CCR7, and possibly CD27, were associated with the expression of Eomes, the transcription factor responsible for effector function development and memory cell generation (Banerjee et al., 2010; Cruz-Guilloty et al., 2009). This is consistent with previous studies showing that IL-2 signals direct an effector/effector memory differentiation and induce Eomes expression in mice (Manjunath et al., 2001; Pipkin et al., 2010; Weninger, Crowley, Manjunath, and von Andrian, 2001). With the overall reduced expression of Tcf-1 and FoxO1, and moderately enhanced expression of T-bet and Eomes in the cells generated with the IL-2-based condition, this transcription factor profile also indicates a status of further differentiation.

Although loss of the differentiation-associated receptors is usually linked to an increment in cytolytic effector functions (Appay et al., 2002; Romero et al., 2007), populations grown in the IL-2-based condition were found to have either similar or lower effector molecule expression and cytokine secretion compared to those grown in IL-7-based conditions. Reduced effector functions include production of granzyme B and perforin, and TNF secretion. These apparent reductions in effector functions are not due to functional exhaustion associated with the IL-2-induced extensive proliferation, since there was an absence of expression for exhaustion markers, and production of the effector molecules and cytokines was still relatively robust (Wherry et al., 2007). Therefore, it is likely that the long term exposure to IL-21 together with IL-12 priming in the IL-7-based expansion conditions had augmented the expression of at least some of the effector molecules, especially IFN-γ and TNF, as described in Chapter 5. IL-2-induced apoptosis upon restimulation may also have partially contributed to reduced cytokine secretion and reduced production of effector molecules.
6.4.2 Addition of IL-15 to support secondary expansion and survival of M2 cells

IL-15 supplementation successfully supported secondary expansion and survival of the in vitro-generated memory cells. This implies that the previously observed incapability of M1 populations to expand upon restimulation was due to a lack of adequate survival signals to support the accumulation of cells, that M1 populations are functional for recall responses, and that IL-2 autocrine signalling during naïve CD8⁺ T cell stimulation is sufficient to generate competent memory cells. IL-15 supplementation during secondary expansion of the M1 population was also found to reduce CCR7 and CD28 expression, and induce CD56 expression on rested M2 populations. However, it had no significant effects on the expression of CD62L, CD27, the exhaustion markers PD-1 and 2B4, or the replicative senescence-associated marker, CD57 (Brenchley et al., 2003). In terms of transcription factor expression, the lengthened duration of IL-15 exposure together with IL-2 supplementation during the primary expansion was found to moderately enhance the expression of Eomes and reduce FoxO1. No expression of T-bet or Tcf-1 was found in any of the rested M2 populations, and the expression of Lef-1 was similar across all conditions.

These results indicate that IL-15 plays a role in the regulation of qualitative attributes of CD8⁺ memory cells, with homing and costimulatory capabilities being partially affected by selected markers. Although the role of IL-15 in the homeostasis and survival of T cells is well established, different studies had reported either a significant role (Sandau, Kohlmeier, Woodland, and Jameson, 2010; Schluns, Williams, Ma, Zheng, and Lefrancois, 2002) or a lack of effects (Becker et al., 2002; Wherry et al., 2002a) for IL-15 in the generation of effector CD8⁺ T cells or CD8⁺ T cell differentiation. There have been difficulties with the use of both in vivo and in vitro systems to establish whether the diminished percentage of effector cells found in IL-15 signalling-deficient CD8⁺ T cell pools were due to an absence of differentiation, or to a lack of survival of differentiated cells, or both. Our studies, where alterations of differentiation-associated phenotypic marker expression and an enhancement of expansion were observed with IL-15, indicate that this cytokine plays a role in regulating both survival and differentiation. The phenotypic alterations following IL-15 supplementation are different to IL-2-supported expansions, where a wider range of differentiation-associated markers was affected and a distinctive effector differentiation lineage was specified. It appeared that IL-15-directed limited differentiation and affected the expression of only a selected subset of differentiation-associated markers. This helps to explain discrepancies in previous reports for IL-15’s effects on differentiation. In cases where only one or two selected memory markers were investigated, the effects of IL-15 may or may not have been observed. IL-15-induced CD56 expression is consistent with previous observations where IL-15 was found to upregulate NK cell receptor expression on human CD8⁺ T cells. This correlated with augmented production of effector molecules (Correia, Costa, Uhrberg, Cardoso, and Arosa, 2011; Pittet, Speiser, Valmori, Cerottini, and Romero, 2000). In summary, these data indicate that IL-15 plays a specific role in the differentiation of CD8⁺ T cells, through regulation of distinct cellular attributes.
Chapter 7: General discussion

Durable and effective immunity requires the optimal generation of memory cells with the capabilities to persist and exert effector functions to confer host protection. The integration of the signals during naïve cell activation plays an important role in the programming of the differentiation pathway of the stimulated T cells and thus governs the type and strength of the immune responses, as well as regulating the generation of the memory cells. Many clues for the regulation of CD8⁺ T cell differentiation have been provided by murine models over the last few decades. However, knowledge of the factors that programme naïve cells and govern the optimal generation of memory cells in humans is limited. We aimed to study how human naïve CD8⁺ T cells are programmed, and the effects of this programming on the differentiation and generation of memory cells. First, we established a unique culture system where human naïve CD8⁺ T cells can be stimulated in vitro, expanded and allowed to rest into memory populations. These memory populations were then able to be restimulated to assess their cellular attributes. We then used this system to explore the programming effects of cytokine exposure during naïve cell activation. We found three-day cytokine exposure during naïve CD8⁺ T cell activation programmed the quality of the memory cells generated, with alterations to cell surface phenotype, cytokine secretion profiles and expression of lytic molecules. We then found specific cellular attributes could be programmed by a combination of the priming cytokines, generating memory cells with optimal characteristics. Subsequently, we were also able to investigate and correlate the programming conditions and the cellular attributes of the generated memory populations to their expression of transcription factors, and gain insights into the molecular regulation of human CD8⁺ T cell differentiation.

7.1 IL-7 culture system

During the establishment of the in vitro model system, we confirmed that IL-7 provides the optimal survival signals for naïve and memory populations with minimal effects on cellular attributes in comparison to a range of γc cytokines, both for cells at rest and during expansion. We have successfully expanded naïve CD8⁺ T cells with IL-7 over 3 weeks, and rested them into memory populations ("M1" cells) with an early differentiation phenotype and minimal expression of activation markers. These memory cells could then be restimulated without activation-induced death, so their function could be tested. The absence of exogenous IL-2 supplementation in this culture system indicates that we can avoid the IL-2-driven terminal differentiation and AICD sensitivity commonly observed in CD8⁺ T cells cultured with continuous IL-2 (Kalia et al., 2010; Lenardo, 1991; Pipkin et al., 2010). Therefore, we have established a unique in vitro system, whereby the programming of human CD8⁺ T cells by signals received during their priming can be studied by examining the resting memory cells they produce.

With the IL-7 in vitro system, we found naïve CD8⁺ T cells were capable of expanding with endogenous IL-2 following TCR and CD28 stimulations regardless of exposures to third signals, implying signals 1 and 2
are sufficient to trigger an autonomous programme for proliferation. The secretion of IL-2 by the stimulated naïve cells indicated that the IL-2 autocrine signalling critical for the generation of functional memory cells was available (Feau et al., 2011; Williams et al., 2006). Our in vitro system also showed a striking difference in the expansion of naïve cells from that of the first generation of memory cells. Expansion of both cell populations was supported by endogenous secretion of IL-2. However, while IL-7 was sufficient to allow survival of memory T cells following the stimulation of naïve cells, it was not sufficient to support their survival following the stimulation of first generation “M1” memory cells. Additional IL-2 supplementation during primary expansion did not support M1 survival either, but IL-15 availability during secondary expansion did. Our results concur with recent evidence in mice showing IL-15 has an important role in the survival of secondary effector cells, and the generation of the secondary memory populations (Hervas-Stubbs et al., 2012; Sandau et al., 2010; Soudja, Ruiz, Marie, and Lauvau, 2012). Because of our unique system where generations of memory cells can be compared in a controlled fashion, we believe this is the first time similar results have been observed in humans.

7.2 Cell surface phenotype

Following the stimulation and expansion of human naïve CD8+ T cells with our IL-7 in vitro model, the first generation memory “M1” populations were able to be rested and maintained in vitro with IL-7 as the only available γc cytokine. Examination of the surface phenotypes of the M1 populations found a general absence of exhaustion and activation markers, confirming the resting and functional status of the M1 populations by phenotypic expression. The majority of the M1 populations were also found to express phenotypic markers correlated with an early differentiation state, including CCR7, CD62L, CD28 and CD27 that are associated with survival, proliferative capacity, and effector capability. Somewhat surprisingly, CD45RA was also prominently expressed – whereas much of the literature has regarded the switch from CD45RA to CD45RO expression as a definitive phenotypic change between naïve and central memory cells (Appay et al., 2002; Champagne et al., 2001). However, the phenotype of the M1 cells we generated closely resembles the recently described stem cell-like memory (SCM) cells in ex vivo human PBMCs with the expression of CD45RA, CCR7 and costimulatory receptors (Gattinoni et al., 2011). SCM cells possess optimal survival and self-renewal capabilities compared with the CCR7+ CD45RA-CD28+CD27+/− EMRA cells. This is consistent with recent evidence that indicated CD45RA re-expression is not strictly correlated with terminal differentiation (Henson, Riddell, and Akbar, 2012). Such re-expression of CD45RA on non-terminally differentiated antigen-experienced cells is thought to have developed in the presence of homeostatic cytokines following the withdrawal of antigen (Henson et al., 2012), consistent with our culture conditions. The generation of CD45RA+ antigen-specific memory cells has also been observed in human vaccination studies that possessed memory cell characteristics including high proliferative potential, long term persistence and polyfunctionality (Ahmed and Akondy, 2011; Akondy et al., 2009). The generation of these M1 populations indicate that human naïve CD8+ T cells can be expanded successfully in vitro without the use of exogenous IL-2, and generate memory cells with very early differentiation status.
The addition of cytokines to the IL-7 culture system during the first 3 days of T cell priming had moderate effects on the phenotype of the rested memory cells generated three weeks later. The three-day IL-12 exposure upon naïve cell activation was found to increase the proportion of M1 cells that had lost CCR7 and CD45RA expression, resulting in the highest percentages of CM, EM and EMRA cells across the M1 populations, although these cells maintained high CD27 expression. This enhanced loss of CCR7 and CD45RA expression is consistent with previous observations on IL-12-exposed human cells 7 days following primary stimulation (Ramos et al., 2009), and supports the observations in mice where IL-12 drives effector and terminal differentiation (Joshi et al., 2007; Keppler and Aichele, 2011).

