Chronic cytomegalovirus infection drives the accumulation of memory T cells with low functional avidity during ageing

Submitted by Stephen James Griffiths

2011

For the degree of Doctor of Philosophy
Department of Immunology and Molecular Pathology, University College London

I, Stephen James Griffiths, confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

Signed:

Abstract

Immune senescence is associated with a predisposition to infections, poor vaccination responses and early mortality in older individuals. Furthermore, evidence that chronic cytomegalovirus (CMV) infection is a key driver of immune senescence is becoming increasingly recognised. This thesis aimed to investigate the hypothesis that large $CD8^+$ T cell expansions (TCEs) caused by chronic CMV infection during ageing may be instrumental in this association.

Data presented here shows CMV-specific $CD8^+$ TCEs that accumulate during ageing are predominately of the $CD45RA^+$ memory phenotype. However, these cells exhibit low Ki-67 positivity and low Bcl-2 levels directly *ex-vivo,* in addition to poor proliferation and low telomerase activity in response to activation. This indicates they are not accumulating through increased proliferation or resistance to cell-death, and may represent a population close to senescence. These CMV-specific $CD8^+CD45RA^+$ memory T cells were also found to display a lower functional avidity for peptide, with higher activation threshold compared with CMV-specific $CD8^+CD45RO^+$ T cells. Furthermore, IL-15 was shown to cause CMV-specific $CD8^+CD45RO^+$ memory T cells to proliferate and re-express CD45RA *in-vitro*; adding to existing evidence indicating a role for IL-15 in the homeostatic, rather than antigenic, driven generation of $CD8^+CD45RA^+$ memory T cells from a $CD45RO^+$ memory T cell pool.

The possible impact of these CMV-specific TCEs during ageing is highlighted by the finding that old CMV positive individuals had significantly shorter T cell telomere lengths than old CMV negative individuals. Therefore, the accumulation of TCEs is likely to impact the CD8 compartment of healthy individuals in two ways; by restricting immune space and also lowering the overall telomere length of the compartment through the accumulation of highly differentiated $CD8^+$ TCEs. Both of these have been shown to increase susceptibility to infection. This study therefore provides further evidence for the detrimental effects of CMV infection and its role in driving immune senescence.

Dedication

This thesis is dedicated to Harriet Filmer; who has supported me throughout this process and without whose love, kind nature and good humour this work would not have been possible. Thank you.

Acknowledgements

I would like to thank my supervisors, professors Arne Akbar and Vince Emery, for giving me the opportunity to conduct this research, and for their continual guidance and advice throughout. I also thank the Medical Research Council for funding the project.

My gratitude goes out to everyone in the Akbar group, especially: Jo Masters, with whom I began most of my training and initial research; Sian Henson for invaluable discussions, help with lab troubles and for reviewing the drafts of this thesis; Natalie Riddell for comments and discussions on this work; Milica Vukmanovic-Stejic for discussions; Valentina Libri for assisting with cell sorting and other work; Richard Macaulay, for interesting discussions, both scientific or otherwise. I would also like to thank everyone in the virology department of the Royal Free Hospital; especially Claire Jolly and Richard Milne for examining my MPhil/PhD transfer. I am also indebted to the invaluable support from others in the Windeyer, especially: Carolyn McElvaney, Isabel Lubeiro, Biljana Nikolic, and Pamela Manfield.

I would also like to thank our collaborators: Florian Kern and Raskit Lehmann at the Brighton and Sussex medical school; Janko Nikolich-Zugich and Anne Wertheimer at the University of Arizona center on aging; Michael Kemeny at the National University of Singapore; Paul Klenerman and Matthias Hoffmann at Oxford University. Finally, a very big thank you to my family and friends, and those volunteer donors!

List of publications

Griffiths, S.J., Masters, J., Henson, S.M, Libri, V., Lachmann, R., Fuhrmann, S., Wertheimer, A., Kemeny, M.D., Nikolich-Zugich, J., Klenerman, P., Kern, F., Emery V., and Akbar, A.N. (2011). Cytomegalovirus drives the accumulation of $CD8^+$ T_{EMRA} cells exhibiting a low functional avidity profile (in preparation).

Griffiths, S.J., Emery V., and Akbar, A.N. (2011). The use of quantum dot technology for the expanded application of Flow-FISH (in preparation).

van deBerg,P.J., **Griffiths,S.J**., Yong,S.L., Macaulay,R., Bemelman,F.J., Jackson,S., Henson,S.M., ten Berge,I.J., Akbar,A.N. and van Lier,R.A. (2010). Cytomegalovirus infection reduces telomere length of the circulating T cell pool. Journal of Immunology.*184*, 3417-3423.

Henson,S.M., Franzese,O., Macaulay,R., Libri,V., Azevedo,R.I., Kiani-Alikhan,S., Plunkett,F.J., Masters,J.E., Jackson,S., **Griffiths,S.J**, Pircher,H.P., Soares,M.V. and Akbar,A.N. (2009). KLRG1 signaling induces defective Akt (Ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. Blood.*113(26),* 6619-28.

Table of Contents

Abstract **3**
Dedication **5**
Acknowledgements **6**
List of publications **7**
List of Figures **13**
List of tables **15**
List of Abbreviations **16**
1 Introduction **18**
1.1 Ageing 18
1.1.1 Theories of ageing 19
1.1.2 Measuring cellular ageing – Telomere length 20
1.1.3 Telomerase 21
1.1.4 Ageing and infectious disease susceptibility 21
1.1.5 Ageing of the immune system – Immune senescence 22
1.2 T cells 23
1.2.1 T cell generation 23
1.2.2 TCR structure 24
1.2.3 T cell activation 25
1.2.3.1 Antigen presenting cells 26
1.2.3.2 T cell interaction with APC 27
1.2.3.3 Co-stimulation 28
1.2.3.4 T cell signalling events following activation 28
1.2.4 Generation of effector and memory T cells 29
1.2.5 Redressing the balance after resolution of infection 29
1.2.5.1 The extrinsic apoptotic pathway 30
1.2.5.2 The intrinsic apoptotic pathway 30
1.2.6 Differentiation pathways of effector and memory T cells 31
1.2.7 The identification of T cells using pMHC multimeric complexes 33
1.2.7.1 Affinity vs. Avidity 33
1.2.7.2 Identifying high avidity T cells using mutated tetramers 34
1.3 T cell subpopulations 36
1.3.1 Defining T cell subpopulations 36
1.3.1.1 CD45R isoform expression 36
1.3.1.2 CD27 and CD28 co-stimulatory molecule expression 37
1.3.1.3 Homing and adhesion molecule expression 37
1.3.2 Central memory (T_{CM}) and effector memory (T_{EM}) cells 38
1.3.3 $CD45RA^{+}$ Memory (T_{EMRA}) cells 39
1.3.4 Subpopulation consensus issues 40
1.4 The impact of ageing on T cells 42
1.4.1 Decreased naive T cell production during ageing 42
1.4.2 Increased naive T cell turnover during ageing 42

1.4.3 Expansion of memory T cells with ageing 43
1.4.4 Age-related restriction of the TCR repertoire 43
1.4.5 Inverted CD4:CD8 ratio 44
1.4.6 Down-regulation of CD27 and CD28 expression 44
1.4.7 T cell defects at a cellular level 45
1.4.8 T cell senescence vs. exhaustion 45
1.4.9 The immune risk phenotype (IRP) 48
1.5 The impact of ageing on other components of the immune system 49
1.5.1 The innate immunity and immune senescence 49
1.5.2 B cells and immune senescence 50
1.6 Cytomegalovirus 51
1.6.1 Discovery of CMV 51
1.6.2 Seroprevalence of CMV 51
1.6.3 CMV pathology 52
1.6.4 Latency 52
1.6.5 Is CMV reactivating in healthy individuals? 53
1.6.6 Immune responses to CMV 54
1.6.7 CMV immune evasion strategies 54
1.6.8 CMV and the immune risk phenotype 55
1.6.9 CMV-specific T cell differentiation 56
1.6.10 CMV-specific $CD8^+$ T cell clonal expansions 57
1.6.11 Are CMV-specific T cell expansions useful? 57
1.6.12 The Impact of T cell expansions 58
1.6.13 CMV, inflammation and the Inflamm-ageing hypothesis 59
1.7 Aims 60
2 General Materials and Methods 61
2.1 Donors 61
2.1.1 Donors and Blood sample collection 61
2.1.2 PBMC isolation 61
2.1.3 Determination of donor CMV status 62
2.1.4 Viable Cell Count 63
2.1.5 Cryopreservation of PBMCs. 63
2.2 Flow Cytometric Analysis 64
2.2.1 Surface staining PBMCs 64
2.2.2 Tetramer staining 65
2.2.3 Intracellular staining 65
2.2.4 Intranuclear staining 66
2.3 Isolation and sorting of populations from PBMCs 66
2.3.1 Negative selection of $CD8^+$ T cells using MACS isolation kit. 66
2.4 Measurement of telomere length and telomerase activity 68
2.4.1 Telomere length via Flow-FISH 68
2.4.2 Measuring telomere length in CMVpp65-specific cells 69
2.4.3 Measuring telomerase activity – the TRAP assay 69
2.5 Functional analysis 70

2.5.1 $CD8^+$ T cell subpopulation multiparametric flow cytometry 70
2.5.2 Activation responses of CD45RA vs. CD45RO CMV-specific $CD8^+$ T cells ... 70
2.5.3 Polyfunctionality of normal vs. high avidity CMV-specific $CD8^+$ T cells..... 71
2.6 IL-15 experiments..... 71
2.6.1 CD45RA re-expression experiments 71
2.6.2 Measuring IL-15 mRNA following treatment with CMV lysate or IFN-α 72
2.7 Statistical Analysis 73
3 Phenotyping $CD8^+$ and CMV-specific $CD8^+$ T cells according to age.....74
3.1 Introduction..... 74
3.2 Aims..... 74
3.3 Results 75
3.3.1 Selecting suitable donors and steps for phenotypic analysis 75
3.3.2 Investigating the differentiation status of $CD8^+$ T cells 78
3.3.2.1 Differentiation of $CD8^+$ T cells by CD45RA and CD27 expression..... 78
3.3.2.2 $CD8^+$ T cell subpopulation profiles by age group 79
3.3.2.3 Age-related differentiation of $CD8^+$ T cells..... 80
3.3.2.4 CMV accelerates age-related differentiation of $CD8^+$ T cells 80
3.3.3 CMV-specific $CD8^+$ T cell expansion during ageing..... 86
3.3.3.1 CMV-specific $CD8^+$ T cells expands during ageing 86
3.3.3.2 CMV-specific $CD8^+$ T_{EMRA} cells dominate in older individuals..... 88
3.3.4 Investigating the rate of proliferation by $CD8^+$ T_{EMRA} cells 92
3.3.4.1 Using Ki-67 expression as a marker for proliferation 92
3.3.4.2 $CD8^+$ T_{EMRA} cells show low Ki-67 93
3.3.5 Apoptosis resistance of $CD8^+$ and CMVpp65-specific $CD8^+$ T cells..... 97
3.3.5.1 Bcl-2 expression as an indicator of susceptibility to apoptosis..... 97
3.3.5.2 $CD8^+$ T_{EMRA} cells show little resistance to apoptosis..... 98
3.4 Discussion..... 102
4 CMV infection and telomere length attrition during ageing 107
4.1 Introduction..... 107
4.2 Aims..... 108
4.3 Results 109
4.3.1 Measuring telomere lengths..... 109
4.3.2 CMV accelerates age-associated telomere attrition in T cells 111
4.3.2.1 Telomere lengths in total lymphocytes..... 111
4.3.2.2 Telomere lengths in $CD4^-CD8^-$ lymphocytes..... 112
4.3.2.3 Telomere lengths in $CD4^+$T cells 112
4.3.2.4 Telomere lengths in $CD8^+$T cells 113
4.3.3 Using Quantum dot technology in the Flow-FISH assay..... 118
4.3.3.1 Qdots show superior heat stability during flow-FISH..... 118
4.3.4 Telomere lengths within $CD8^+$ T cell subpopulations 122
4.3.4.1 Telomere lengths within $CD8^+$ T cell subpopulations 122
4.3.4.2 Impact of CMV on $CD8^+$ T cell subpopulation telomere lengths..... 124
4.3.5 Telomere lengths within CMV-specific $CD8^+$ T cells 126
4.3.5.1 Telomere lengths within CMV-specific $CD8^+$ T cells..... 126