In contrast, three-day IL-4 and IL-21 priming were found to generate M1 populations with the highest expression of homing molecules and costimulatory receptors, suggesting IL-4 or IL-21 exposure promoted an early differentiation status and durability of the M1 populations. For IL-4 priming, this phenotype is consistent with the higher CD28 expression found on human Tc2 clones (Vukmanovic-Stejic et al., 2000). For IL-21 priming, this is also consistent with the higher expression of CCR7, CD62L and CD27 found on human tumour-specific CTLs stimulated with T-cell APCs genetically modified to produce IL-21 (Kaka et al., 2009), and the enhanced expression of CD28 found on human cells 7 days following stimulation with continuous IL-21 (Alves et al., 2005; Ansen et al., 2008; Li et al., 2005). A number of other reports have observed either a maintenance or reduction of CCR7, CD62L and CD27 expression on human CD8+ T cells (Ansen et al., 2008; Li et al., 2005; Singh et al., 2011). However, these studies have employed IL-2 in their systems, which is likely to have an influence on the phenotypic expression of CD8+ T cells via its capability to drive differentiation (Kalia et al., 2010; Pipkin et al., 2010). The maintenance of the memory marker expression with IL-21 priming in the absence of IL-2 signalling is consistent with its general role observed to restrict differentiation in mice (Cox et al., 2011; Hinrichs et al., 2008; Yi, Ingram, and Zajac, 2010), providing a potential mechanism for the reduction of memory erosion in chronic infections by CD4+ T cell help (Elsaesser et al., 2009; Frohlich et al., 2009; Yi et al., 2009).

The combined exposure of IL-12 plus IL-21 was observed to maintain the overall SCM-like phenotype, where the extended exposure of IL-21 further enhanced CD28 expression, which is consistent with previous observations (Alves et al., 2005; Ansen et al., 2008; Li et al., 2005). The increased expression of CD28 will be able to provide enhanced costimulatory signals upon restimulation and contribute to better survival of restimulated cells via Bcl-xL induction (Boise et al., 1995).
Chapter 7

7.3 Function

7.3.1 Cytokine secretion profiles

With the IL-7 in vitro system, we found the stimulated naïve CD8\(^+\) T cells are able to produce IL-2 and IFN-\(\gamma\) upon TCR stimulation without third signals, providing evidence for their inherent capability to exert effector functions and functional memory development (Feau et al., 2011; Williams et al., 2006). This is consistent with observations in mice, where murine cells stimulated in vitro were found to display basal effector activities upon both primary and secondary stimulations (Curtsinger et al., 2003b). And indeed, we have also found the M1 populations primed with or without third signals produce a myriad of effector cytokines and chemokines upon secondary stimulation, indicating their autonomous capability to develop basal cytokine productions following TCR and costimulatory signals (signals 1 and 2) during naïve activation.

We observed a broad range of cytokines secreted by the M1 populations primed in the absence of third signals, encompassing both type 1 and type 2 cytokines: these included the effector cytokines IL-2, IFN-\(\alpha\) and TNF, type 2 reaction cytokines IL-4, IL-5, IL-13 and GM-SCF, as well as IL-10. This pattern may represent an uncommitted phenotype due to the lack of third signal cytokines upon priming. The capability to co-produce IL-2, IFN-\(\gamma\) and TNF has been previously correlated with an early differentiation state (Seder et al., 2008) and indicates superior quality to survive (Rutishauser and Kaech, 2010), and promote cytotoxic activities (Betts et al., 2006; Harari et al., 2006; Imai, Ikeda, Tawara, and Shiku, 2009; Seder et al., 2008; Wilde et al., 2012). The general secretion of IL-10 across our M1 populations is likely to be a negative feedback control resulted from strong TCR stimulations, where IL-10 has been observed to be promoted in active effector cells (Altin, Goodnow, and Cook, 2012; Gabrysova et al., 2009; Saraiva et al., 2009; Trandem et al., 2011), potentially to reduce immunopathology (Cope, Le Friec, Cardone, and Kemper, 2011; O'Shea and Paul, 2010; Saraiva and O'Garra, 2010). This restriction of inflammation and immune reactions has been observed to be required for optimal CD8\(^+\) T cell memory formation (Cui, Liu, Weinstein, Craft, and Kaech, 2011; Foulds, Rotte, and Seder, 2006).

Upon exposure of naïve CD8\(^+\) T cells to third signal cytokines for the first three days following TCR stimulation, the M1 populations generated three weeks later were found to be programmed to change their behaviour. Patterns of cytokine secretion upon secondary stimulation were consistently skewed by the third signals delivered to the naïve cells, providing what we believe is the first experimental evidence for successful programming of human memory CD8\(^+\) T cells by third signals at priming. M1 populations primed in the presence of IL-12 were found to significantly enhance type 1 cytokine secretions upon secondary stimulation, IL-4 priming enhanced the type 2 cytokines, and IL-21 priming significantly reduced IL-6, delayed IFN-\(\gamma\) production and upregulated IL-10 secretion, as discussed below.
7.3.1.1 With IL-12 priming

We found a three-day exposure to IL-12 upon naïve CD8^+ T cell activation programmed the memory cells to increase production of all type 1 cytokines (including IFN-γ, IL-2, MIP-1α and TNF) upon secondary stimulation 21 days later. A number of murine models consistently observed the same effect of IL-12 on subsequent IFN-γ secretion (Agarwal et al., 2009; Chowdhury et al., 2011; Cui et al., 2009; Curtisinger et al., 1999; Lisiero et al., 2011; Ramos et al., 2009; Schmidt and Mescher, 1999). Similar effects have also been reported in some previous studies of human CD8^+ cells, although in a slightly different context, where cells primed in the presence of IL-12 were restimulated within the first week (Chowdhury et al., 2011; Ramos et al., 2009), before they had rested and established a memory phenotype, as in our IL-7-supported model.

These short-term human models had also shown increases in IL-2 and TNF secretion following priming in the presence of IL-12, by comparisons with neutral (Chowdhury et al., 2011; Ramos et al., 2009) or Tc1 (Halverson, Schwartz, Carter, Gress, and Fowler, 1997) conditions, respectively. This enhanced secretion of IL-2 by IL-12 priming may have contributed to the more differentiated attributes of the IL-12-primed M1 populations, since IL-2 drives effector differentiation (Kalia et al., 2010; Pipkin et al., 2010). This effect may have been augmented by the upregulation of CD25 expression by IL-12 (Lisiero et al., 2011; Pipkin et al., 2010; Valenzuela, Schmidt, and Mescher, 2002).

An increased IL-10 production was also observed to be programmed by IL-12 priming, which has not been previously reported for human CD8^+ T cells. However, consistent observations were found in murine models (Saraiva et al., 2009), where IL-10 was found to aid the survival of effector CD8^+ T cells and enlarge antigen-specific populations potentially by limiting inflammation (Lee, Lee, and Chang, 2007).

7.3.1.2 With IL-4 priming

Three-day IL-4 exposure during naïve cell priming was observed to programme an upregulated secretion of IL-4, IL-5, IL-13, GM-CSF and IL-10, as well as a downregulated production of IFN-γ, upon secondary stimulation 21 days later. The skewing towards a type 2 response is consistent with previous studies in human CD8^+ T cells exposed to continuous IL-4, as assessed by IL-4, IL-5 and IL-10 production (Halverson et al., 1997; Stanciu, Roberts, Lau, Coyle, and Johnston, 2001; Vukmanovic-Stejic et al., 2000). However we are not aware of any other studies that have demonstrated programming of human CD8^+ T cells after such short exposure to IL-4 during priming.

Murine models of both CD8^+ and CD4^+ T cells have clearly demonstrated that IL-4 polarizes cytokine secretion towards type 2 cytokines (Cerwenka, Carter, Reome, Swain, and Dutton, 1998; Croft, Carter, Swain, and Dutton, 1994; Kemp et al., 2005; Le Gros, Ben-Sasson, Seder, Finkelman, and Paul, 1990; Mosmann and Coffman, 1989; Sad, Marcotte, and Mosmann, 1995). In CD4^+ T cells, Th2 differentiation requires both IL-4 and IL-2-induced STAT-5 activation (Cote-Sierra et al., 2004; Le Gros et al., 1990; Zhu
et al., 2003), and exogenous IL-2 has always been added to culture media when Tc2 cells are generated in vitro in the reported studies in the literature. The enhanced secretion of the type 2 cytokines in our system without IL-2 supplementation indicates that either the endogenous IL-2 secretion was sufficient for autocrine signalling, or the continuous presence of IL-7 provided a substitute for the activation of STAT-5 signalling.

The reduction in IFN-γ secretion we observed is consistent with previous observations of human cells following continuous IL-4 signalling (Vukmanovic-Stejic et al., 2000), or repeated TCR stimulations in the presence of IL-4 (Stanciu et al., 2001). Murine CD4+ T cell studies have suggested a potential mechanism for this reduction of IFN-γ expression, where the IL-4-induced GATA-3 activities were found to repress IFN-γ production (Usui, Nishikomori, Kitani, and Strober, 2003; Yagi et al., 2010). Even so, memory cells that had been primed in the presence of IL-4 in our system still secreted IFN-γ, albeit at reduced levels. This is perhaps not surprising, given that even in murine systems, strong polarisation towards a type 2 secretion profile requires addition of anti-IFN-γ to cultures as well as IL-4 (Sad et al., 1995). Indeed, CD8+ T cells cultured under Tc2 conditions have often demonstrated continued secretion of IFN-γ, with or without neutralising antibodies (Cerwenka et al., 1998; Croft et al., 1994; Kemp et al., 2005; Stanciu et al., 2001; Vukmanovic-Stejic et al., 2000). Naïve CD8+ T cells express Runx3 (Taniuchi et al., 2002), a transcription factor responsible for the induction of Eomes, IFN-γ and lytic molecules in CD8+ T cells (Cruz-Guilloty et al., 2009) and the inhibition of IL-4 in CD4+ T cells (Djuretic et al., 2007). Similarly, CD8+ T cells have heightened expression of ROG (repressor of GATA-3), which represses IL-4 production and GATA-3-induced transactivation (Miaw, Choi, Yu, Kishikawa, and Ho, 2000; Omori et al., 2003). This transcription factor profile makes it unsurprising that IFN-γ secretion is still detected in our system, despite successful skewing towards a Tc2 phenotype.

7.3.1.3 With IL-21 priming

We found that three-day exposure to IL-21 during CD8+ T cell priming programmed the cells to reduce secretion of IL-6, increase secretion of IL-10, and delay secretion of IFN-γ upon secondary stimulation 21 days later. Some published work with human cells has reported that continuous IL-21 exposure increased IFN-γ secretion, however this was either in the context of continuous IL-21 exposure with IL-2 or IL-15 supplementation (Alves et al., 2005) or repeated TCR stimulations (Ansen et al., 2008; Kaka et al., 2009), and is therefore difficult to directly compare to our experimental system. The reduction of IFN-γ production we observed immediately following the secondary stimulation is consistent with murine in vitro systems where no additional γc cytokines were supplemented, and the expression of IFN-γ assessed within 4 days of continuous IL-21 exposure (Casey and Mescher, 2007; Hinrichs et al., 2008). Another murine study found IL-21 had opposing effects on IFN-γ production in acute versus chronic LCMV infections (Elsaesser et al., 2009). This evidence suggests that the effect of IL-21 on the production of IFN-γ by CD8+ T cells is context-dependent, and may be dynamically regulated by synergistic activities with γc cytokines and characteristics of TCR stimulations. Our data is therefore of interest because it suggests for the first time

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that even brief exposure to IL-21 during a single TCR stimulation can programme subsequent CD8$^+$ memory T cells to delay secretion of IFN-$\gamma$.

The enhancement of IL-10 secretion in memory T cells primed in the presence of IL-21 has not previously investigated in human CD8$^+$ T cells, but it is consistent with previous observations in murine CD8$^+$ and CD4$^+$ T cells (Ansari et al., 2011; Spolski, Kim, Zhu, Levy, and Leonard, 2009; Spolski and Leonard, 2010). It is also in line with the critical role of IL-21 to sustain CD8$^+$ T cell functions for the control of chronic viral infections (Elsaesser et al., 2009; Frohlich et al., 2009; Yi et al., 2009) via the restriction of overt inflammation with the anti-inflammatory properties of IL-10. The regulation of IL-6 secretion by IL-21 has not previously been investigated. Our evidence that priming in the presence of IL-21 significantly reduces IL-6 secretion on secondary stimulation is consistent with IL-21 acting to dampen inflammation, both by reducing the inflammatory effects of IL-6 (McGeough et al., 2012), and augmenting IL-10 secretion.

When IL-21 was added in addition to IL-12 during priming, we observed the subsequent memory cells reduced secretion of IL-6 and enhanced production of IL-10 – effects of IL-21 – while the enhancing effects of IL-12 exposure on production of IL-2, TNF and MIP-1$\alpha$ were maintained. This provides evidence that cytokines available during priming can have additive effects on human CD8$^+$ T cells, as they can in mice (Casey and Mescher, 2007). Interestingly, in our work, extended exposure to IL-21 beyond the priming period had no further effect on the secretion of IL-10, and maintained the production of IL-2 and IFN-$\gamma$, while moderately enhancing the secretion of TNF, and possibly further downregulating the production of IL-6. This suggests the specific time frame of IL-21 exposure has subtle effects on cytokine secretion by human CD8$^+$ T cells, although overall extended IL-21 exposure beyond the priming period maintains polyfunctionality, proliferative capabilities and effector functions.

7.3.1.4 Plasticity of cytokine secretion profiles in programmed cells

Following the successful programming of naïve CD8$^+$ T cells observed by the specific skewing of cytokine secretion profiles, we found that programmed “M1” memory cells retained plasticity, and were able to alter their cytokine secretion profiles during secondary stimulation in the presence of different cytokines. M1 populations primed in the presence of IL-12 (Tc1 skewing) were able to upregulate the type 2 cytokines IL-5 and IL-13 with exposure to IL-4 during restimulation; conversely, M1 cells primed in the present of IL-4 (Tc2 skewing) were able to upregulate the type 1 cytokines IFN-$\gamma$, TNF and MIP-1$\alpha$ with exposure to IL-12 during restimulation. The persistent capability of the IL-4-primed M1 populations to upregulate type 1 cytokines on IL-12 exposure was consistent with their persistent T-bet expression and secretion of type 1 cytokines, despite their skewing towards Tc2. However, it was interesting to have also found the M1 populations skewed for a type 1 profile retained the potential for type 2 cytokine secretion. As GATA-3 expression is essential for the production of IL-5 and IL-13 (Zhu et al., 2004), these data indicate that Tc1 programming by IL-12 priming did not “close” the GATA-3 gene locus or permanently silence the cytokine genes that were not promoted during the primary stimulation. This is similar to previous observations in
murine CD4$^+$ T cells, where the cytokine genes associated with the specific priming conditions were found to have enhanced accessibility by stable histone hyperacetylation, while hypoacetylation did not lead to irreversible silencing of the non-expressed cytokine genes (Messi et al., 2003).

For a number of secreted cytokines, including IL-2, IFN-γ, IL-5, IL-6, IL-13 and IL-10, an interactive effect between the cytokine programming during the primary and secondary stimulations has been found to regulate their production, indicating an adjusted memory response according to environment of the accumulated stimulations. TNF, MIP-1α and GM-CSF secretions appeared to be more closely regulated by the proximal priming signals upon the secondary stimulations, suggesting more flexible gene locus configurations. At the same time, the IL-4-primed M1 populations upregulated IL-4 secretion regardless of the secondary cytokine exposure, while the same was not observed for the secretion of IL-5 and IL-13. One possible mechanism for this phenomenon is a more stable “open” conformation of the IL-4 gene locus and chromatin structure following naïve cell programming, where the IL-4 gene locus was found to be demethylated and histone hyperacetylated in mouse CD4$^+$ T cells (Messi et al., 2003; Santangelo, Cousins, Winkelmann, Triantaphyllopoulos, and Staynov, 2009; Yamashita et al., 2004).

We have provided evidence for the first time that programmed memory CD8$^+$ T cells retain plasticity for cytokine secretion responses depending on the changing environmental conditions upon successive stimulations. This is consistent with the accumulating evidence for the lineage plasticity observed in CD4$^+$ T cells (Murphy and Stockinger, 2010; O'Shea and Paul, 2010; Oestreicher and Weinmann, 2012a), and underscores the potential of programmed cells to adapt to new immune reaction contexts. This may be important for aged humans and mice, as memory cells are more and more relied upon for immune reactions during aging (Ahmed et al., 2009a; Decman et al., 2012), as well as for therapeutic interventions for disease, where “wrongly” programmed T cells could potentially be “re-programmed”.

7.3.2 Cytolytic molecule expression

Upon the investigations of granzyme B and perforin expression, we have observed an absence of continuous granzyme B and perforin expression in the rested M1 populations that was induced upon secondary stimulations, implicating an early differentiation status of the M1 populations (Araki et al., 2009b; Weng, Araki, and Subedi, 2012) with a capability to exert cytolytic effector functions. The lytic molecules were found to be generated by the M1 cells upon secondary stimulations with or without exposures to third signal cytokines during naïve activation, indicating their production as an inherent property of CD8$^+$ T cells. The addition of various priming cytokines during naïve cell activation resulted in largely comparable expressions of both granzyme B and perforin across the M1 populations, but nevertheless, IL-12 priming appeared to have programmed the M1 populations to express the most consistent, high levels of granzyme B, whereas IL-27 exposure appeared to have programmed reduced granzyme B expression. The heightened levels of granzyme B and perforin production were maintained with the addition of IL-21 to IL-12 priming, indicating a persistent effect of IL-12 on their expression.
A recent study in human CD8+ T cells showed that within the first week of primary stimulation, the presence of IL-12 in culture enhanced granzyme B expression on restimulation (Agarwal et al., 2009; Chowdhury et al., 2011; Rao et al., 2010). Murine models have shown that IL-12 exposure enhances the cytolytic and anti-tumour capabilities of CD8+ T cells (Chang et al., 2004; Curtsinger, Gerner, Lins, and Mescher, 2007; Lisiero et al., 2011). One murine study has found IL-12 to promote histone hyperacetylation at the granzyme B gene loci, suggesting that the opening of the chromatin structure following IL-12 signalling allowed its enhanced transcription upon the following TCR stimulations (Agarwal et al., 2009). Our data would be consistent with direct effects of IL-12 on the human granzyme B locus, and by analogy the perforin locus, and show that only 3 days exposure during priming is sufficient to exert this effect. However, IL-12 is clearly not required to enable expression of either of these molecules during restimulation of human CD8+ T cells, since both molecules are expressed in cells never exposed to IL-12.