4.4 Discussion 130
5 The generation of CMV-specific CD8$^+$ T cell expansions during ageing 133
5.1 Introduction 133
5.2 Aims 135
5.3 Results 136
5.3.1 Proliferative responses of CD8$^+$ T cell subpopulations to stimulus 136
5.3.1.1 CD8$^+$ T_{EMRA} cells show poor proliferative responses 136
5.3.1.2 CMV-specific CD8$^+$ T_{EMRA} cells show poor proliferative responses 137
5.3.2 Telomerase activity of CD8$^+$ and CMV-specific CD8$^+$ T cells 139
5.3.2.1 Measuring telomerase activity using the TRAP assay 139
5.3.2.2 CD8$^+$ T_{EMRA} cells show low level telomerase activity 140
5.3.2.3 CMV-specific CD8$^+$ T_{EMRA} cells show low level telomerase activity 140
5.3.2.4 CD8$^+$ T_{EMRA} cells show evidence of high DNA damage 142
5.3.3 Proliferative and phenotypic responses to IL-15 cytokine 144
5.3.3.1 IL-15 maintains the survival and proliferation of CD8$^+$ T cells 146
5.3.3.2 IL-15 causes CD45RA re-expression 146
(B) 147
5.3.3.3 IL-15 causes CMV-specific T cells to proliferate 148
5.3.3.4 IL-15 causes CMV-specific T cell CD45RA re-expression 148
5.3.3.5 CMV lysate and IFN-α increases production of IL-15 150
5.4 Discussion 152
6 The functional quality of CMV-specific CD8$^+$ T cells during ageing 156
6.1 Introduction 156
6.2 Aims 158
6.3 Results 159
6.3.1 CD8$^+$ T cell subpopulation functional responses to activation 159
6.3.1.1 CD8$^+$ T_{EMRA} cells show low frequency responses to activation 159
6.3.2 CMV-specific CD45RA vs. CD45RO functional responses 161
6.3.2.1 CMV-specific CD45RA$^+$ T cells show low frequency responses 162
6.3.3 Investigating CMV-specific CD8$^+$ T cell avidity for antigen 164
6.3.3.1 Using mutated 'CD8null' tetramers to identify high avidity T cells 164
6.3.4 CMV-specific CD8$^+$ T cell avidity and ageing 165
6.3.4.1 Low avidity NLV-specific CD8$^+$ T cell populations in the old 165
6.3.5 The relationship between avidity and CD45RA re-expression 167
6.3.5.1 CD45RA expression is associated with low avidity 167
6.3.6 Co-receptor availability on CD8$^+$ and CMV-specific CD8$^+$ T cells 170
6.3.6.1 CD8null tetramers identify T cells with superior polyfunctionality 171
6.4 Discussion 175
7 General Discussion 180
7.1 CMV and the Immune Risk Phenotype 180
7.2 Senescence vs. exhaustion in T_{EMRA} cells 182
7.3 Generation of T_{EMRA} cells 186
7.4 Mechanism and purpose for reversion 189
7.5 The impact of CMV on telomere length 190

7.6 Clinical relevance of the study 191
7.7 Future studies..... 195
7.7.1 Generation of high vs. low avidity T cell populations 195
7.7.2 Investigating other CMV antigens 195
7.7.3 Alternate senescence pathways..... 196
7.7.4 Telomere length analysis using quantum dots. 196
Bibliography 197

List of Figures

Figure 1-1. Models of T cell differentiation pathways. 32
Figure 1-2. Antigen-specific $CD8^+$ T cell staining using normal or mutated (CD8null) tetramers. 35
Figure 1-3. The 3 phases of senescence versus exhaustion 46
Figure 3-1. Donor selection process 76
Figure 3-2. CMV positive screening and selection for tetramer analysis 77
Figure 3-3. Gating method to identify $CD8^+$ T cell subpopulations from PBMCs 79
Figure 3-4. Representative density plots of $CD8^+$ T cell subpopulation profiles by age group 79
Figure 3-5. Distribution of naive ($CD45RA^+CD27^+$) $CD8^+$ T cells and the impact of age and CMV serostatus. 82
Figure 3-6. Distribution of central memory ($CD45RA^-CD27^+$) $CD8^+$ T cells and the impact of age and CMV serostatus. 83
Figure 3-7. Distribution of effector memory ($CD45RA^-CD27^-$) $CD8^+$ T cells and the impact of age and CMV serostatus. 84
Figure 3-8. Distribution of effector memory CD45RA re-expressing ($CD45RA^+CD27^-$) $CD8^+$ T cells and the impact of age and CMV serostatus. 85
Figure 3-9. The CMVpp65-specific $CD8^+$ T cell population expands with age 87
Figure 3-10. Differentiation status of CMVpp65-specific $CD8^+$ T cells in young and old donors. 89
Figure 3-11. Relative size of each CMVpp65-specific $CD8^+$ T subpopulation as a percentage of total CD8+ T cell subpopulation in young and old donors. 90
Figure 3-12. Relative size of CMVpp65-specific $CD8^+$ T subpopulations as a percentage of total CD8+ T cells in young and old donors. 91
Figure 3-13. Representative example of Ki67 staining directly *ex-vivo* in one individual 94
Figure 3-14. Ki-67 within total $CD8^+$ and CMVpp65-specific $CD8^+$ T cells..... 95
Figure 3-15. Ki-67 within $CD8^+$ and CMVpp65-specific $CD8^+$ T cell subpopulations..... 96
Figure 3-16. Bcl-2 staining directly *ex-vivo* 99
Figure 3-17. Bcl-2 levels in $CD8^+$ and CMV-specific $CD8^+$ T cells..... 100
Figure 3-18. Bcl-2 expression in $CD8^+$ and $CD8^+$ CMVpp65-specific T cell subpopulations 101
Figure 4-1. The Flow-FISH procedure 110
Figure 4-2. The impact of age and CMV serostatus on telomere lengths in total lymphocytes 114
Figure 4-3. The impact of age and CMV serostatus on telomere lengths in $CD4^-CD8^-$ lymphocytes 115
Figure 4-4. The impact of age and CMV serostatus on telomere lengths in $CD4^+$ T cells 116
Figure 4-5. The impact of age and CMV serostatus on telomere lengths in $CD4^+$ T cells 117
Figure 4-6. Histograms depicting original fluorochromes versus Qdots 119

Figure 4-7. The use of Quantum dot technology in the flow-FISH protocol 121
Figure 4-8. Using Qdots to investigate telomere lengths within $CD8^+$ T cell subpopulations 123
Figure 4-9. Telomere lengths of $CD8^+$ T cell subpopulations grouped by age and CMV status..... 125
Figure 4-10. Determining telomere lengths of CMV-specific $CD8^+$ T cell subpopulations 127
Figure 4-11. Telomere lengths of $CD8^+$ CMVpp65-specific CD45RA and CD45RO memory subpopulations 129
Figure 5-1. Proliferative responses of sorted $CD8^+$ and CMV-specific $CD8^+$ T cell subpopulations to stimulation..... 138
Figure 5-2. Telomerase levels in $CD8^+$ and CMV-specific $CD8^+$ T cell subpopulations .. 141
Figure 5-3. DNA damage levels as indicated by γ-H2AX expression on CD8+ T cell subpopulations 143
Figure 5-4. Schematic of IL-15 reversion experiments 145
Figure 5-5. Proliferative and phenotypic responses of $CD8^+$ T cell subpopulations to IL-15 treatment..... 147
Figure 5-6. Homeostatic proliferation and CD45RA re-expression on $CD8^+$ and CMVpp65-specific $CD8^+$ CD45RA negative T cells during IL-15 treatment..... 149
Figure 5-7. IL-15 production following the stimulation of total PBMCs with either IFN-α or CMV lysate..... 151
Figure 6-1. Analysing polyfunctional responses of $CD8^+$ T cell subpopulations to activation 160
Figure 6-2. Outline for CMV-specific CD45RA vs. CD45RO $CD8^+$ T cell activation experiments..... 161
Figure 6-3. Functional response of CMV-specific CD45RA vs. CD45RO memory $CD8^+$ T cell populations..... 163
Figure 6-4. Normal vs. mutated (CD8 Null) tetramers..... 164
Figure 6-5. Identification of normal and high avidity populations within donors..... 166
Figure 6-6. $CD45RA^+$ memory CMVpp65-specific $CD8^+$ T cells are associated with low avidity..... 168
Figure 6-7. Dual populations identified with TPR-specific tetramer show alternate phenotypes 169
Figure 6-8. CD8 co-receptor expression in global $CD8^+$ and CMV-specific $CD8^+$ T cell subpopulations 170
Figure 6-9. Norm vs. high affinity polyfunctional comparison experiments..... 172
Figure 6-10. CMVpp65-specific $CD8^+$ T cell functional responses identified by avidity tetramers 174
Figure 7-1. Hypothesis for the generation of CMV-specific TEMRA cells during ageing 188

List of tables

Table 1-1. Examples of cellular damage that may contribute to ageing 19
Table 1-2. Subsets of human dendritic cells 26
Table 1-3. Members of the BCl-2 family involved in apoptosis. 31
Table 1-4. Phenotypic markers defining T cell subpopulations. 41
Table 1-5. Parameters of the IRP 48
Table 1-6. HCMV genes involved in immune evasion 55
Table 2-1. Details and concentrations of antibodies used 64
Table 4-1. Original vs. New Flow-FISH set-up on the BD LSR I 122

List of Abbreviations

AIDS	Acquired immune deficiency syndrome
APC	Allophycocyanin
APC	Antigen presenting cell
CMV	Cytomegalovirus
DAG	Diacylgylcerol
DDR	DNA damage response
DKC	Dyskeratosis congenita
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
Flow-FISH	Flow cytometric detection of fluorescence in situ hybridization
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HSV	Herpes simplex virus
IE	Immediate early
IFN	Interferon
IL	Interleukin
IRP	Immune risk phenotype
kB	kilo base
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LCMV	Lymphocytic choriomeningitis virus
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

NK cell	Natural killer cell
NLV	NLVPMVATV HLA-A*02 restricted epitope of CMVpp65
NONA	Nonagenarian longitudinal studies
OCTO	Octogenarian longitudinal studies
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
pMHC	Peptide MHC complex
RNA	Ribonucleic acid
RTE	Recent thymic emigrants
SARS	Severe acute respiratory syndrome
SIV	Simian immunodeficiency virus
SSC	Side scatter
TCE	T Cell Expansion
T_N	Naive T cell
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memory CD45RA expressing T cell
TCR	T cell receptor
TNF	Tumour necrosis factor
TPR	TPRVTGGGAM HLA-B*07 restricted epitope of CMVpp65
T_{reg} cell	Regulatory T cell
VZV	Varicella zoster virus
WT	Wild type

1 Introduction

Medical advances during the 20th century have contributed to the extension of life-expectancy by a staggering 30 years in western countries, with developing countries showing the most rapid gains (Kirkwood, 2008, Christensen et al., 2009). However, predictions that 40% of the population in Europe and America will be over 60 years of age by 2050 (Lutz et al., 1997) raises concerns of how public healthcare systems will cope with an increasingly aged population (Christensen et al., 2009). The incidence of age associated illnesses is predicted to become increasingly common, putting pressure on healthcare systems and resources (Adhikari et al., 2010). Longer life expectancy also presents a new challenge to the immune system, as it must continue to protect for many more years than our ancestors required. A lifetime of antigenic burden may complicate a variety of clinical conditions in the upper limits of life expectancy (Franceschi et al., 2007, Soderberg-Naucler, 2006). Now more than ever, it is important to identify these age-related changes and determine their influence on the quality and duration of life.

1.1 Ageing

Ageing is characterised by the declining ability to respond to stress, increased homeostatic imbalance, and increased risk of age associated diseases, with the inevitable consequence being death. However, the exact mechanisms of ageing remain insufficiently understood.

1.1.1 Theories of ageing

Generally, theories of ageing fall into one of two categories: programmed or stochastic theories. Programmed theories propose ageing is due to changes in gene expression, which control maintenance, repair and defence responses, and is advantageous to the species as a whole, rather than the individual, by having a finite lifespan (Ljubuncic and Reznick, 2009). Stochastic theories propose the lifelong accumulation of a wide variety of molecular and cellular damage causes ageing (Kirkwood, 2008), which are summarised in table 1.1. However, this categorisation of theories has been questioned (Rattan, 2006), and ageing is now generally regarded to result from multiple causes.

DNA damage	RNA damage	Protein damage	Membrane damage
Copying/repair errors	Transcription errors	Misfolding	Oxidation
Telomere shortening	Aberrant slicing	Synthesis error	
Mitochondrial mutations		Post-translational error	
Viral gene disruption		Aberrant aggregation	
Epigenetic modifications		Impaired catabolism	

Table 1-1. Examples of cellular damage that may contribute to ageing (Adapted from Kirkwood, 2008).

The evolutionary aspects of ageing have also been hotly debated. Darwin's theory of natural selection is based on the concept that random and heritable variations of biological traits will lead to the reproduction of those who are preferentially fit to survive in the environment. However, as Peter Medawar formalised in his mutation accumulation theory - the force of natural selection weakens with increasing age through 'real hazards of mortality' (predation, disease, accidents); therefore if a 'genetic disaster' occurs later in life, its effects may be completely unimportant (Medawar 1946).

1.1.2 Measuring cellular ageing – Telomere length

Research into cellular senescence has long attracted both popular and scientific interest. The dominant view of the early 20th century was that cells could grow indefinitely, highlighted in Alexis Carrel's publication 'on the permanent life of tissues outside the organism' (Carrel, 1912). It was not until 1962, that Leonard Hayflick challenged the dogma by showing that normal cells had a limited capacity for replication - since termed 'the Hayflick limit' (Hayflick, 1965), and also referred to as replicative senescence. The sensing mechanism that limits this lifespan is the telomere. Telomeres are composed of the simple sequence TTAGGG, which is repeated many hundreds to thousands of times at the chromosome ends (Hodes et al., 2002). 50-100 base pairs of this telomeric DNA are lost per cell division due to the inability of DNA polymerase to fully replicate the ends of chromosomes. With increasing divisions, a decrease in telomere length results; eventually leading to DNA instability and apoptosis, or cell-arrest through p53 (Rufer et al., 1999). Indeed, a hallmark of senescent cells is the diminished ability to respond to cellular stress and overall physical and oxidative stress (Effros et al., 2005). Telomere length can therefore be indicative of the residual replicative capacity of the cell, making it an important biomarker in the context of senescence. Most importantly, mounting evidence suggests short telomeres in lymphocytes are a risk factor for mortality in cardiovascular disease, Hepatitis C infection, and age-related diseases such as Alzheimer's disease (Samani et al., 2001, Calado and Young, 2009, Brouilette et al., 2007, Brouilette et al., 2003). One study has also correlated short telomeres with an eight fold increase in susceptibility to death by infection (Cawthon et al., 2003).

1.1.3 Telomerase

Telomere attrition can be suppressed by the enzyme telomerase, a ribonucleoprotein complex that adds back the telomeric repeat sequence directly to the single stranded telomeric overhang (Akbar and Vukmanovic-Stejic, 2007). Lymphocytes are among a small number of somatic cells that can up-regulate telomerase, which compensates for telomere attrition (Hiyama et al., 1995, Weng et al., 1996). This suggests an important role in the preservation of immune memory (Hodes et al., 2002, Beverley, 2010). However, repeated stimulation can eventually lead to loss of telomerase activity in lymphocytes (Plunkett et al., 2007, Henson et al., 2009, Lobetti-Bodoni et al., 2010, Hodes et al., 2002). Telomerase therefore represents a possible target for the reversion of age-related damage, which has already been demonstrated in mouse studies (Jaskelioff et al., 2011). Dysfunctions of telomerase can also lead to malignancy, with current estimates suggesting 85% of tumour cells are the result of telomerase up-regulation (Cesare and Reddel, 2010). The significance of telomerase was recognized by awarding the 2009 Nobel Prize of Physiology and Medicine to its discoverers: Elizabeth Blackburn, Carol Greider and Jack Szostak.

1.1.4 Ageing and infectious disease susceptibility

Age-associated susceptibility to a variety of pathogens including pneumonia, meningitis, sepsis, urinary tract infections, influenza, and Respiratory Syncytial Virus have been documented (Gorina et al., 2008, Fry et al., 2005, Akbar et al., 2004, Nikolich-Zugich, 2008). Viral reactivation is also more common in old individuals; the best example being

Varicella-Zoster Virus (VZV) (LaGuardia and Gilden, 2001), but there are also reports of Epstein-Barr virus (EBV) and cytomegalovirus (CMV) reactivations (Scott et al. 1994;(McVoy and Adler, 1989). Furthermore, novel pathogens in particular, are known to cause greater disease severity in the old; Severe Acute Respiratory Syndrome (SARS) was rarely fatal in the young, but caused >50% mortality in the old (Donnelly et al., 2003). Also, West Nile Virus, an emerging virus in the US, showed 80% of adult infections under 50 experienced an asymptomatic infection, with <1% developing severe disease, whereas lethality was 10 fold higher in the over 50's, and a staggering 40-50 times higher in the over 70's (Chowers et al., 2001, Mostashari et al., 2001, O'Leary et al., 2004). Individuals with premature ageing syndromes such as Dyskeritosis congenita, who mimic many age-related changes to the immune system seen in the old, are also more susceptible to infections (Sedgwick & Boder, 1991).

1.1.5 Ageing of the immune system – Immune senescence

The extent of immunological memory to a previous pathogenic challenge is remarkable, and can persist for a lifetime without re-challenge. This is exemplified by the fact that 90% of those given the smallpox vaccine up to 75 years ago, still maintain immunity (Hammarlund et al., 2003). However the immune system undergoes significant changes during ageing, including a major reduction in T cell generation through thymic involution, and senescence of the memory pool through repeated homeostatic proliferation. Poor vaccination responses are also commonplace in the old, highlighted by the fact that seasonal influenza vaccine has an efficacy of just 30-40% in the over 65's

(Kovaiou et al., 2007, McElhaney and Dutz, 2008, Seder et al., 2008, Weinberger et al., 2008). Such age-associated impairments of the immune system, collectively called immune senescence, are therefore thought to contribute to the increased incidence in age-associated susceptibility to infection.

1.2 T cells

Before discussing the impact of ageing on T cells (section 1.4), an overview of T cells will be given. This section outlines: what T cells are and how they are generated; how T cells are activated; the generation of effector and memory T cells; the maintenance of T cell numbers; and what constitutes a quality T cell response.

1.2.1 T cell generation

T cells are generated from bone marrow derived haematopoietic progenitors, but undergo selection and maturation in the thymus (Kindt et al., 2007). Haematopoietic progenitors enter the thymus from the blood (Schwarz and Bhandoola, 2006), and undergo extensive cell division within the thymus, to generate a large population of immature thymocytes (Kindt et al., 2007). Immature thymocytes each make a distinct T cell receptor (TCR), by a process of random gene rearrangement (Kruisbeek, 1993). TCR recognizes antigen only when presented by major histocompatibility complex (MHC) molecules, on antigen presenting cells (APCs)(Kindt et al., 2007). Thymocytes undergo a selection procedure by interaction with APCs in the thymus. Positive selection selects for T cells capable of interacting with self MHC presented by thymic stromal cells

(Kruisbeek, 1993). Only those thymocytes interacting with adequate affinity for self antigen and MHC receive a survival signal and are positively selected, whereas those with insufficient affinity die by apoptosis (Starr et al., 2003). The two main subsets of T cell are the $CD4^+$ and $CD8^+$ T cell, which recognize antigen presented by MHC class II and MHC class I respectively. The fate of a thymocyte as a $CD4^+$ or $CD8^+$ T cell is dependent on whether they are presented self peptide by MHC II or MHC I respectively, during positive selection (Starr et al., 2003). Thymocytes which survive positive selection then migrate towards the thymic medulla, at which point they are again presented with self antigen in complex with MHC by APCs including dendritic cells and macrophages. Thymocytes that interact too strongly at this point undergo apoptosis by a process of negative selection. The vast majority of thymocytes will die at this stage (Starr et al., 2003). Therefore, only interactions with intermediate reactivity survive selection, and are exported into the periphery as recent thymic emigrants, which mature post thymically to form naïve T cells (Shortman et al., 1990). This results in a naive T cell repertoire that is sufficiently diverse and reactive towards a vast array of foreign antigens, but is also non-reactive to self antigens (Nikolich-Zugich et al., 2004).

1.2.2 TCR structure

The TCR is a heterodimer composed of two different protein chains: 95% of T cells display the αβ heterodimer and 5% the γδ heterodimer (Kindt et al., 2007). Each chain of the TCR is a member of the immunoglobulin superfamily; consisting one N-terminal immunoglobulin (Ig) variable (V) domain (binding MHC and presented antigen, pMHC)

and one Ig constant (C) domain (a trans-membrane spanning region and a short cytoplasmic tail at the C terminal end involved with intracellular cell signalling events) (Rudolph et al., 2006). αβ TCR diversification mainly occurs in the thymus, by stochastic recombination of non-contiguous gene segments, with further diversity enhanced by imprecise joining of nicked segments, addition of non-germline nucleotides by DNA-repair machinery, and pairing of different α and β segments (Nikolich-Zugich, 2008). Binding of the variable domain to pMHC is largely dependent on the complementarity determining regions (CDRs). There are 3 CDRs on each vB protein chain, with CDR3 showing the greatest diversification (Kindt et al., 2007). Diversification results in the T cell repertoire i.e. a pool of T cells specific for a vast array of antigens. Once activated, a naïve T cell will proliferate and differentiate into effector or memory T cells, which generate immune responses against cells infected by pathogen or altered self (cancerous) cells - referred to as cell-mediated immunity.

1.2.3 T cell activation

The activation of naive $CD4^+$ and $CD8^+$ T cells are generally similar, and are initiated by co-ordinated signalling from two main sources: the interaction of TCR with processed antigen presented by MHC on the APC, and co-stimulatory molecule signalling (Smith-Garvin et al., 2009). It is also the role of various accessory and adhesion molecules to stabilise and sustain this interaction, and for cytokines to direct the responses during and following activation (Kindt et al., 2007).

1.2.3.1 Antigen presenting cells

As all cells express either MHC class I or II, strictly speaking they are all antigen presenting cells (APCs). However, by definition, those cells displaying MHC class I only are referred to as target cells (for $CD8^+$ T cells) and those displaying MHC class II (to $CD4^+$ T cells) are APCs (Kindt et al., 2007). Although a variety of cells may act as APCs, three types are classified as professional APCs: dendritic cells (DCs), macrophages, and B cells. Non-professional APCs are cells induced to express MHC class II and co-stimulatory molecules transiently during an inflammatory response, and include fibroblasts in the skin, glial cells in the brain, pancreatic β cells, and various epithelial and endothelial cells (Nickoloff and Turka, 1994). DCs are among the most effective APCs, as they constitutively express high levels of MHC class II and co-stimulatory molecules. DCs can further be divided on the basis of their anatomical location, phenotype and function as depicted in table 1.2.

	Plasmacytoid DCs	**Myeloid DCs**	**Langerhans cells**	**Interstitial DCs**
Location	Blood	Blood	Epidermis	Dermis and other tissues
Phenotype	$CD11c^-CD1a^+$ $CD1c^-CD123^{high}$ $CD304^+$	$CD11c^+CD1a^+$ $CD1c^+CD123^{low}$ $CD304^-$	$CD11c^+CD1a^+$ $CD207^+$	$CD11c^+CD1a^-$ $CD68^+$ coagulation factor XIII A chain
T cell priming	Yes	Yes	Yes	Yes
B cell activation	Yes	Yes	Weak	Yes
IFN-α production	High	Yes	Yes	Yes

Table 1-2. Subsets of human dendritic cells (adapted from Lambotin et al., 2010).

DCs will initially exist as immature DCs; unable to activate T cells as they lack the requisite molecules such as CD40, CD54, and CD86 (Banchereau and Steinman, 1998). However, these cells are efficient at capturing antigen, and can take up particles or microbes by a variety of methods, including: phagocytosis, pinocytosis, c-type lectin receptors and pattern recognition receptors (PPRs) such as toll-like receptors (TLRs) (Banchereau and Steinman, 1998, Lambotin et al., 2010). Antigen capture triggers full maturation and mobility of the DC to T cell rich areas of the lymph nodes, during which time antigen is processed and displayed together with MHC, on the surface of the DC (Banchereau and Steinman, 1998). DCs migrate to lymphoid tissues such as the spleen and lymph nodes, where they complete their maturation and release chemokines to attract circulating T cells and B cells (Adema et al., 1997).

1.2.3.2 T cell interaction with APC

The interaction between the TCR complex on the surface of the T cell and antigen presented with MHC on the APC is a highly specific one. However, other molecules strengthen this interaction, including the co-receptors CD4 and CD8 that bind to conserved regions on MHC class II and class I respectively. These co-receptors prolong engagement and recruit essential molecules inside the cell which are involved in signalling (Rudolph et al., 2006). The TCR also associates with CD3 on the surface of the T cell, forming a TCR-CD3 complex. The CD3 accessory molecule participates in signal transduction following interaction of the TCR with antigen, but does not itself influence antigen binding (Smith-Garvin et al., 2009).