The reduction of granzyme B and perforin expression following IL-27 priming is consistent with its inhibitory roles (Stumhofer and Hunter, 2008; Wojno and Hunter, 2012). Although some previous studies have found an upregulation of granzyme B expression by IL-27, either a simultaneous IL-2 signalling was present (Morishima et al., 2005), or high concentrations of IL-27 were necessary in the absence of costimulatory signals (Schneider, Yaneva, Beauseigle, El-Khoury, and Arbour, 2011).

**7.3.3 Minimal effects with IFN-α, IL-18, IL-23 or IL-27 priming**

Four other cytokines that we have also investigated during naïve CD8+ T cell priming were found to result in either only marginal effects (IFN-α, IL-23) or no effects (IL-18, IL-27) in cellular attributes of the memory cells.

In our system, we have found IFN-α priming to induce minor increases in the production of IFN-γ, TNF, IL-5, IL-13 and IL-10, encompassing both type 1 and type 2 cytokines, and no significant effects on the expression of the lytic molecules. This was initially surprising, given the role it shares with IL-12 in murine systems in providing third signals to naive CD8+ T cells for the development of fully functional effector and memory cells (Agarwal et al., 2009; Curtsinger et al., 2005; Huber and David Farrar, 2011; Kolumam et al., 2005; Xiao et al., 2009). However, recent human studies have resulted in contrasting results, where two studies reported minimal effects following naïve cell stimulation in the presence of IFN-α (Chowdhury et al., 2011; Ramos et al., 2009), while two other studies reported an induction of proliferative capacity and effector functions (Hervas-Stubbs et al., 2012; Hervas-Stubbs et al., 2010). Much lower quantities of TCR stimulus were employed in the IFN-α sensitive reports in comparison to the IFN-α insensitive studies, suggesting IFN-α may have acted to strengthen weak TCR signals. In human CD4+ T cells, IFN-α signalling is insufficient to drive Th1 development due to a lack of stable T-bet expression (Ramos, Davis, George, and Farrar, 2007). All these data suggest that the effects of IFN-α on human T cells may differ.
substantially from those of IL-12, and that our observations may simply reflect that IFN-α cannot substitute for IL-12 in programming human CD8^+ T cells, despite their similar effects in murine models.

We found exposure to IL-23 during priming programmed upregulation of IFN-γ, TNF, MIP-1α and IL-10 production upon secondary stimulation of the M1 populations. This profile was similar, though smaller in scale, to the cytokine profile induced by IL-12 exposure, and is in line with the shared receptor subunit and related roles of IL-23 with IL-12 (Henry et al., 2010; Khader et al., 2005; Vignali and Kuchroo, 2012). Previous observations of primary stimulation of human CD8^+ T cells showed similar effects of IL-23 on IFN-γ and IL-10 secretion (Vanden Eijnden et al., 2005). As IL-23R is not expressed in naïve CD8^+ T cells, and its upregulation upon TCR stimulation in the presence of IL-23 is gradually enhanced over the period of at least 6 days, lengthened exposure of IL-23 may be required to observe more prominent effects (Vanden Eijnden et al., 2005). However, the unique role of IL-23 to induce IL-17 production in both CD4^+ and CD8^+ T cells (Aggarwal, Ghilardi, Xie, de Sauvage, and Gurney, 2003; Cric, El-behi, Cabrera, Zhang, and Rostami, 2009; Curtis, Way, and Wilson, 2009; Khader et al., 2005; Langrish et al., 2005; Vanden Eijnden et al., 2005) was not observed in our system (data not shown). A variation in the required combination of cytokines have been reported to induce the differentiation of IL-17-producing CD4^+ T cells across murine and human models, where the use of serum containing medium has contributed to the different requirements by providing trace amounts of cytokines (Korn, Bettelli, Oukka, and Kuchroo, 2009). The absence of IL-17 production in our system indicates additional cytokine supplantations are necessary. Indeed, one study reported to have successfully induced human Tc17 cells in vitro has used a cocktail of IL-1β, IL-6, IL-23 and TGFβ (Kondo, Takata, Matsuki, and Takiguchi, 2009). Also, as Tcf-1 has been recently found to inhibit the expression of IL-17 (Yu, Sharma, Ghosh, and Sen, 2011), its expression in our M1 populations may have contributed to the absence of IL-17 production.

IL-18 exposure during priming had minimal effects on the cytokine profile of the subsequent M1 populations. In mouse CD4^+ T cells, it has been found that STAT-4 epigenetically modifies the IL-18R1 gene locus for its full expression (Yu, Chang, Ahyi, and Kaplan, 2008; Yu, Thieu, and Kaplan, 2007), and consistently, IL-18 was observed to synergise with IL-12 signalling to induce optimal IFN-γ and TNF secretion in both CD4^+ and CD8^+ T cells (Balasubramani et al., 2010; Blom and Poulsen, 2012; Freeman et al., 2010; Robinson et al., 1997). Our observations suggest that human naive CD8^+ T cells may also require an accompanying signal in order for IL-18 to programme subsequent memory cell function.

We have also observed no alterations in cytokine secretion profile following priming in the presence of IL-27. Both pro-inflammatory and anti-inflammatory effects have been previously observed with IL-27 (Hunter, 2005; Wojno and Hunter, 2012). However, in murine studies the type 1 differentiation and anti-tumour effects IL-27 induced in CD8^+ T cells appears to require either the presence of IL-12, where IL-27 has been found to upregulate IL-12R (Lucas, Ghilardi, Li, and de Sauvage, 2003; Morishima et al., 2005), or the presence of IL-2 (Morishima et al., 2005; Salcedo et al., 2009; Sun, Dodd, Moser, Sharma, and Braciale, 2011). Another recent study have also revealed a requirement of IL-2 for the production of IL-10 following
exposure to IL-27 for both murine and human CD8\(^+\) T cells (Sun et al., 2011). A human study has reported an induction of IFN-\(\gamma\) and T-bet by IL-27 on CD8\(^+\) T cells without the presence of other cytokines, but it has also revealed that the IL-27 effects were only apparent at concentrations of 100ng/ml or higher with only anti-CD3 stimulation (Schneider et al., 2011). This indicates that the effects of IL-27 may only be apparent with minimal activation stimuli and that the high concentrations of IL-27 may be compensating for absent costimulatory signals \textit{in vitro}. At the same time, the reduced granzyme B production observed with IL-27 exposure suggests a possible dose-dependent effect of IL-27, where low doses may induce an inhibitory effect.

### 7.4 Transcription factor expression

Following the generation of rested, programmed human CD8\(^+\) memory T cells \textit{in vitro}, we set out to characterise their transcription factor protein expression profile by immunocytochemistry and to compare them against CD8\(^+\) memory cell subsets purified \textit{ex vivo}. We believe this is the first time that the protein expression of these transcription factors has been studied by immunocytochemistry in human CD8\(^+\) T cells. This series of experiments provided an array of interesting and often surprising observations, in part because these transcription factors have mostly been studied at the transcript level rather than the protein level in existing literature; in addition, any previous studies of protein expression relied on intracellular flow cytometry, and therefore failed to distinguish between nuclear and cytoplasmic protein. Our results provided new information on the regulation of transcription factor protein expression and their cellular localization in human CD8\(^+\) T cells upon differentiation, TCR stimulation, and cytokine programming. To put the programming results in context, we first need to consider how transcription factor expression changed during CD8\(^+\) T cell differentiation and TCR activation.

#### 7.4.1 With CD8\(^+\) T cell differentiation

We sorted CD8\(^+\) T cell memory subsets \textit{ex vivo} and observed that as these subsets became more differentiated, there was a downregulation of Tcf-1 and Lef-1, a downregulation of FoxO1, and an upregulation of Eomes protein expression (summarised in Figure 7–1). Importantly, each of these transcription factors was fairly homogeneous within a particular memory subset, indicating a good correlation between the expression level of each of these transcription factors and their differentiation status. Unexpectedly we found T-bet protein to be expressed in only a very small proportion of all memory subsets with no differential expression between different subsets.

The reduction of Tcf-1 and Lef-1 protein expression we observed with CD8\(^+\) T cell differentiation is consistent with the previous microarray data and protein expression examined in human CD8\(^+\) T cells by flow cytometry (Gattinoni et al., 2011; Willinger et al., 2006), but for the first time we confirmed that these proteins were localized to the nucleus, and therefore active. Our results were therefore in line with the role
of Tcf-1 and Lef-1 in the generation and persistence of functional CD8$^+$ memory T cells observed in mice (Zhou and Xue, 2012).

The transcription factor FoxO1 is associated with a quiescent state, and we have provided the first evidence for its downregulation with differentiation in human CD8$^+$ T cells. This is consistent with the role of FoxO1 in supporting the expression of lymphoid organ homing receptors and the maintenance of survival signals in naïve cells (Kerdiles et al., 2009), which are known to decline upon differentiation. We also observed that expression of FoxO1 was not completely abrogated in the EM and EMRA cells, but a clear nuclear exclusion of FoxO1 was detected, which is likely to indicate lost functional activity. This indicates that both nuclear localization and degradation are important mechanisms for the regulation of FoxO1 upon CD8$^+$ T cell differentiation, and that the promotion of cellular quiescence by FoxO1 (Stahl et al., 2002) may only be of significance for cells very early in differentiation.