1.2.3.3 Co-stimulation

Signalling solely through the TCR results in a non-responsive state known as anergy, therefore co-stimulation is required for productive activation (Smith-Garvin et al., 2009). Co-stimulation may be provided by various molecules, but two families of receptors, the CD28 receptor family and the tumour necrosis factor (TNF) receptor family, are key players in providing co-stimulation. Various studies have shown the importance of CD28, which binds CD80 and CD86 on APC's, in promoting T cell proliferation, survival, metabolism and cytokine production (reviewed in(Acuto and Michel, 2003). The TNF receptor family includes CD27, CD40, 4-1BB and OX-40. CD27 for example, binds CD70 on APCs, and is important in the generation, survival, maintenance, and quality of a T cell response (Duttagupta et al., 2009).

1.2.3.4 T cell signalling events following activation

The first step of intracellular signalling following TCR ligation is the activation of src (lck and fyn) protein tyrosine kinases (PTKs), which leads to the phosphorlyation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of CD3 (van der Merwe and Dushek, 2011). This triggers the recruitment of ZAP-70 and subsequent cascade of phosphorylation events (Smith-Garvin et al., 2009). Among such events are the activation of phospholipase C γ-1 (PLCγ-1) dependent pathways, including Ca^{2+} and Diacylglycerol (DAG) induced responses, cytoskeletal rearrangement and integrin activation pathways (van der Merwe and Dushek, 2011). Co-stimulatory molecules such as CD28 also act to augment these responses (Acuto and Michel, 2003).

1.2.4 Generation of effector and memory T cells

Upon successful activation, the T cell will undergo rounds of repeated division 2-3 times per day for 4-5 days, enhanced by an increase in IL-2 levels, generating a progeny of T cell effector clones. Naive $CD8^+$ T cells become cytotoxic effector cells, targeting intracellular pathogens and altered self cells; whereas $CD4^+$ T cells largely provide effector cytokine help to $CD8^+$ T cell and B cell responses (Kindt et al., 2007). On resolution of the infection, the vast majority of the expanded population of effector T cell clones are removed, and a population of memory $CD4^+$ and $CD8^+$ T cells are formed, providing protection in the event of re-challenge.

1.2.5 Redressing the balance after resolution of infection

Following the successful clearance of a pathogen, up to 95% of the expanded T cell clones are removed, as they no longer benefit the host and may produce large amounts of potentially detrimental cytokines (Krammer et al., 2007). At the same time, immunological memory must be formed in the event antigen is re-encountered. A T cell's fate will be determined both by the survival signals it receives (through TCR, co-stimulatory molecules, adhesion molecules and cytokines) and the apoptotic signals it receives (Adams and Cory, 1998). Caspases are the executioners of apoptosis, a family of proteases having a major role in regulating most mechanisms of apoptotic cell death, including extrinsic cell-death-receptor-dependent apoptosis and intrinsic mitochondria-dependent apoptosis (Krammer et al., 2007). These two pathways have different origins of initiation, but can converge downstream of activation at the point of caspase release.

1.2.5.1 The extrinsic apoptotic pathway

The extrinsic apoptotic pathway is initiated by ligation of the cell-death receptors containing 'death domains' on the surface of cells. These include TNF receptor, CD95 (FasL or apo-1), TNF related apoptosis inducing ligand receptor (TRAIL) 1 and TRAIL 2, and death receptor 3 (DR3) or DR6 (Ashkenazi and Dixit, 1998). Pro-apoptotic signals are delivered by these receptors through a death inducing signalling complex (DISC) formation on the plasma membrane e.g. CD95 triggering by CD95L, results in the formation of the CD95 DISC. This initiates downstream caspase activation leading to apoptosis of the T cell.

1.2.5.2 The intrinsic apoptotic pathway

The intrinsic apoptotic pathway differs from the extrinsic apoptotic pathway in that it is regulated by the balance of pro- and anti-apoptotic signals predominately from the B cell lymphoma 2 (Bcl-2) family (Adams and Cory, 1998, Aggarwal and Gupta, 1998, Chao and Korsmeyer, 1998). A number of BCl-2 family members have been discovered and are summarised in table 1.3. The intrinsic apoptotic pathway involves mitochondrial permeabilisation, causing the release of mitochondrial contents including cytochrome C. Together with apoptotic-protease activating factor 1 (APAF-1), this initiates apoptosome formation and caspase release, leading to the destruction of the cell through apoptosis (Adams and Cory, 1998, Chao and Korsmeyer, 1998).

Pro-apoptotic	Anti-apoptotic
Bax (Oltvai et al., 1993)	Bcl-2 (Chao and Korsmeyer, 1998)
Bak (Chittenden et al., 1995)	Bcl-X_L (Boise et al., 1993)
Bad (Yang et al., 1995)	Mcl-1 (Zhou et al., 1997)
Bcl-X_S (Boise et al., 1993)	Bcl-w (Gibson et al., 1996)
Bik (Boyd et al., 1995)	A1 (Lin et al., 1996)
Bid (Wang et al., 1996)	

Table 1-3. Members of the BCl-2 family involved in apoptosis (with references).

1.2.6 Differentiation pathways of effector and memory T cells

It remains unclear whether effector or memory T cells arise independently from each other or if they are the same cells at various stages of differentiation. The two main models proposed for the differentiation of T cells are the linear and divergent pathways (figure 1.1). As their name's suggest, the linear pathway proposes a direct lineage from naive to effector, then to memory, T cells (Opferman et al., 1999, Sallusto et al., 1999) whereas the divergent pathway proposes effector and memory cells are generated as daughter cells from one cell (Chang et al., 2007, Manjunath et al., 2001). Evidence exists to support both models, and are reviewed elsewhere (Kalia et al., 2010), however a few lines of evidence will be mentioned here. Support of a linear differentiation pathway comes from studies using lymphocytic choriomeningitis virus (LCMV) infection, which shows memory $CD8^+$ T cells develop from a greatly expanded population of effector $CD8^+$ T cells (Kaech et al., 2002, Murali-Krishna et al., 1998). Additionally, adoptive transfer experiments have shown memory $CD4^+$ T cells can be generated from antigen activated effector $CD4^+$ T cells *in-vivo* (Garcia et al., 1999). Support for a divergent

pathway comes from elegant experiments by Change et al., in which they showed activated naive T cells exhibit unequal partitioning of proteins that mediate cell signalling and cell fate, leading to asymmetrical division of the two daughter cells, which are differentially fated phenotypically and functionally to become effector or memory T cells (Chang et al., 2007).

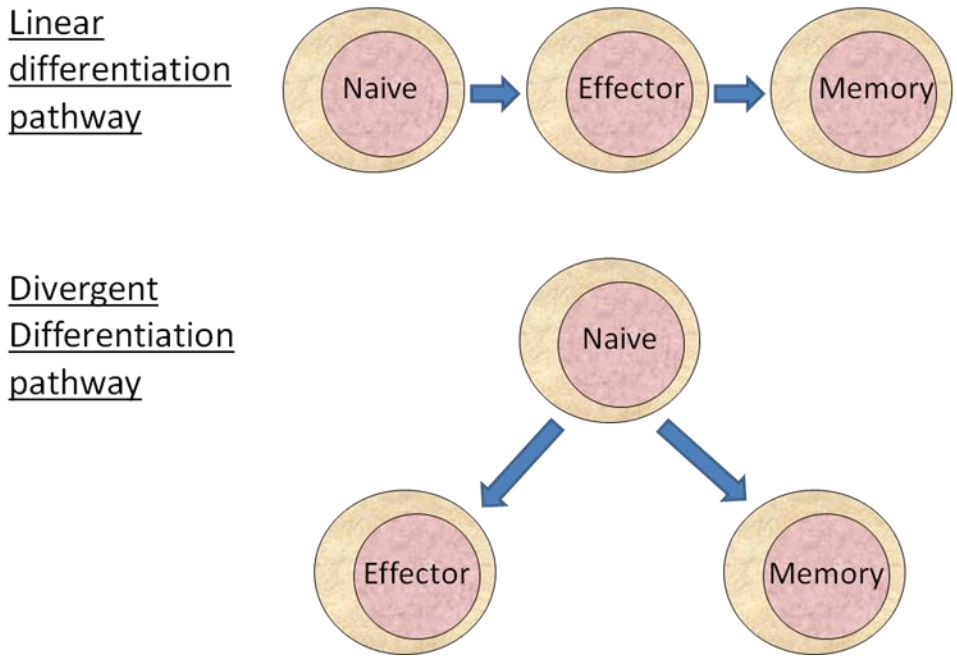


Figure 1-1. Models of T cell differentiation pathways.

Two models proposing the differentiation pathway of T cells are shown. The linear pathway (top) proposes a direct lineage from naive to effector then to memory T cells, whereas the divergent pathway (bottom) proposes effector and memory cells are generated as daughter cells at the same time from one single cell.

1.2.7 The identification of T cells using pMHC multimeric complexes

HLA-peptide multimeric complexes allow for the direct *ex-vivo* visualization of antigen-specific T cells by flow cytometry (Klenerman et al., 2002, Ogg and McMichael, 1998, Altman and Davis, 2003). Specific peptide is engineered and folded in the presence of MHC to construct single pMHC monomers. These pMHC monomers are therefore recognized by T cells specific for that peptide. The TCR-pMHC interaction is rather weak however, therefore single pMHC monomers are biotinylated (using Bir A enzyme) then conjugated (using streptavidin) together as four, five or more multimeric units to generate tetramers, pentamers or streptamers respectively. These provide multiple TCR-pMHC interactions which increases the chance of binding, and therefore of staining, the T cell of interest (figure 1.2a). Although, it is currently believed three (or even two) pMHC monomer complexes are sufficient for staining (Wooldridge et al., 2009). Multimers are usually fluorescently labeled during streptavidin conjugation to facilitate T cell identification by flow cytometry (Altman and Davis, 2003).

1.2.7.1 Affinity vs. Avidity

The terms affinity and avidity are often used interchangeably, however they are distinct (Stone et al., 2009). Affinity is defined as the strength of the interaction between one ligand and its receptor e.g. TCR affinity for peptide presented by MHC (pMHC). TCR affinity for peptide is determined during T cell generation (section 1.2.2). Avidity is defined as the sum strength of multiple interactions e.g. TCR binding to pMHC is strengthened by additional interactions between the T cell and APC (Smith-Garvin et al., 2009). These include: co-receptor binding e.g. CD4 and CD8 bind conserved region of

MHC; co-stimulatory molecules e.g. CD27 and CD28 on T cells interact with CD70 and CD80/CD86 on APCs; adhesion molecules e.g. LFA-1 on T cell bind cellular adhesion molecules on APCs (Smith-Garvin et al., 2009, Stone et al., 2009)

1.2.7.2 Identifying high avidity T cells using mutated tetramers

T cells recognising the same antigen presented by MHC can vary in their sensitivity by several orders of magnitude (Alexander-Miller, 2000, Alexander-Miller et al., 1996, Walker et al., 2010). Furthermore, it has been shown that high-avidity $CD8^+$ T cells are the most efficient at eliminating viruses (Alexander-Miller et al., 1996, Almeida et al., 2007, Derby et al., 2001). Although, one study has shown that low-avidity $CD8^+$ T cells may be more beneficial in chronic infections (Harari et al., 2007), necessitating further investigation into the role of T cell avidity in immune control during acute and chronic viral infections. High avidity T cell populations may be identified experimentally by stimulating T cells with titrating amounts of peptide, and identifying cells responding at the lowest concentrations as the high avidity T cells (Almeida et al., 2007, Harari et al., 2007, Villacres et al., 2003). In the present study, specially engineered 'CD8null' tetramers were used that contain a mutation in the α3 domain (D227K/T228A) of the conserved MHC binding site (Walker et al., 2010, Melenhorst et al., 2008). This mutation weakens CD8 co-receptor binding to MHC class I that normally stabilises the peptide-TCR interaction. Therefore, $CD8^+$ T cells binding mutated tetramer are less reliant upon CD8, and have higher avidity for peptide. A high avidity population can therefore be identified and compared with the normal population which binds regular, un-mutated tetramer (figure 1.2).

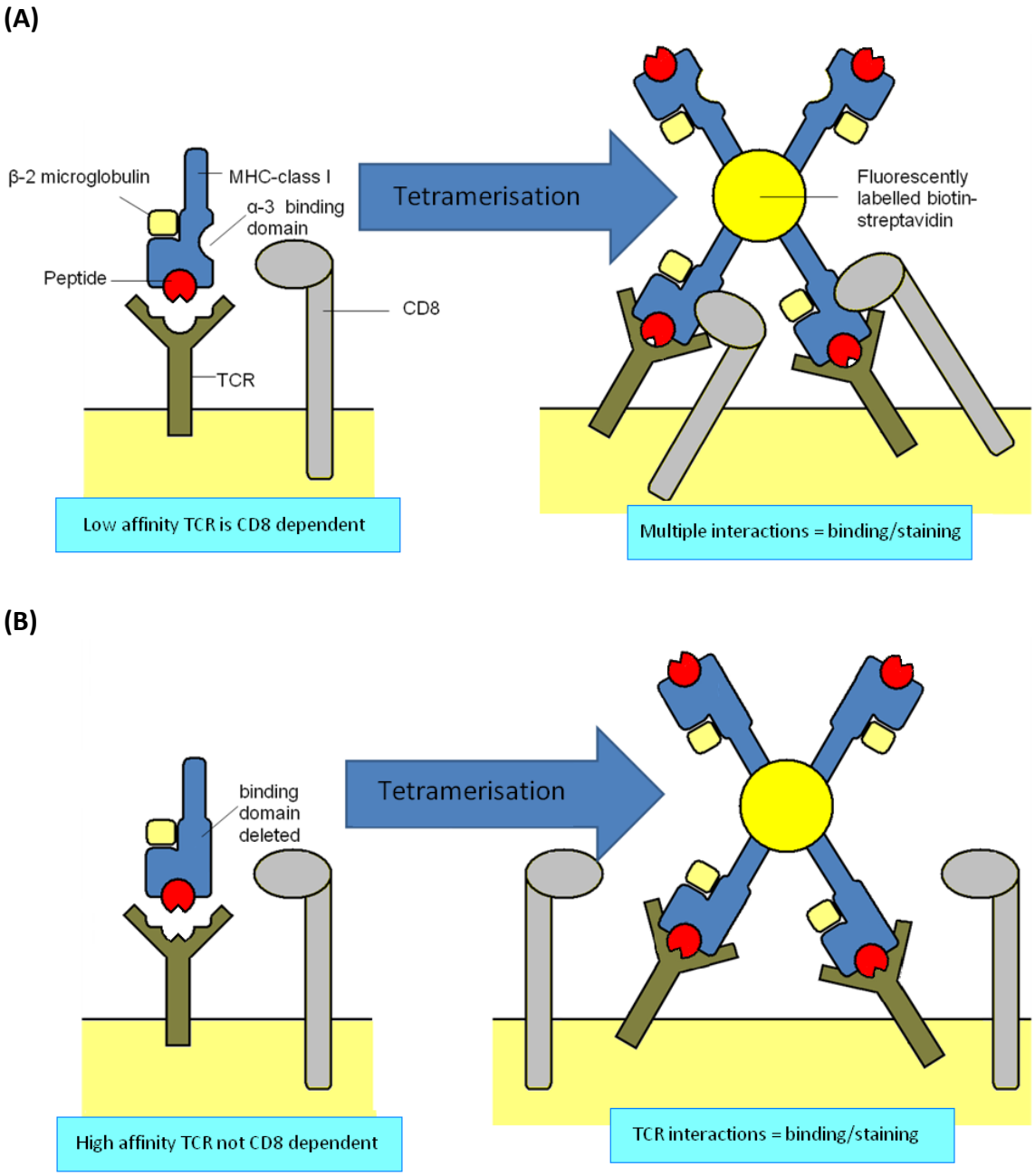


Figure 1-2. Antigen-specific CD8$^+$ T cell staining using normal or mutated (CD8null) tetramers.
(A) Normal tetramers are composed of four MHC class I monomers engineered to present a specific peptide recognised by a specific CD8$^+$ T cell. As with regular MHC class I molecules, these contain the α-3 binding domain which interacts with CD8 co-receptor for stabilisation. In this example, the TCR has low affinity to pMHC. When monomers are conjugated to a fluorescently labelled streptavidin molecule via biotin, tetramers are formed (tetramerisation). This creates multiple interactions which are strong and most CD8$^+$ T cells specific for that antigen are stained with the aid of CD8. (B) Mutated (CD8null) tetramers are composed of the same antigen-specific MHC class I monomers, but contain an α-3 binding domain mutation (D227K/T228A) which results in an inability to bind CD8. Therefore, as shown in this example, only T cells with high affinity TCRs will bind the tetramer without CD8 help. Either the normal or CD8null tetramer binding populations can then be identified by flow-cytometry.

1.3 T cell subpopulations

T cells are commonly classified into subpopulations, based on their phenotypic and functional characteristics. These give an insight into the role of the T cell, or its relative point at various stages of differentiation, which reflect changes in expression of genes controlling cell cycle, survival, effector functions and migration.

1.3.1 Defining T cell subpopulations

T cells subpopulations are based on various criteria, so it is important to understand which markers are commonly used for classification and why.

1.3.1.1 CD45R isoform expression

CD45R, also called the common leukocyte antigen, was the first and prototypic receptor-like protein tyrosine phosphatase to be identified (Thomas, 1989). It consists of an intracellular domain, a transmembrane region and an extracellular domain, which exists in different isoforms; CD45RA being the largest and CD45RO the smallest isoforms (Hermiston et al., 2003). CD45R is expressed on all haematopoietic cells and covers up to 10% of the cell surface, making it one of the most abundant cell surface glycoproteins (Thomas, 1989). Its ligand has yet to be identified, but the main intracellular targets of CD45 phosphatase activity are the Src-family kinases (predominately $p56^{Lck}$ and $p59^{Fyn}$ in the T cell) which initiate signal transduction upon TCR engagement (McNeill et al., 2007). Experiments using monoclonal antibodies revealed expression of the higher molecular weight isoform, CD45RA, was indicative of naïve T cells, and the subsequent

shift in expression to the lower molecular weight isoform, CD45RO, following activation was indicative of an antigen experienced T cell (Akbar et al., 1988).

1.3.1.2 CD27 and CD28 co-stimulatory molecule expression

CD27 and CD28 were described previously as important co-stimulatory molecules in T cell activation (section 1.2.3.3). However, these molecules are also useful markers for defining T cell subpopulations; as their expression is down-regulated in response to repeated stimulation, and therefore indicative of T cells at various stages of differentiation (Appay et al., 2002, Azuma et al., 1993, Hamann et al., 1997). This is exemplified by the fact that long term culture of T cells result in the entire population eventually becoming $CD28^-$ (Effros et al., 1994). Interestingly, $CD4^+$ T cells differentiate and lose the expression of CD27 first and then CD28, whereas $CD8^+$ T cells show CD28 then CD27 down-regulation (Appay et al., 2002, Khan et al., 2002b). This may reflect preferential use of either co-stimulatory pathway.

1.3.1.3 Homing and adhesion molecule expression

Lymphocytes continually circulate in the blood and lymph in surveillance of foreign antigen. Different subpopulations of T cells will also migrate differentially to various tissues. Naive T cells for example, are unable to mount an immune response until activation, and therefore do not require homing to inflammatory sites. Instead they need to interact with DC's in secondary lymphoid tissues. The route of trafficking by a T cell is determined by the homing (or trafficking) receptors on its surface.

Some homing receptors recognise specific vascular addressins expressed by the high endothelial venules' (HEV's) of different secondary lymphoid tissues, thus promoting migration into the secondary lymphoid tissue. Others homing receptors interact with vascular addressins on endothelium at sites of inflammation, therefore promoting peripheral migration. There are multiple receptors involved in the homing of T cells (Woodland and Kohlmeier, 2009), but some commonly used for classification include CD62L (l-selectin) and CCR7. CCR7 is a chemotactic receptor, belonging to the G-protein coupled receptor family, which binds CCL19 and CCL21, directing migration to the lymphoid tissues (Woodland and Kohlmeier, 2009). CD62L is an adhesion molecule, which, in addition to CCR7, is required for the migration of T cells to the lymph nodes (Butcher and Picker, 1996). Naive T cells therefore express CCR7 and CD62L.

1.3.2 Central memory (T_{CM}) and effector memory (T_{EM}) cells

By dividing the antigen experienced $CD45RO^+$ memory $CD4^+$ or $CD8^+$ T cells further by CCR7 expression, two main subpopulations were found to have distinct functional properties (Sallusto et al., 1999). T cells lacking CCR7, rapidly respond to antigen by producing effector cytokines, including IFN-γ, IL-4, IL-5, and perforin. These cells were termed effector memory T cells (T_{EM}) as they migrate to inflamed tissues, using the alternate receptors CCR1, CCR3 and CCR5. T cells expressing CCR7 were termed central memory T cells (T_{CM}), as they lacked immediate effector function and had low activation threshold, but could home to the lymph nodes for efficient DC communication (Lanzavecchia and Sallusto, 2005, Wherry et al., 2003). Furthermore, CD62L expression

is found to largely overlap with CCR7 expression (Sallusto et al., 1999) and is therefore sometimes used in place of CCR7. By using radioactive isotope labelling of proliferating cells *in-vivo*, the turnover rate of $CD4^+$ naïve, T_{EM}, and T_{CM} cells were determined as 1 year, 15 days and 48 days respectively (Macallan et al., 2004). This highlights that T_{EM} in particular, are short lived effectors that may require continual replenishment.

1.3.3 $CD45RA^+$ Memory (T_{EMRA}) cells

The switch from a naive $CD45RA^+$ to a memory $CD45RO^+$ T cell is reversible, and a population of the memory pool may comprise these $CD45RA^+$ memory T cells. These can be identified by tetramers, confirming their specificity for antigen (Appay and Rowland-Jones, 2004, van Leeuwen et al., 2002, Faint et al., 2001). They are delineated from naive T cells by their lack of expression of CD27 and CCR7; in which case they are termed T_{EMRA} cells (Hamann et al., 1999b, Sallusto et al., 2004, van Leeuwen et al., 2002). Compared to naïve CD45RA expressing T cells, T_{EMRA} cells show signs of repeated stimulation i.e. low expression of the co-stimulatory molecules CD27 and CD28, indicative of repeated cell division (Wills et al., 2002), and express various effector-like markers (van Leeuwen et al., 2002, Wills et al., 2002).

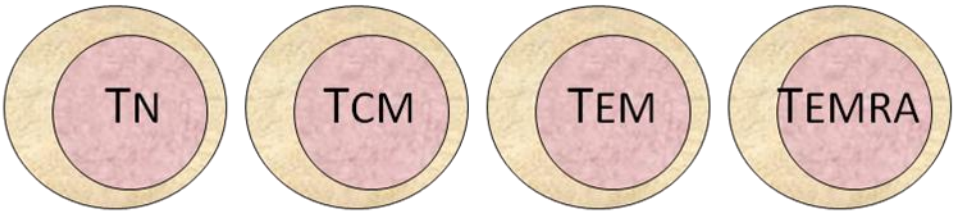
It was initially proposed that T_{EMRA} cells were terminally differentiated (Champagne et al., 2001). However, evidence now suggests that T_{EMRA} cells may instead represent a stable population within the T cell memory pool. *In-vitro* studies have shown that stimulated CMV-specific and EBV-specific $CD8^+$ T_{EMRA} cells can undergo significant clonal

expansion, up-regulate CD45RO expression, secrete perforin and IFN-γ, and show direct *ex-vivo* cytotoxicity (van Leeuwen et al., 2002, Faint et al., 2001, Wills et al., 2002). Although lacking CD27 and CD28 expression, stimulated CMV-specific T_{EMRA} cells can increase expression of CD137 (4-1BB), CD278 (ICOS) and homing receptors CCR5 and CXCR3 (Waller et al., 2007). The significance of this is apparent when CD137L treated T_{EMRA} cells are given CD137L blocking antibody and a subsequent drop in proliferation (40-60%) is observed compared to controls (CD137L treated, no blocking antibody), an effect not observed with CD80, CD86 or CD275 (Waller et al., 2007). This suggests highly differentiated T_{EMRA} cells could be using other co-stimulatory signalling pathways besides the classical CD27/CD28 co-stimulatory pathways.