Expression of Eomes protein was found to increase with differentiation, albeit with some donor variation, consistent with the increase of Eomes transcription found in microarray studies of human CD8$^+$ T cells (Gattinoni et al., 2011; Willinger et al., 2005). We also observed that when naïve cells were expanded in IL-2, with consequent loss of CCR7 expression, Eomes expression was also upregulated. This is also consistent with the augmentation of Eomes activity with CD8$^+$ T cell differentiation.

In contrast, T-bet protein was rare across all the ex vivo memory subsets, and this was unexpected, as microarray studies have found T-bet transcription to increase with human CD8$^+$ T cell differentiation (Gattinoni et al., 2011; Willinger et al., 2005), and T-bet is known to be responsible for CD8$^+$ T cell effector differentiation in murine models (Intlekofer et al., 2007; Intlekofer et al., 2005; Sullivan et al., 2003). However, as discussed below, nuclear T-bet protein was readily detected in cells that had recently received TCR stimulation, and this expression was gradually ablated as the cells came to rest under IL-7 support. Hence the small number of cells with prominent T-bet staining in the ex vivo memory subsets is consistent with the small number of cells in the circulation that show recent signs of activation; conversely the vast majority of human CD8$^+$ T cells in the peripheral blood are in a resting state (Appay et al., 2002), and our data suggests that even if they have recently been stimulated, nuclear T-bet staining will have faded within days post-stimulation. The absence of T-bet expression found in the IL-2-expanded, differentiated and rested, CD8$^+$ T cells regardless of their CCR7, CD28 and CD27 expression is also consistent with this idea. The difference between the transcriptional and protein expression data in the differentiated cells suggests a potential post-transcriptional regulation mechanism for the protein expression and nuclear localization of T-bet. Functionally, enhanced T-bet transcription with CD8$^+$ differentiation that is withheld from protein expression until TCR signals are received would still allow T-bet upregulation over time to improve effector functions upon TCR engagement as the T cells differentiate.

As Eomes and T-bet are known to share the contribution of effector CD8$^+$ T cell function development (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005), this suggests that Eomes could potentially be
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responsible for CD8\(^+\) T cells’ constitutive expression of effector molecules as they differentiate, whereas T-bet is primarily responsible for the effector functions immediately following TCR stimulations. This is consistent with the role of Eomes found in the promotion of memory cell development and maintenance (Banerjee et al., 2010; Rao et al., 2010; Zhou et al., 2010), and the association of T-bet expression with effector cells (Intlekofer et al., 2007; Takemoto et al., 2006).

Figure 7–1 Proposed transcription factor expression regulations upon human CD8\(^+\) T cell differentiation, activation and cytokine signalling

A summary of the regulations of transcription factor protein expression we have observed across our investigations and their transcriptional expression data in the literature. Solid lines, protein expression; dash lines, mRNA transcription data in the literature; dotted red line, hypothesised differentiation position of the M1 populations. \(\uparrow\), cytokine induced transcription factor expression upregulation; \(\downarrow\), cytokine induced transcription factor expression downregulation; \(=\), similar transcription factor expression; \(*\), consistent evidence in the literature.
7.4.2 With rested M1 populations

With the knowledge of the transcription factor expression profiles in the ex vivo CD8⁺ memory subsets, we were able to map the differentiation status of our rested M1 populations according to their transcription factor expression. In the rested M1 populations, we found a general expression of Tcf-1, Lef-1 and FoxO1 across the M1 populations generated with various priming conditions, with very little Eomes expression. This transcription factor expression profile is the most similar to the ex vivo naïve or CM cells (marked by dotted red line in Figure 7–1) and indicates an early differentiation status of the M1 populations. As discussed below, T-bet was rarely expressed in resting M1 cells, consistent with data from the memory subsets sorted ex vivo.

Visualization of the transcription factor expression by ICC provided direct evidence for the nuclear localization of Tcf-1 and Lef-1 in these cells. The expression of Lef-1 and Tcf-1 in the in vitro-generated M1 populations at rest confirms their early memory cell phenotype and also correlates with early differentiation status as previously observed in murine studies (Gattinoni et al., 2010; Gattinoni et al., 2009; Zhao et al., 2010). Tcf-1 has been found to enable the optimal expression of CD62L, CCR7, CD127, CD122 and Bcl-2, which direct lymphoid organ homing and enhance survival capabilities (Zhou et al., 2010), and it is also responsible for the cells’ proliferative potential and the capability to undergo secondary expansions (Jeannet et al., 2010; Zhao et al., 2010; Zhou et al., 2010). All these features are entirely consistent with our results. Interestingly, Tcf-1 expression appeared to be lost faster than Lef-1 expression upon differentiation, as the M2 populations were found to lack Tcf-1 expression, where Lef-1 expression persisted.

The resting M1 populations were observed to prominently express FoxO1, with most expression localized to the cytoplasm. This finding is consistent with nuclear exportation following FoxO1 phosphorylation induced by TCR stimulation and γc cytokine signalling (Hedrick, Michelini, Doedens, Goldrath, and Stone, 2012; Peng, 2008), and indicates that FoxO1 does not readily shuttle back into the nuclei upon resting of memory cells with continuous IL-7 signalling. Due to its localization in the cytoplasm, whether these prominent FoxO1 protein expression contributes to the expression of homing molecules (Kerdiles et al., 2009; Stein, 2009), the promotion of quiescence by inhibiting cell cycling (Stahl et al., 2002), or the generation of memory cells (Rao, Li, Gubbels Bupp, and Shrikant, 2012) in the M1 populations – as previously observed in murine naïve CD8⁺ T cells – requires further investigation. In M2 populations, which were generated with the supplementation of IL-15 following IL-7-based primary expansions with or without IL-2, FoxO1 expression was reduced in comparison to the M1 populations, correlating with their reduced expression of CCR7 and CD62L (Kerdiles et al., 2009; Stein, 2009), but also potentially altering their ability to rapidly commit to cell cycling.

The lack of Eomes expression in resting M1 cells is consistent with our data on human memory cells sorted ex vivo. Some murine in vivo studies have shown upregulation of Eomes transcription as naïve
CD8\(^+\) T cells become memory cells following one round of antigenic challenge (Intlekofer et al., 2007; Intlekofer et al., 2005; Takemoto et al., 2006), however the fold changes of mRNA were very small, and protein expression was not investigated. The minimal expression of Eomes found in the rested M1 populations is consistent with their early differentiation status and an absence of continuous expression of effector molecules.

### 7.4.3 With CD8\(^+\) T cell activation

We have observed the transcription factor expression patterns of the M1 populations to change temporarily upon TCR stimulation. Tcf-1 and Lef-1 expression was found to downregulate with TCR engagement and was re-expressed in the following few days, whereas T-bet and GATA-3 were found to be upregulated upon TCR stimulation and downregulated in the following 3-4 days. At the same time, we have also provided evidence for the regulation of T-bet expression by the strength of TCR stimulation. Differences between activated and rested T cells in the expression of these genes have only occasionally been discussed in any existing literature, and there is very little protein-based data with which to compare our results.

Expression of Tcf-1 and Lef-1 transcripts have previously been noted to downregulate with antigenic stimulation and become partially restored as memory cells are formed in both human and murine T cells (Willinger et al., 2006; Zhao et al., 2010). So the concept that expression of these transcription factors may cycle with activation status is not novel, although it has only rarely been discussed compared with the frequent finding that gradual loss of their expression is linked to differentiation. Combining these two phenomena leads us to propose the schema shown in Figure 7–1, which has not previously been crystallised in this way.

The upregulation of T-bet expression upon TCR stimulation in a dose-dependent manner – and its loss on reversion to a resting state, supported by IL-7 – was initially more surprising. However, some murine studies support these findings. *In vivo* studies have observed an upregulation of T-bet transcription as murine CD8\(^+\) T cells activate and enter the effector phase, followed by a decline in T-bet transcription in memory cells following clearance of infection (Intlekofer et al., 2007; Intlekofer et al., 2005; Takemoto et al., 2006). Some *in vitro* studies using murine cells have also observed rapidly upregulated T-bet transcription and protein translation on antigen stimulation, followed by a gradual decline in expression once peak stimulation has been reached (Cruz-Guilloty et al., 2009; Rao et al., 2010). The immediate production of IFN-γ by murine CD8\(^+\) T cells following T-bet activation may also contribute to further T-bet expression by autocrine signalling (Curtsinger, Agarwal, Lins, and Mescher, 2012), but whether other endogenous cytokines also played a role requires further investigation. So again, while Figure 7–1 presents a novel schema for the expression of T-bet across subsequent rounds of antigen exposure, there is some support for this concept from some murine literature.
If T-bet protein expression does indeed cycle between rounds of stimulation as we propose, it has some interesting implications. First, we observed this strong expression of T-bet following TCR stimulation in the absence of any third signal cytokines. This may present a mechanism for the intrinsic induction of effector function in CD8⁺ T cells as opposed to the necessity of IFN-γ/STAT-1 or IL-12/STAT-4 signalling reported for Th1 differentiation in CD4⁺ T cells (Afkarian et al., 2002; Zhu et al., 2012). Secondly, our data suggest increased antigen dose and enhanced TCR stimulation augmented T-bet expression during activation, and this could represent a direct mechanism for how increased antigenic load induces enhanced effector functions (Beuneu et al., 2010; Henrickson et al., 2008b). Similarly, the complete loss of nuclear T-bet protein as activated cells revert to early memory cells could account for the loss of lytic granule expression in these rested cells.

Finally regarding T-bet and activation, it must be noted that the continuous STAT-5 signalling via IL-7 in the rested M1 populations may have contributed to the repression of T-bet expression as found previously in murine T cells (Rao et al., 2012; Zhu et al., 2003). However the lack of T-bet expression by the vast majority of human CD8⁺ T cells ex vivo confirms that lack of nuclear T-bet protein – whether induced by exposure to homeostatic cytokines or not – is the dominant phenotype of human CD8⁺ T cells in vivo, and not an in vitro artefact induced by IL-7.