1.3.4 Subpopulation consensus issues

The simultaneous measurement of a variety of markers enables the distinction of a multitude of T cell subpopulations. However, there is yet to be a consensus on which subpopulation classification system is best to use (Appay et al., 2008). This can cause confusion and difficulty when comparing results between research groups. In this thesis, subpopulations of T cells will be defined by the markers CD45RA and CD27, as based on work performed in our lab and others (Dunne et al., 2005, Hamann et al., 1997, Libri et al., 2011, van Lier et al., 2003). In our experience, delineating on the basis of CD27 expression gives a clearer dissection of the four subpopulations, compared with CCR7. CD27 is also more relevant to the present study, in terms of ageing and differentiation, rather than CCR7, which is indicative of homing properties. Furthermore, CD27 re-

expression does not occur on CD27 negative memory T cell populations (Hintzen et al., 1993) whereas CCR7 re-expression following antigenic stimulation has been reported (Schwendemann et al., 2005, van Leeuwen et al., 2005). However, there is considerable homology between CD27 and CCR7 or CD62L expression (Sallusto et al., 2004, Sallusto et al., 1999, Appay et al., 2008, Fierro et al., 2008), meaning a fair comparison is possible, as summarised in table 1.4.



	Naive	Central memory	Effector memory	CD45RA re-expressing memory
CD45RA	High	Low	Low	High
CD45RO	Low	High	High	Low
CD27	High	High	Low	Low
CD28	High	High	Low	Low
CCR7	High	High	Low	Low
CD62L	High	High	Low	Low

Table 1-4. Phenotypic markers defining T cell subpopulations.

1.4 The impact of ageing on T cells

Immune senescence can impact the innate immune response (discussed later in section 1.5), however it is the defects in T cell immunity that are the most marked and best documented (Nikolich-Zugich, 2008). These defects can occur at a single-cell or cell population level and will be discussed here.

1.4.1 Decreased naive T cell production during ageing

The progressive involution of the thymus during ageing leads to decreasing production of naive T cells. Involution begins approximately one year after birth, and by middle-age most parenchymal tissue is replaced by fat, although functional thymic tissue remains at least into the sixth decade of life (Linton and Dorshkind, 2004). Although the exact mechanism for thymic involution remains poorly understood, the cause may be a loss or disruption of interaction between developing thymocytes and the supportive thymic stoma (Lynch et al., 2009). With involution of the thymus comes a decrease in recent thymic emigrants (RTE), which may put pressure on homeostatic mechanisms needed to sustain a diverse naive T cell repertoire through ageing (Nikolich-Zugich, 2008).

1.4.2 Increased naive T cell turnover during ageing

Compared with memory T cells, naive T cells rarely undergo division, instead being homeostatically maintained by weak TCR triggering and cytokines (Boyman et al., 2009). However, experiments with aged rhesus macaques have shown naive T cells have significantly higher rates of turnover, with no subsequent increase in numbers (Cicin-

Sain et al., 2010). Furthermore, in humans, $CD4^+$ T cell homeostatic proliferation was seen to double in the over 70's (Naylor et al., 2005). This suggests naive T cells may be under added pressure to survive during old age, and risk being lost through senescence.

1.4.3 Expansion of memory T cells with ageing

The older the individual becomes the more pathogens they are likely to have encountered, and hence the more memory T cells they have generated. However, there are large clonal expansions of T cells specific for a restricted number of epitopes, especially within the $CD8^+$ T cell compartment, reported in ageing mice and humans (Callahan et al., 1993, Posnett et al., 1994, Ku et al., 1997, Schwab et al., 1997). Current evidence suggests these non-malignant expansions are the result of chronic pathogen stimulation by viruses, including Cytomegalovirus (further discussed in section 1.6).

1.4.4 Age-related restriction of the TCR repertoire

The compounding effect of decreased naive T cell numbers and accumulation of memory T cells during ageing is thought to shrink the overall diversity of the T cell pool by old age (Nikolich-Zugich et al., 2004, Naylor et al., 2005, Ahmed et al., 2009). Observations of TCR diversity during ageing suggests it remains constant in youth, but later in life TCR vβ usage becomes uneven and reduction of CDR3 diversity occurs (Vargas et al., 2001, Schwab et al., 1997). This could have consequences, as a diverse T cell repertoire is essential for mounting immune responses to new pathogens. Indeed, increased susceptibility to new infections is a hallmark of age-related immune decline

(section 1.1.4). Although direct evidence for this is lacking in humans, experiments in animal models show a restricted TCR diversity can result in impaired immunity to viruses (Messaoudi et al., 2004, Yager et al., 2008, Cicin-Sain et al., 2010).

1.4.5 Inverted CD4:CD8 ratio

The normal CD4:CD8 ratio in adults is usually around 2:1, in cord blood for comparison it is higher at around 3:1 (Brzezinska, 2005). Interestingly, inversion of the CD4/CD8 ratio alone was predictive of survival rate in a UK sample of old people (Huppert et al., 2003) and predictive of individuals with poor T cell proliferative responses to mitogens (Peres et al., 2003). Inverted CD4:CD8 have also been reported in some older individuals in care homes (Cretel et al., 2010) and those experiencing high levels of job stress (Bosch et al., 2009), an association which became stronger with increased age.

1.4.6 Down-regulation of CD27 and CD28 expression

CD27 and CD28 are co-stimulatory molecules found on the surface on T cells (section 1.3.1.2). Virtually all T cells in umbilical cord blood express CD27 and CD28 (Azuma et al., 1992, Brzezinska, 2005). However, the fraction of $CD4^+$ and $CD8^+$ T cells expressing CD27 and/or CD28 significantly decreases during ageing (Boucher et al., 1998, Nijhuis et al., 1994, Fagnoni et al., 1996, Fletcher et al., 2005, Wikby et al., 2005, Kovaiou et al., 2005, Posnett et al., 1994). These $CD27^-CD28^-$ T cells represent a population which have undergone repeated stimulation, as they have shorter telomere lengths relative to $CD27^+CD28^+$ T cells (Monteiro et al., 1996, Fletcher et al., 2005, Soares et al., 2004) and

show defective telomerase activity (Plunkett et al., 2007). CD27/CD28 expression also gradually decreases during long-term *in-vitro* T cell culturing (Brzezinska, 2005, Effros et al., 1994), which seems to confirm those findings *in-vivo*.

1.4.7 T cell defects at a cellular level

T cells in old individuals have been associated with a variety of functional defects. One of the first to be documented was low IL-2 production upon activation (Gillis et al., 1981, Chen et al., 1986), but has since been extended to include low activation-induced IFN-γ production (Murasko and Goonewardene, 1990), impaired activation (Schindowski et al., 2002), and signalling defects through lipid rafts (Larbi et al., 2010). Studies in mice have also shown ageing results in declining activation of a range of substrates including tyrosine phosphorylation of CD3 ζ-chains and various kinase activities (Miller et al., 1997).

1.4.8 T cell senescence vs. exhaustion

Repeated proliferation and/or stimulation of T cells throughout life can result in T cell senescence or exhaustion. However, although these terms are often used interchangeably, there is evidence to suggest they may be distinct processes (Akbar and Henson, 2011). Generally, either process can be divided into 3 phases; each phase on course to a different pathway (figure 1.3).

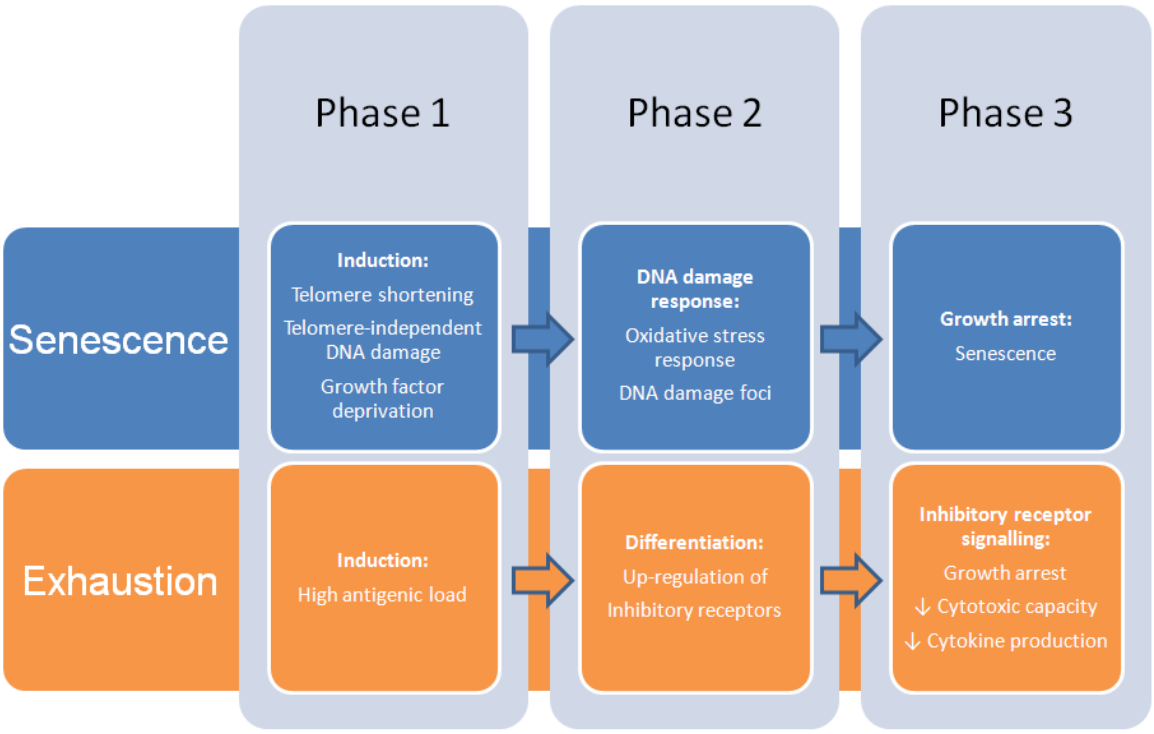


Figure 1-3. The 3 phases of senescence versus exhaustion (adapted from Akbar and Henson, 2011)

Cellular senescence can be induced (phase 1) either by telomere erosion or DNA damage, caused by reactive oxygen species (ROS), chromatin perturbation and activation of stress pathways in response to growth factor deprivation (Campisi and d'Adda di Fagagna, 2007, d'Adda di Fagagna, 2008, Passos and Von Zglinicki, 2006). These telomere-dependent, and telomere-independent, inducers of senescence lead to phase 2: the DNA damage response (DDR), which involves the recruitment of DNA repair proteins (Akbar and Henson, 2011, Campisi and d'Adda di Fagagna, 2007). This causes temporary growth arrest, however, if the DNA damage cannot be repaired, cell cycling ceases and a permanent state of senescence (phase 3) is reached (Campisi and d'Adda di Fagagna, 2007, d'Adda di Fagagna, 2008). A well characterised marker of T cell senescence is CD57 a.k.a HNK-1, a terminally sulfated glycan carbohydrate epitope

(glycoepitope) that was first described in 1981 on human natural killer (HNK) cells (Abo and Balch, 1981). CD57 is associated with high susceptibility to activation-induced cell death and an inability to undergo cell division (Focosi et al., 2010), a proliferative defect which cannot be overcome by cytokine help (Kern et al., 1996). Another marker of senescence is killer-cell lectin-like receptor G-1 (KLRG-1). KLRG-1^{+} cells are unable to undergo clonal expansion following activation (Voehringer et al., 2002), due in part to KLRG-1 inducing defective Akt phosphorylation and proliferation (Henson et al., 2009).

T cell exhaustion is defined by the progressive loss of function and is induced (phase 1) by high antigenic load, such as during chronic viral infection or cancer (Akbar and Henson, 2011). Under conditions of high antigenic load, T cells are driven further to differentiation and begin to express inhibitory receptors (phase 2). Signalling through these receptors can lead to decreased cytotoxic capacity, decreased cytokine production and eventually growth arrest (phase 3). A well characterised inhibitory receptor is programmed death receptor 1 (PD-1); a member of the CD28 receptor family that has been shown to be highly expressed on $CD8^{+}$ T cells during chronic infection (Barber et al., 2006, Blackburn et al., 2009). Importantly, blocking PD-1 has been shown to restore T cell function in mice and humans (Barber et al., 2006, Lages et al., 2010, Nakamoto et al., 2009, Trautmann et al., 2006). Other inhibitory receptors shown to induce T cell exhaustion include cytotoxic T lymphocyte antigen 4 (CTLA4), T cell immunoglobulin domain and mucin domain protein 3 (TIM3), and lymphocyte activation gene 3 (LAG3) (Blackburn et al., 2009, Sakuishi et al., 2010, Wherry et al., 2007).