In contrast to Tcf-1, Lef-1 and T-bet, the expression of FoxO1 protein appears largely unaffected by TCR stimulation other than its stronger correlation with differentiation status, although its bright cytoplasmic staining makes the assay harder to interpret than the “on/off” nuclear expression pattern of the other transcription factors.

We did not investigate the expression of Eomes immediately following TCR stimulation, but this would clearly be of interest given that nuclear T-bet is so clearly linked to activation status in our data.

### 7.4.4 With third signal programming

In our experimental system, exposure of human CD8⁺ T cells to IL-4 for only 3 days during priming skewed their cytokine secretion towards type 2 cytokines 21 days later. The observation that these same cells uniquely expressed GATA-3 provided clear evidence that we had transcriptionally programmed the T cells with exposure to a third signal during priming alone. However, once again the pattern of GATA-3 expression was initially surprising, since it was only detected once the programmed M1 cells were restimulated with antigen. This dependence on antigenic stimulation for nuclear expression of a transcription factor mirrored our observations of the kinetics of nuclear T-bet expression, as described above. However in contrast to GATA-3, T-bet was expressed in all M1 cells, regardless of their exposure to third signal cytokines during priming. Hence T-bet induction seemed to be a default transcriptional programme in our system, while GATA-3 expression was exclusively dependent on exposure to IL-4 during priming.
7.4.4.1 GATA-3

GATA-3 is well known as the master regulator for Th2 responses in CD4+ T cells (Pai, Truitt, and Ho, 2004; Zhang, Cohn, Ray, Bottomly, and Ray, 1997; Zheng and Flavell, 1997; Zhu et al., 2004). However, its examination in CD8+ T cells has been limited. GATA-3 transcription has been shown to be upregulated with type 2 polarizing conditions in murine CD8+ T cells (Apte et al., 2010; Serre et al., 2010), but it has not been investigated in human CD8+ T cells under Tc2 conditions. In our hands, we have found GATA-3 to be upregulated in the M1 populations primed in the presence of IL-4 upon secondary stimulation, but not in the M1 cells primed under different conditions. In the IL-4-primed M1 populations, GATA-3 returned to basal expression by day 4 following restimulation. The prominent GATA-3 specifically expressed in the IL-4-primed M1 cells upon restimulation without further IL-4 signalling strongly suggests a memory of the type 2 polarizing conditions from the brief exposure of IL-4 three weeks prior to restimulation, providing evidence for a transcriptional programming of GATA-3 without the requirement for either constitutive expression of GATA-3 or constitutive secretion of IL-4. This indicates that the positive feedback amplification pathway of IL-4/IL-4R/STAT-6/GATA-3 (Zhu, 2010) is likely to be restricted to activated cells, and the previously observed autoactivation of GATA-3 transcription in mice (Ouyang et al., 2000) does not translate to continuous protein expression in human cells switching between activation and reversion to resting memory cells. Mouse studies with CD4+ T cells have suggested epigenetic regulation maintains open DNA and chromatin structures (Allan et al., 2012; Santangelo et al., 2009; Wei et al., 2009) for the re-expression of GATA-3 and IL-4 upon the secondary stimulation following the binding of Th-2-supporting transcription factors, including STAT-6, NF-kB1, Jag1/Notch and STAT-5 (Allan et al., 2012; Murphy and Reiner, 2002; Zhu, 2010). However our data suggests that while epigenetic regulation may enable GATA-3 expression in IL-4-programmed human CD8+ T cells, protein translation and/or translocation to the nucleus does not take place until the cell is stimulated with antigen.

The upregulation of GATA-3 expression in the IL-4-primed M1 populations correlated with their elevated levels of type 2 cytokine secretions, providing evidence that the programmed GATA-3 expression by IL-4 priming in human CD8+ T cells is very likely to have contributed to the secretion of type 2 cytokines, similar to CD4+ T cells. The diminished IFN-γ secretion by the IL-4-primed M1 cells may also be associated to the expression of GATA-3: IL-4 signalling and GATA-3 expression in CD4+ T cells were found to inhibit IFN-γ production (Usui et al., 2003; Yagi et al., 2010; Zhu et al., 2004), potentially regulated via the Runx3-Eomes-IFN-γ pathway (Zhu, 2010), although CD8+ T cells have not previously been investigated.

The upregulation of GATA-3 expression and type 2 cytokine secretion have been found to be abrogated by high peptide concentrations and strong TCR stimulations in CD4+ T cells via the activation of the Erk pathway (Milner, Fazilleau, McHeyzer-Williams, and Paul, 2010; Yamane, Zhu, and Paul, 2005). It has been suggested that IL-4 exposure reduces TCR signalling to favour a type 2 differentiation either via a downregulation of CD8 expression (Kienzle, Baz, and Kelso, 2004), or via a suppression of IL-12 production by DCs that reduces activation of CD4+ T cells (Everts et al., 2009; Steinfeld et al., 2009).
Although nuclear GATA-3 expression was only detected in cells primed in the presence of IL-4 by ICC, flow cytometry suggested there was some GATA-3 protein expression in all M1 cells tested, regardless of how they were primed. One of the common features these cultures shared was strong TCR signals delivered via anti-CD3/CD28 beads, so it is unlikely that any low level background expression of GATA-3 protein was due to weak antigenic signal. In murine CD4+ T cells, endogenous GATA-3 transcripts have been found at low levels in naïve cells (Usui et al., 2003). Therefore, it is possible that low levels of endogenous GATA-3 expression had been maintained by the IL-7-induced constitutive STAT-5 signalling in our in vitro model, as STAT-5 has been found to maintain GATA-3 expression (Guo et al., 2009). Only low levels of GATA-3 are required in the presence of activated STAT-5 to promote Th2 differentiation in CD4+ T cells and epigenetically modify the IL-4 gene locus to promote its production (Cote-Sierra et al., 2004; Zhu et al., 2003). Therefore it seems likely that the basal secretion of the type 2 cytokines across the M1 populations relates to an inherent property of human CD8+ T cells when maintained in IL-7 perhaps related to STAT-5 signalling and some basal expression of GATA-3 that is not detectable on immunocytochemistry.

### 7.4.4.2 T-bet

T-bet was originally described as the master transcription factor responsible for Th1 differentiation in CD4+ T cells (Szabo et al., 2000; Szabo et al., 2002), then later found to be required for the effector differentiation in CD8+ T cells following antigen stimulation and inflammatory signals (Joshi et al., 2007; Sullivan et al., 2003). Following secondary stimulation of our M1 cells primed under different conditions, nuclear T-bet expression was similar regardless of whether the cells had been primed with or without exposure to IL-12 or IL-4. This result was unexpected as T-bet expression is known to upregulate in murine CD4+ T cells under type 1 conditions (exposure to IL-12 and/or IFN-γ) (Afkarian et al., 2002; Schulz, Mariani, Radbruch, and Hofer, 2009; Szabo et al., 2000) and also in murine CD8+ T cells stimulated with peptides with concomitant IL-12 signals (Joshi et al., 2007; Pipkin et al., 2010; Rao et al., 2010; Takemoto et al., 2006). However it is worth noting that we could detect strong IFN-γ secretion by naïve cells under the antigenic stimulation we used, even in the absence of third signal cytokines, and at least some murine data suggests that IFN-γ can substitute for IL-12 for the induction of T-bet expression (Curtsinger et al., 2012). We also showed a correlation of T-bet expression with the concentration of anti-CD3 antibodies used in the most recent TCR stimulation. In support of our data, a recent microarray study of human naïve CD8+ T cells activated in vitro with high concentrations of plate-bound anti-CD3 and anti-CD28 antibodies with or without IL-12 have also found no alterations in the levels of T-bet transcription (Chowdhury et al., 2011). It therefore remains possible that expression of T-bet in human CD8+ T cells is not epigenetically programmed strictly by IL-12, perhaps in contrast to GATA-3 by IL-4. However it remains unclear whether T-bet expression in human CD8+ T cells depends on achieving threshold of TCR stimulation during priming, or any of the other signals such as IFN-γ that may be available in culture systems like ours.
Nevertheless, the similar expression of T-bet across the differentially primed M1 populations provided interesting insights for the function of T-bet in these M1 populations. The equally comparable perforin and granzyme B expression across the M1 populations are consistent with the previously reported association between T-bet and these two lytic molecules (Makedonas et al., 2010; Smith et al., 2012). However, although T-bet has been found to be responsible for IFN-γ secretion in murine CD8⁺ T cells (Rao et al., 2010) and to transactivate the expression of TNF and MIP-1α in human type 1 CD4⁺ T cells (Jenner et al., 2009), the similar T-bet expression between the M1 populations suggest the upregulation of IFN-γ, TNF and MIP-1α secretions by the IL-12-primed cells may be contributed by other factors. A microarray study of human naïve CD8⁺ T cells activated in vitro with IL-12 priming found enhanced Eomes transcription (Chowdhury et al., 2011), which is able to direct IFN-γ production (Cruz-Guilloty et al., 2009). However, since we did not measure Eomes expression in relation to the different third signal cytokines provided during priming, further investigation will be required to determine whether it is responsible for the enhanced secretion of type 1 cytokines observed in cells primed in the presence of IL-12.

On the other hand, our data suggest IL-21 may have the potential to downregulate T-bet expression. Investigation of T-bet expression 5 days following naïve cell stimulation found T-bet expression only in the “IL-2 only” condition in comparison to the “IL-21” and “IL-21 + IL-2” conditions; prominent T-bet expression was also found in naïve cells stimulated in the presence of IL-7 alone 4 days following stimulation. This evidence suggests an intrinsic upregulation of T-bet with TCR stimulation regardless of γc cytokine signalling and indicates the T-bet expression was unlikely to be upregulated by IL-2. Previous studies in murine naïve CD8⁺ T cells report minimal effects of IL-2 signals on T-bet transcription (Kalia et al., 2010; Pipkin et al., 2010), supporting the concept that IL-21 exposure rather than IL-2 exposure was responsible for modulating T-bet expression in our experiments. Further comparative studies will be required to confirm whether IL-21 does indeed programme cells to express lower levels of T-bet on activation. Previous microarray studies investigating the effects of IL-21 have not reported significant alterations in T-bet transcription (Hinrichs et al., 2008). If IL-21 does not affect T-bet transcript levels, our observations may be due to post-transcriptional regulation of T-bet, or to a synergistic effect of a combination of cytokines.

7.4.4.3 T-bet and GATA-3 co-expression

In our studies of CD8⁺ T cells, we found T-bet to be largely expressed in both IL-12- (Tc1) and IL-4-primed (Tc2) populations, while GATA-3 was specifically expressed in the IL-4-primed cells. This led to the observation of an evident co-expression of GATA-3 and T-bet by flow cytometry in the IL-4-primed M1 populations, which correlates with the persistent capability of the IL-4-primed population to produce type 1 cytokines. Based on the regulation of type 1 and type 2 cytokines by T-bet and GATA-3, respectively, this data suggests that the cytokine secretion profiles with a mixture of type 1 and 2 cytokines is not a mere consequence of two mutually exclusive subsets of cells with differential expression, but single cell capabilities to secrete both types of cytokines.
Interestingly, these observations were in contrast to the regulation of transcription factor expression for Th1 and Th2 polarization in CD4\(^+\) T cells, where T-bet expression was found to repress GATA-3 and IL-4 expression in CD4\(^+\) cells (Usui et al., 2006; Zhu et al., 2012) and inhibit GATA-3 from binding to target genes by protein interaction, including the transactivation of IL-5 (Hwang, Szabo, Schwartzberg, and Glimcher, 2005; Oestreich and Weinmann, 2012b). In our data, expression of T-bet did not prevent the upregulation of GATA-3 and a strong skewing towards a type 2 secretion profile in M1 cells that had been primed in the presence of IL-4, including an ≈100-fold enhancement of IL-5 secretion. This would indicate that human CD8\(^+\) T cells can express GATA-3 and skew its cytokine profile towards type 2 cytokines at the same time as expressing T-bet and producing type 1 cytokines and effector molecules. Human cells secreting both sets of cytokines have long been identified in the literature, especially at early stages of differentiation, and the regulation of cytokine secretion in CD8\(^+\) T cells differs from CD4\(^+\) T cells, for example due to the expression of ROG (Repressor of GATA-3) in CD8\(^+\) T cells (Omori et al., 2003). However this phenomenon deserves further investigation to establish how the molecular mechanisms differ from what has previously been reported in murine systems.

### 7.4.4.4 Tcf-1 and Lef-1

Following the observation of downregulated Tcf-1 expression on naïve cell expansion in the presence of IL-7 alone (further discussed later), Tcf-1 was found to be expressed in M1 populations generated with IL-4, IL-12 and/or IL-21 third signal exposure. This suggests that these cytokines rescued Tcf-1 expression to allow generation of memory precursors and functional memory cells (Jeannet et al., 2010; Zhou and Xue, 2012; Zhou et al., 2010). IL-21 was previously observed to elevate Tcf-1 transcription in both human and murine CD8\(^+\) T cells (Hinrichs et al., 2008; Kaka et al., 2009). Our study confirms Tcf-1 protein expression is increased in memory cells generated with exposure to IL-21.

In the comparison of Tcf-1 and Lef-1 expression between IL-4- and IL-12-primed M1 populations, we found expression of both transcription factors to be higher in the IL-4-exposed cells. This is consistent with the reduced Tcf-1 transcription observed in murine CD8\(^+\) T cells upon stimulation with IL-12 (Agarwal et al., 2009). Tcf-1 and Lef-1 expression have previously been correlated with early differentiation (Gattinoni et al., 2011; Willinger et al., 2006) and Tcf-1 was found to contribute to CCR7 and CD62L expression (Zhou et al., 2010). In our data, the lower expression of Tcf-1 and Lef-1 in cells primed in the presence of IL-12 correlated with lower expression of CCR7, suggesting IL-12 was accelerating differentiation. By contrast, we observed that Tcf-1 was most highly expressed in cells primed in the presence of IL-4, which strongly expressed GATA-3 on activation, along with a type 2 cytokine profile and reduced IFN-γ. Studies of mouse CD4\(^+\) T cells have found that Tcf-1 is required for the optimal expression of GATA-3 and IL-4, and negatively regulates IFN-γ production (Yu et al., 2009).
7.4.5 With γc cytokine supplementation

With the supplementation of IL-2 into the IL-7 in vitro model as well as a direct comparison of naïve cells expanded in IL-2, IL-7 and IL-15 (in the absence of other cytokines acting as third signals), we observed that γc cytokines themselves regulate the expression of some transcription factors in human CD8⁺ T cells. We found Tcf-1 expression to be downregulated by IL-2 and IL-7, while Eomes was upregulated by IL-2.

A recent study has revealed that Tcf-1 and Lef-1 have synergistic roles to direct the generation of memory precursors, memory cells, production of TNF and recall capacities (Zhou and Xue, 2012), but the more extensive loss of CTL functions by Tcf-1-deficient CD8⁺ T cells in comparison to Lef-1-deficient cells suggests a more important role of Tcf-1 in CD8⁺ T cell differentiation (Zhou and Xue, 2012). This is consistent with the more variable Tcf-1 expression intensity we observed across the M1 populations, depending on their exposure to the γc cytokines in culture as well as the priming cytokines, and suggests that Tcf-1 is the more important regulator of CD8⁺ T cell function development and memory generation influenced by these cytokines. Tcf-1 expression was found be abrogated in naïve CD8⁺ T cells expanded in IL-7 or IL-2 alone, but not in IL-15. Consistent with our observations, Tcf-1 transcription has been previously observed to downregulate with IL-7 incubation in murine T cells (Yu, Erman, Park, Feigenbaum, and Singer, 2004). We found a further reduction in expression of Tcf-1 with IL-2 supplementation in the IL-7 in vitro model. The reduced Tcf-1 expression in cells exposed to IL-2 is likely to correlate with their decreased capability to expand upon secondary stimulation as previously observed in mice (Jeannet et al., 2010; Zhou and Xue, 2012). The extended exposure to both IL-7 and IL-2 is likely to have also contributed to the loss of Tcf-1 expression in the M2 populations. It will now be interesting to examine whether these cells’ recovery in proliferative responses to antigenic stimulation induced by exposure to IL-15 relates to its effects on Tcf-1. In contrast to Tcf-1, we observed strong Lef-1 expression across the M1 and M2 populations regardless of culture and priming conditions. In a previous murine study, Lef-1 transcription was downregulated together with Tcf-1 following IL-7 incubation (Yu et al., 2004), so the differential regulation of Tcf-1 and Lef-1 in our studies will be of considerable interest to follow up.

The moderate upregulation of Eomes we have observed in the IL-2-expanded populations with IL-12 priming is consistent with previous observations, since IL-2 has been observed to enhance Eomes expression (Hinrichs et al., 2008; Pipkin et al., 2010). Previous human studies have also observed an upregulation of Eomes with IL-12 signalling (Chowdhury et al., 2011), although IL-12 exposure can downregulate Eomes in mice (Pipkin et al., 2010). By comparison, expansion of naïve human CD8⁺ T cells in IL-12 and IL-21 induced much lower expression of Eomes than expansion in IL-2. Eomes downregulation by IL-21 has previously been observed in murine studies (Hinrichs et al., 2008). In general these findings are consistent with IL-2 driving differentiation by increased expression of Eomes, and IL-21 counteracting that differentiation by suppressing Eomes expression.
A direct comparison between the γc cytokines found similar nuclear exportation of FoxO1 by naïve CD8⁺ T cells expanded with IL-2, IL-7 and IL-15. This is consistent with the Akt-mediated FoxO1 phosphorylation and nuclear exportation downstream of γc cytokine signalling as previously reported (Peng, 2008). From our studies, however, we cannot distinguish the effects of γc cytokine signalling on FoxO1 nuclear exportation apart from TCR signalling and CD8⁺ T cell differentiation, which were found to play evident roles in FoxO1 regulation as discussed above, and requires further investigation.

7.5 Future directions

The studies carried out as part of this thesis have provided many interesting observations that can add to the current literature with regards to programming and differentiation of human CD8⁺ T cells. However many questions raised remain answered, and require further investigation. Firstly, following the observations of an absence, or only moderate, effects of IFN-α, IL-18, IL-23 and IL-27 exposure during naïve CD8⁺ T cell activation, it would have been of interest to examine whether combination of these cytokines with IL-12, or other cytokine, exposure produce additional effects on human CD8⁺ T cells as suggested in the murine models, and as we observed with the combination of IL-12 and IL-21 exposure. Secondly, the series of investigations into transcription factor expression that provided a number of surprising results for human CD8⁺ T cells requires additional experimentation to provide definitive support for some of the models of transcription factor regulation presented in this thesis. The functional and mechanistic differences between T-bet and GATA-3 in the regulation of type 1 and type 2 skewing in CD8⁺ T cells will be of particular interest to investigate further. Our data suggest fundamental differences with CD4⁺ T cells, as well as considerable plasticity of the programmed cells. Also, the role of T-bet following IL-12 exposure in human CD8⁺ T cells requires further confirmation, and whether other transcription factor contributes to the enhanced Tc1 functionality we observed in the memory cells generated in the presence of IL-12 priming. The rare T-bet expression observed across the ex vivo memory cell subsets regardless of differentiation status also poses interesting questions regarding its relationship to T cell activation and subsequent effector function.