1.4.9 The immune risk phenotype (IRP)

The age-related changes to T cells described thus far are therefore important biomarkers indicative of immune ageing. How these biomarkers are associated with age-related susceptibility to infections and poor vaccination responses have therefore been investigated. One such investigation is the ongoing Swedish OCTO and NONA longitudinal studies (Wikby et al., 2005, Wikby et al., 2002, Wikby et al., 2006), which measure various immunological parameters in the very old (80+ and 90+ years of age respectively) to determine their impact on life expectancy. Upon conclusion of the study, a set of parameters defining an immune risk phenotype (IRP) were proposed, and those individuals within the IRP had decreased survival rates of up to 4 years compared to those outside the IRP. Furthermore, follow up studies have shown groups of long lived individuals over 100 years of age are frequently outside the IRP (Strindhall et al., 2007, Derhovanessian et al., 2010). Many of the significant associations identified in the studies were found to relate to T cells (table 1.5). The significant association with cytomegalovirus seropositivity will be discussed further in section 1.6.

Immune Risk Phenotype
CD4:CD8 ratio <1
Poor T cell proliferative responses
CD28↓
CD57↑
KLR-G1↑
IL-2↓
IFN-γ↓
CMV^{pos}

Table 1-5. Parameters of the IRP (adapted from Pawelec et al., 2004)

1.5 The impact of ageing on other components of the immune system

1.5.1 The innate immunity and immune senescence

Innate immunity comprises four types of defence: the anatomical, physiological, phagocytic and inflammatory barriers. Anatomical barriers include the skin and mucous membranes. Physiological barriers include temperature e.g. Fever (inhibits pathogen growth), pH (e.g. stomach acid), lysozymes (cleave bacterial cell walls) and interferon (induces antiviral state in surrounding uninfected cells). Phagocytic barriers including blood monocytes, neutrophils and tissue macrophages, which internalize, digest and kill whole microorganisms. Finally there are inflammatory barriers: tissue damage causes local leakage of vascular fluid, containing serum proteins with antimicrobial activity, and inflammatory mediators which recruit phagocytic cells into the area.

In comparison to the lymphoid progenitors, there is no decrease in the numbers of myeloid progenitors during ageing which give rise to many of the cells involved in innate immunity (Beerman et al., 2010). In fact, there are reports suggesting increased numbers and hyper-activity of these cells in the old (Gomez et al., 2008, Kovacs et al., 2009, Shaw et al., 2010). Instead, 'innate immune senescence' usually reflects dysregulation. For example, decreased TNF-α production by dermal macrophages impairs T cell migration during immune responses to Candida in old skin, whereas *ex-vivo,* macrophages function properly (Agius et al., 2009). This demonstrated dysregulation of macrophages in the tissue environment, rather than impaired function.

1.5.2 B cells and immune senescence

B cells are generated and mature within the bone marrow. A mature B cell leaves the bone marrow expressing membrane-bound immunoglobulin (mIg) with a single antigenic specificity. The mIg associates with the disulphide linked heterodimer Ig-α/Ig-β which is involved in cell signalling, to collectively form the B cell receptor or BCR (Kindt et al., 2007). When a naïve B cell encounters antigen specific for its BCR, it divides rapidly, differentiating by a process of affinity maturation for antigen, class switching, and formation of effector (plasma) and memory B cells. Plasma cells produce and secrete antibody in large amounts which attach to pathogens initiating their destruction or facilitating their removal by phagocytes. Plasma B cells die after a few days, however memory B cells have much longer lifespan and continue to express the bound BCR and patrol the organism in the event of re-challenge.

Although total B cell numbers do not decline with age (Weksler and Szabo, 2000), the memory B cell compartment increases with age and shows decreased CD27 co-stimulatory molecule expression (Colonna-Romano et al., 2003), in a similar manner to T cells. Furthermore, B cells from older individuals are stimulated 70% less efficiently than B cells from young individuals (Aydar et al., 2002). However, It is believed that many of these age-related impairments in B cell activation result from a lack of T cell help, and the change in the cytokine environment with ageing (Weiskopf et al., 2009).

1.6 Cytomegalovirus

Cytomegalovirus (CMV) is associated with driving T cells to greater differentiation (Akbar and Fletcher, 2005, Appay et al., 2002, Fletcher et al., 2005, Khan et al., 2002b, Pawelec et al., 2004). CMV seropositivity was also found to be a significant factor in the immune risk phenotype (IRP), a cluster of immune parameters associated with decreased survival in the very old (section 1.4.9). This section describes CMV and evidence linking CMV with immune senescence.

1.6.1 Discovery of CMV

As far back as 1881, descriptions of large "protozoan like" cells in the kidneys of still-born babies were reported (Ribbert, 1904). With the advent of advanced cell culture techniques, the virus causing these cytomegalic cells with intranuclear inclusions was isolated, and hence named Cytomegalovirus (Craig et al., 1957).

1.6.2 Seroprevalence of CMV

CMV is shed in bodily fluids, resulting in infection of others through close contact (Hanshaw et al., 1968). Seroprevalence is approximately 50-90%, with the majority of the population being infected by adulthood; highlighted by a one per cent cumulative rise in chance of infection per year of age (Griffiths, 1997). The rate of infection is also highly influenced by geographical location and socio-economic status (Ho, 2008). The success of the virus is highlighted by the incidence of CMV seropositivity being so ubiquitous it reaches even the most isolated tribal communities (Black et al., 1974).

1.6.3 CMV pathology

Upon acute infection of a healthy host, symptoms are rare, but may include mononucleosis (Taylor, 2003). The virus then establishes a life-long chronic yet sub-clinical infection, entering a state of latency, when infectious virions are undetectable (Sinclair, 2008). In immune-compromised individuals however, CMV is a major cause of morbidity and mortality, with various pathological manifestations. In AIDS patients for example, CMV disease commonly presents as retinitis or enteritis (Harari et al., 2004). In organ transplant recipients, CMV is the most common viral infection: causing fever, hepatosplenomegaly, myalgias, leukopenia and thrombocytopenia with or without specific organ dysfunction (Emery, 2001). This can indirectly affect the host's immune response, leading to acute rejection, graft loss, and onset of other opportunistic infections (Strippoli et al., 2006). Primary CMV infection is also the leading cause of birth defects and developmental disabilities (Hyde et al., 2010).

1.6.4 Latency

CMV belongs to a family of viruses of the genus herpesviridae, from the greek herpein, meaning "to creep" - in reference to the latent, recurring infection typical of this group of viruses. CMV is part of the subfamily betaherpesvirinae, which establishes latency predominately in monocytes, as compared with the alphaherpesvirinae herpes-simplex virus (HSV) and varicella-zoster virus (VZV) or the gammaherpesvirinae EBV, which establish latency predominately in neuronal cells and B cells respectively (Sinclair, 2008). However, many other cell types are permissive for CMV infection *in-vivo*; including

epithelial cells, endothelial cells, granulocytes and lymphocytes (Emery, 2001, Sinclair and Sissons, 2006, Revello and Gerna, 2010), creating a potentially large reservoir of latent virus. Evidence suggests latent CMV virus in monocytes may become reactivated during differentiation into macrophages (Soderberg-Naucler et al., 1997, Soderberg-Naucler et al., 1998). CMV may also disseminate throughout the body as a result of adhesion molecule interaction between leukocytes and infected endothelial cells, creating fusion points for virion transmission (Revello and Gerna, 2010).

1.6.5 Is CMV reactivating in healthy individuals?

CMV related pathology in otherwise healthy individuals has been reported (McVoy and Adler, 1989), although reports are seldom, indicating CMV infection is kept under control. Analysis for the presence of CMV DNA in the monocytes of old volunteers however revealed 56% had detectable CMV DNA, with significantly higher percentages of CMV-pp65 specific $CD8^+$ T cells than those without detectable CMV DNA (Leng et al., 2011). CMV IgM titers were also found to be negative for all subjects, indicating that recent primary CMV infection was unlikely and reactivation therefore the cause. CMV may also be reactivating at other sites of the body, including the kidneys, as CMV can be detected in the urine of older CMV positive individuals (Stowe et al., 2007). Furthermore, 'abortive reactivations' occur frequently for herpesviruses such as EBV and CMV (Kurz et al., 1999, Laichalk and Thorley-Lawson, 2005). In this process, viruses start gene replication within the cell but abort before generation of infectious virus. Frequent abortive reactivation result in repetitive immune stimulation (Virgin et al.,

2009) and may account for an expansion of clones without detection of CMV in the blood or appearance of CMV associated symptoms.

1.6.6 Immune responses to CMV

CMV elicits a strong immune response *in-vivo*, triggering early B cell production of CMV-specific neutralising antibodies targeting surface glycoprotein B (gB/UL55) (Emery, 2000). Immunisation with CMV-specific gB induces neutralising antibody responses which can protect animals from an otherwise lethal CMV infection (Schleiss, 2008). Evidence also suggests Natural killer (NK) cells are important in immune responses to CMV. Neonatal mice are rendered susceptible to lethal infection if NK cells ablated (Brown et al., 2001, Polic et al., 1998). In humans, CMV-related disease during organ transplantation is correlated with poor NK cell protection (Gandhi and Khanna, 2004, Quinnan et al., 1982). However, it is the cell-mediated branch of the immune response, in particular $CD8^+$ T cells, which control CMV replication most efficiently (Moss and Khan, 2004, Gandhi and Khanna, 2004, Harari et al., 2004). $CD8^+$ T cells can target a broad range of CMV peptides, but the major targets are the pp65 matrix protein (UL83) and the immediate-early (IE) genes (Sylwester et al., 2005, Emery, 2000, Kern et al., 2000, Khan et al., 2002a).

1.6.7 CMV immune evasion strategies

CMV is a DNA virus with a large genome encoding over 200 genes (Chee et al., 1990, Emery, 2001). It has co-existed with eukaryotic cells for millions of years (Maffei et al.,

2008) and this is exemplified by the vast array of immune evasion strategies it has developed (Wiertz et al., 1997, Emery, 2001). Many of these disrupt antigen processing and presentation within CMV infected cells, thereby avoiding recognition by patrolling immune cells. Some CMV genes devoted to immune evasion and their mechanisms of action are summarised in table 1.6.

HCMV gene	Immune evasion strategy
US2/US11	Destroy newly synthesized MHC class I molecules
US3	Retains MHC class I molecule in endoplasmic reticulum
US6	Blocks peptide transport by transporter associated with antigen (TAP)
US28	Acts as promiscuous chemokine receptor
UL18	MHC class I homolog. Acts as a decoy for natural killer (NK) cells
UL83 (pp65)	Phosphorlyates immediate early (IE) protein leading to IE presentation being suppressed, due to lack of processing by proteasome

Table 1-6. HCMV genes involved in immune evasion
Abbreviations: unique short (US) and unique long (UL).

1.6.8 CMV and the immune risk phenotype

One hundred per cent of those in the immune risk phenotype (IRP) group in the NONA study were CMV positive versus eighty four per cent in the non-IRP group (Wikby et al., 2002). The role of CMV as a causal factor in the IRP may be due to the accumulation of CMV-specific $CD8^+$ T cell expansions (TCEs) which the virus elicits (see 1.6.10). These could contribute to the skewing of the CD4:CD8 ratio, a parameter in IRP and itself a predictor of early mortality in the old (Huppert et al., 2003). Furthermore, there is a decrease in naïve CD8 T cells in the old, but it is the increase of effector memory T cells that cause the inflated CD8 numbers (Pawelec et al., 2006a). A decrease in the expression of the co-stimulatory molecules CD27 and CD28, and increased CD57

expression, on T cells are also biomarkers of the IRP, and incidentally also linked to CMV driven differentiation and senescence in T cells. This will be discussed in more detail in the following sections.

1.6.9 CMV-specific T cell differentiation

MHC class I tetramers have been particularly useful in studying virus-specific $CD8^+$ T cell populations during the progress of infection, and different virus specific $CD8^+$ T cells display varying degrees of differentiation. For example, Influenza-A-specific memory $CD8^+$ T cells show an early differentiation phenotype; reflecting the acute nature and clearance of an Influenza infection (Appay et al., 2002). CMV-specific $CD8^+$ T cells however, show the most differentiated phenotype of all virus-specific $CD8^+$ T cells, exhibiting low CD27 and CD28, indicative of repeated activation (Appay et al., 2002, Klenerman and Hill, 2005, Koch et al., 2007, van Lier et al., 2003, Wills et al., 2002).