7.6 Implications for immunotherapy

Although our culture system was focused on the expansion of naïve cells under different conditions, and reading out the phenotype and function of the resulting memory cells, it does have some potential implications for adoptive cell therapy (ACT). Some of our findings may apply to the expansion of memory cells for ACT. In addition, the optimal conditions for expanding naïve CD8⁺ T cells are of increasing interest due to the use of TCR transgenes in ACT.

For decades, standard strategies to expand T cells for ACT employed high dose exogenous IL-2 and repeated strong antigen stimulations to generate large numbers of cells for transfer. It is increasingly clear now that the high dose IL-2 and repeated TCR stimulations compromise successful clinical outcomes due
to the IL-2- and TCR-driven terminal differentiation and corruption of the transferred cells. IL-2-expanded T cells often have reduced capability to persist, expand and exert anti-tumour function in vivo following adoptive transfer. At the same time, an accumulation of preclinical studies and clinical trials have reported enhanced efficacy of less-differentiated cells for ACT, due to enhanced survival, proliferation, and functionality (Gattinoni et al., 2011; Hinrichs et al., 2009; Hinrichs et al., 2011; Kaech and Wherry, 2007). Therefore, the selection and generation of “younger” cells are now thought to be crucial for the next generation of ACT (Klebanoff et al., 2012; Restifo et al., 2012). We have successfully expanded human CD8+ T cells in vitro without the use of exogenous IL-2, and generated expanded memory populations with many quality traits that are associated with minimal differentiation. These features include an SCM-like phenotype, polyfunctionality and transcription factor expression profile that is similar to ex vivo naïve or CM cells with the capacity to direct functional memory development. Specifically, a number of studies have shown that the expression of CD28 and CD27 on antigen-specific CD8+ cells is correlated with longer telomere lengths, better proliferative potential on antigen exposure and longer persistence in vivo in both murine models and melanoma patients, resulting in more effective adoptive immunotherapy on a per cell basis (Klebanoff et al., 2005; Rosenberg et al., 2011; Zhou et al., 2005b). At the same time, the expression of CCR7 and CD62L allows for lymphoid organ homing to receive survival signals and indicates the capability to effectively deploy effector populations upon secondary stimulation (Link et al., 2007; Sallusto et al., 2004; Schuster et al., 2009). All these factors make it worthwhile considering whether our IL-7-based culture system with optimized IL-12 and IL-21 supplementation might be a potential alternative expansion protocol for adoptive transfer.

A clear disadvantage of expanding human T cells in our IL-7-based system, relying on the brief production of endogenous IL-2 to enhance proliferation, is that total T cell yields were clearly much lower than those cultured in continuous IL-2. However we found that a short duration of IL-2 supplementation during the primary expansion with IL-12 and IL-21 priming was able to augment the magnitude of proliferation with minimal alterations to the cellular attributes of the M1 populations subsequently generated. These experiments prove it is possible to expand human T cells under exogenous IL-2, then withdraw the IL-2 without the cells dying from cytokine withdrawal, provided homeostatic cytokines such as IL-7 are available. This concept may have implications for ACT, as an alternative method of generating sufficient CD8+ T cells for transfusion (Klebanoff et al., 2011; Rosenberg et al., 2011) while maintaining an early differentiation status with anti-tumour efficacy (Gattinoni, Klebanoff, and Restifo, 2012).

Further optimizations may be required for the use of this protocol to expand memory cells, and investigate whether additional signals are necessary, such as CD40, CD27, 4-1BB and OX40 costimulatory signals. CD8+ memory cells commonly prefer IL-15 for survival signals – as indicated by the clear need for IL-15 to allow our M1 cells to proliferate optimally in response to restimulation – and although there is evidence of memory cells able to survive with high doses of IL-7 (Kieper et al., 2002), it seems likely that IL-15 supplementation may needed to sustain optimal survival of expanding antigen-specific memory cells. Nevertheless, the concept of restricting IL-2 to a short burst before switching to homeostatic cytokines to
sustain proliferation and survival, as in our IL-7-based model, may prove useful for ACT, especially with the addition of factors such as IL-12 and IL-21 at optimal times.

Even if our results do not translate to the expansion of memory T cells, we may be able to utilise IL-7-based protocols for the expansion of naïve or SCM cells with genetically engineered TCR to confer de novo antigen specificity. The use of TCR-transgenic T cells in ACT is gaining prominence due to its advantages over ex vivo antigen-specific cell isolation and expansion (June et al., 2009; Turtle, Hudecek, Jensen, and Riddell, 2012), particularly the ability to rapidly and consistently generate sufficient antigen-specific T cells from autologous or donor cells. It would be very interesting to directly apply our optimised IL-7 protocol to TCR-engineered naïve cells in vitro and to test their in vivo efficacy in humanized mouse models (Shultz, Brehm, Garcia-Martinez, and Greiner, 2012).

Adoptive immunotherapy with T cells expanded in vitro is often complicated by factors such as the generation of regulatory T cells, restriction of T cell diversity and hyporesponsiveness of anti-tumour or anti-viral T cells due to the suppressive effects of tumour microenvironments or chronic infections (Baitsch, Fuertes-Marraco, Legat, Meyer, and Speiser, 2012; Wherry, 2011). Preclinical and clinical studies of IL-7 therapy have previously found it has useful attributes in immunotherapy. IL-7 has been shown in experimental systems to induce potent immunorestorative effects (Storek et al., 2003) with a resistance to suppressive effects of TGFβ and regulatory T cells and it can also enhance immunotherapy in chronic infections by strengthening cellular functions (Pellegrini et al., 2009). Clinical studies have also demonstrated recombinant human IL-7 (rhIL-7) therapy increases T cell numbers with a preferential proliferation of naïve cells, and broadens TCR repertoire diversity (Sportes et al., 2008). Although it is not yet known whether these advantageous effects of rhIL-7 will translate into improved clinical outcomes, these attributes of IL-7 in clinical studies could help enable its wider use in cell therapy approaches.

7.7 Conclusions

In this thesis, we have successfully established an in vitro system for the investigation of priming signals for the generation of human memory CD8$^+$ T cells. Following the observations of altered cytokine secretion profiles and programmed upregulation of specific transcription factors upon secondary stimulation, we provided evidence for successful programming of human CD8$^+$ T cells. This indicates that our in vitro model is able to be used to explore events around priming for their effects on subsequent epigenetic programming. The capability to rest activated T cells in vitro and restimulate the rested memory cells without large-scale cell death is a vital part of the system, enabling the investigation of cellular attributes in rested memory cells. In our model secondary stimulation is clearly separated in time from the primary stimulation, avoiding the potential confounding factors of previous studies of human cells, when cells were restimulated soon after primary stimulation, when many cells are still activated. Using this IL-7-based in vitro model, we have shed new light on the regulation of memory CD8$^+$ T cell generation by third signals at priming. We have also revealed new detail of how the expression of key transcription factors maps to
differentiation and activation status, as well as the transcription factors that are most sensitive to third signals delivered during T cell priming. We have also adapted this culture system, by optimising the priming cytokine regime as well as the provision of common gamma chain cytokines, to generate memory cells that are minimally differentiated, by both cell surface phenotype and transcription factor profile, and yet able to optimally produce effector cytokines and lytic molecules on re-exposure to antigen. These protocols have potential applications in Adoptive Cell Therapy, especially for the expansion of naive cells genetically modified by TCR transgenes.
Appendix I

Optimisation of representative assay time points for each of the secreted cytokine analytes in CBA assays

To ensure the functionality of the desired CBA assays and to optimise for representative CBA assay time points, a trial run was performed based on the experimental set up in Chapter 4 with a range of cytokine priming conditions. Briefly, naïve CD8⁺ T cells were stimulated in vitro with differential priming conditions using the IL-7 model. After the cells expanded and rested as memory populations, the cultures were restimulated to examine secreted cytokines in the supernatants. A trial screen was first performed across all cytokine priming conditions at 48hr after restimulation. All analytes could be detected and the secretions of IL-4 and IL-5 were found to be superbly distinguishable at 48hr over the range of priming conditions trialled (Figure IA). For all other secreted cytokines analysed, secretions were found over most, if not all, priming conditions. Therefore, IL-4 and IL-5 secretions were chosen to be sampled at 48hr post-restimulation, and a time course was performed for the other cytokine analytes. Four priming conditions with expected differential characteristics were selected for the time course assay (IL-7, IL-12, IL-21 and IL-12 + IL-21). Supernatant harvested every 24hr, for 120hr, was tested for the desired cytokine analytes on the four selected priming conditions. We found that either the 24hr or the 48hr post-restimulation was the peak of each cytokine secretion over the first five days of restimulation in most priming conditions sampled (Figure IB). A representative sample time point was then chosen for each secreted cytokine analyte by their peak of secretion and/or the most distinguishable time point between the four priming conditions (dotted line box in Figure IB, and listed in Table 2-2).
Figure I Optimisation of representative CBA assay time points

Naïve CD8\(^+\) T cells were stimulated with a 1:1 ratio of anti-CD3/CD28 beads for 2 days in the presence of a range of priming cytokines over the first 3 days of stimulation. IL-7 was given to all conditions from day 3 post-stimulation onwards to support expansion and survival of the cultured cells. After 21 days, cells were restimulated in the same manner with IL-7 only and supernatants were harvested from culture every 24hr from the point of stimulation. A. A screen of supernatants from all cytokine priming conditions at 48hr post-restimulation has found the secretion of IL-4 and IL-5 to be distinguishable from expected conditions. B. A selection of 4 priming conditions were screened for targeted cytokine analytes over the time course of 0-120hr post-restimulation. A peak and/or distinguishable time point post-restimulation over the four priming conditions was selected for each cytokine analyte as a representative sample time point (boxed in dotted lines).
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