Many factors associated with a particular virus may influence the state of differentiation of the $CD8^+$ T cells targeting it, including antigenic load and presentation, viral persistence and location, and the influence of viral immune escape mechanisms (Appay and Rowland-Jones, 2004). Indeed, CMV devotes multiple genes to immune evasion, as discussed earlier. The obvious reason for such a differentiated phenotype is that CMV-specific T cells are encountering antigen more frequently. This may be due to the cellular tropism of the virus e.g. HSV resides in neuronal cells and may therefore not be seen as frequently by T cells as is CMV is, which is present in various cell types, including

epithelial and endothelial cells (Revello and Gerna, 2010, Sinclair, 2008). CMV effects are not limited to $CD8^+$ T cells, as $CD8^+$ T cells also require the help of CMV-specific $CD4^+$ T cells, which are also found in high numbers in healthy CMV seropositive individuals and similarly show a highly differentiated phenotype (Koch et al., 2006, Fletcher et al., 2005).

1.6.10 CMV-specific $CD8^+$ T cell clonal expansions

Large CMV-specific T cell expansions (TCEs) are found within CMV positive individuals, and persist for a lifetime (Harari et al., 2004). These TCEs seem highly restrictive, using only one or two vβ gene segments (Khan et al., 2002b, Wallace et al., 2011, Karrer et al., 2003). They are found in both the $CD4^+$ and $CD8^+$ T cell compartments, accounting for up to 50% of the entire repertoire in older CMV positive individuals (Harari et al., 2004, Looney et al., 1999, Wikby et al., 2002). Furthermore, these TCEs are predominately $CD28^-$ and $CD57^+$ T cells indicating they have undergone repeated stimulation and are close to senescence (Khan et al., 2002b, Weekes et al., 1999, Wills et al., 1999).

1.6.11 Are CMV-specific T cell expansions useful?

It could be argued that expanded CMV-specific T cell clones have protective qualities, as severe CMV disease is rarely seen in the immune-competent host (Effros et al., 2005). However, rare reports of CMV disease in healthy individuals have been documented (Crowley et al., 2002, Eddleston et al., 1997, Wreghitt et al., 2003). It has also been suggested that highly differentiated T cells may have a suppressive function, as donor

specific $CD8^+CD28^-$ T cells are often found in the blood of people with successful, stable, transplants, and are absent in those with acute rejection (Colovai et al., 2003, Liu et al., 1998). However, a suppressive role may also explain their association with relatively poor responses of the old to influenza vaccination (Effros et al., 2005, Song et al., 2010). Furthermore, older individuals have higher numbers of $CD8^+$ T cells specific for CMV, but these have been shown to have significantly decreased secretion of IFN-γ and IL-10 when compared to those in the young (Ouyang et al., 2004, Khan et al., 2004). This questions the benefit of having an expanded CMV-specific T cell population.

1.6.12 The Impact of T cell expansions

Age-related susceptibility to infection and increased incidences in reactivation of latent microorganisms have been documented in the old (section 1.1.4). T cells specific for viruses that are infrequently occurring e.g. vaccinia or influenza, or those less accessible to lymphocytes e.g. latent HSV may be at risk of being lost from the T cell pool through age-dependent changes (section 1.4). One theory is TCEs may cause the squeezing out of other T cell specificities through competition for space and growth factors (Akbar et al., 2004, Ouyang et al., 2004). A number of reports support this 'immune space theory'. In mice, those with TCEs have poor responses to HSV challenge, with greater disease severity than those without TCEs (Messaoudi et al., 2004). In rhesus monkeys, those with TCEs had poor vaccine responses to Vaccinia, compared to those without TCEs (Cicin-Sain et al., 2010).

1.6.13 CMV, inflammation and the Inflamm-ageing hypothesis

The inflamm-ageing hypothesis proposes that immune senescence is associated with chronic inflammation, driven by chronic antigenic load (Franceschi et al., 2000, De Martinis et al., 2005). CMV is therefore an obvious candidate in this hypothesis, and evidence to support the involvement of CMV in exacerbating inflammation is abundant (Freeman, 2009, Schmaltz et al., 2005, Simanek et al., 2011, Soderberg-Naucler, 2006, Wikby et al., 2006). Active CMV replication has also been found at sites of inflammation, including atherosclerotic plaques, vascular lesions of coronary transplant vasculopathy, and during inflammatory bowel disease (IBS), arthritis, Sjögren's Syndrome, Hashimoto's thyroiditis, and systemic lupus erythematosus (Freeman, 2009, Soderberg-Naucler, 2006). One of the most frequently reported associations is between CMV and increased risk of mortality from cardiovascular disease (Simanek et al., 2011, Roberts et al., 2010, Blankenberg et al., 2001). Although, whether CMV causes inflammation or is simply a bystander to inflammation is difficult to determine. To illustrate this point, the reactivation of latent CMV within monocytes can be initiated by the differentiation of monocytes to macrophages as a result of inflammatory mediators including TNF-α and IFN-γ (Sinclair, 2008, Soderberg-Naucler, 2006).

1.7 Aims

Evidence suggests CMV may accelerate immune senescence, and it is hypothesised the accumulation of CMV-specific $CD8^+$ T cell expansions (TCEs) during ageing may be instrumental in this association. The purpose of this thesis was to address this hypothesis. The specific aims of this project were to:

1. Phenotypically characterise $CD8^+$ and CMV-specific $CD8^+$ TCEs according to age.
2. Investigate the impact of CMV infection on the rate of telomere length attrition during ageing.
3. Investigate how CMV-specific $CD8^+$ TCEs may be generated.
4. Investigate the functional quality of CMV-specific TCEs with ageing.

2 General Materials and Methods

2.1 Donors

2.1.1 Donors and Blood sample collection

Whole blood (between 10-80ml) was collected in standard heparinised tubes from healthy volunteers. Where data is stratified by age, young is defined as donors between 18-35 years and old donors between 65-95 years. All samples were obtained according to the ethical committee of the Royal Free and University College Medical School. Donors did not have any co-morbidity and were not on any immunosuppressive drugs, they also retained physical mobility and lifestyle independence.

2.1.2 PBMC isolation

Whole blood was collected in standard heparinised tubes and diluted 1:1 with Hanks Balanced Salt Solution (HBSS, Life Technologies, Paisley, UK). 20ml of Ficoll (Amersham Biosciences. Uppsala, Sweden) was added to a 50ml tube and 25 ml of the whole blood/HBSS mix was gently layered on top of the Ficoll surface. These tubes were then centrifuged for 20 minutes at 800xg without any break. Subsequently, the white PMBC layer formed was then extracted carefully with a pasteurette and placed into a new 50 ml tube. This was filled to the 50ml mark with HBSS and centrifuged for 10 minutes at 650xg with the break at position 2. Supernatant was decanted and cell pellet washed again with 50mls of HBSS for 10min at 350xg. Supernatant was then discarded and PBMC pellet re-suspend in complete medium (RPMI, 100 IU Penicillin, 100 µg/ml

streptomycin, 2mM L-glutamine, and 10% foetal calf serum (FCS) [Life Technologies]) and centrifuged for 5 minutes at 650xg. Finally the supernatant was discarded and an appropriate volume of complete medium added for counting.

2.1.3 Determination of donor CMV status

The CMV status of donors was obtained by stimulation of freshly isolated PBMCs (section 2.1.2) with a CMV viral lysate preparation (Fletcher et al., 2005). CMV viral lysate was prepared by infecting human embroyonic lung fibroblasts with the Towne strain of CMV at multiplicity of infection of 2. After 5 days, infected cells were lysed by freeze-thaw cycles. Upon production of the viral lysate, and when a new batch was prepared, a titrating stimulation was performed to determine peak IFN-γ production and therefore the volume of lysate used for stimulation.

For the stimulation, three sterile polypropylene tubes each containing $1x10^6$ PBMCs were prepared. To these were added either a Staphylococcal Enterotoxin B (SEB) lysate (SEB; Sigma-Aldrich, Dorset, UK) for a positive control, a CMV lysate, or an un-stimulated control as a guide for delineating positive and negative populations. All tubes were then placed at 37°C for 2hrs at which point Brefeldin A was added and samples left for a further 12 hrs. Brefeldin A is a fungal metabolite that enhances the staining of intracellular cytokines by interfering with the vesicular transport of cytokines from the rough ER to the golgi complex. This prevents their secretion in antigen activated T cells and instead makes their identification possible. The cells were then surface stained with

anti-CD4, and then intracellularly stained with anti-IFN-γ, as described in section 2.2.3. Uninfected cell lysates did not induce any IFN-γ secretion and there was significant concordance between an IFN-γ^+ response and seropositivity obtained from routine hospital IgG titres (Fletcher et al., 2005).

2.1.4 Viable Cell Count

Cell suspensions were diluted 1:1 with 0.4% trypan blue (Sigma) and 8µl was inserted beneath the cover slip on an improved Neubauer chamber (Weber Scientific, UK Materials) and total cell counts estimated from the mean of 3 grid sample counts. Dead cells were stained blue and were therefore excluded from the counts.

2.1.5 Cryopreservation of PBMCs.

Cells were frozen at 10^7/ml/cryo-vial. After centrifuging cells for 5 mins @650xg and removing all medium, 0.5ml of FCS was added to cell pellet, which was re-suspended and then added to a 1ml cryo-vial and placed in the fridge for 10 minutes. 0.5ml of freezing mix (20% dimehtylsulphoxide [DMSO] in FCS) was added to the 0.5ml cryo-vial containing cells, mixed gently and left in fridge for 20 minutes. Cells were stored inside a passive freezer (MR. FROSTY by Nalgene) containing isopropyl alcohol and then placed at –80^{o}C overnight. Finally, the cryo-vials were transferred to liquid nitrogen for long-term storage. Frozen PBMCs were thawed quickly by exposing to warm medium then being diluted with warm medium and centrifuged immediately for 5 mins @650xg.

2.2 Flow Cytometric Analysis

The details and concentrations of the antibodies used in the following sections are summarised in table 2.1:

Antigen	Source	Clone	Isotype	Concentration
CD8-FITC	BD	SK1	mIgG1	10
CD8-PerCP	BD	SK1	mIgG1	10
CD27-FITC	BD	M-T271	mIgG1	10
CD27-PE	BD	M-T271	mIgG1	10
CD27-APC	eBiosciences	O323	mIgG1	5
Ki67-FITC	BD	B56	mIgG1	10
Bcl-2-FITC	BD	B56	mIgG1	12
HLA-A2-PE	AbD Serotec	BB7.2	mIgG1	10
HLA-B7-FITC	AbD Serotec	BB7.1	mIgG1	10
IFNγ-FITC	Cedarlane	B27	mIgG2b	1
CD45RA-APC	Invitrogen	MEM-56	mIgG2b	5
CD45RA-PE-Cy7	BD	L48	mIgG1	3
CD45RO-APC	Invitrogen	MEM-56	mIgG2b	5
CD4-Biotin	Immunotech	13B8.2	mIgG1	1

Table 2-1. Details and concentrations of antibodies used
Concentrations shown are amount added (µl) per 100µl of 10^6 resuspended cells

2.2.1 Surface staining PBMCs

Following PBMC isolation (section 2.1.2), cell phenotype was analysed by flow-cytometry using a combination of antibodies. Staining was conducted for 30mins at 4°C and washed in PBS (650xg for 5min). Samples were then fixed with 2% paraformaldehyde and acquired on either the BD FACs Calibur or BD LSR/LSRII using cell quest or DIVA software respectively.

2.2.2 Tetramer staining

Tetramer staining was conducted on Human leukocyte Antigen (HLA) matched donor PBMCs with either HLA-A*02 or HLA-B*07 positive individuals using tetramers loaded with CMVpp65 NLVPMVATV or TPRVTGGGAM peptide respectively (a gift from P.Klenerman, Oxford University). PBMCs were isolated (as discussed in section 2.1.2) and cells suspended in approx. 10^6 cells per 100µl. 1µl of tetramer was added per tube and incubated at 37°C for 20mins, then washed in PBS (650xg for 5min). Cells were then surface stained with CD8-PerCP and any other antibodies indicated and/or intracellularly stained in accordance with protocols in sections 2.2.1or 2.2.3 respectively.

2.2.3 Intracellular staining

Intracellular staining for the detection of IFN-γ was performed using the caltag fixation and permeabilisation kit (Caltag Laboratories, Paisley, UK). Approximately 10^6 cells were suspended in 100µl of PBSA (PBS containing 1% bovine serum albumin, BSA) in a FACS tube. Cells were surface stained with anti-CD4 antibody at 4°C in the dark for 30mins. Cells were then washed in PBSA by centrifuge at 650xg for 5min, wash discarded and cells resuspended in 100µl solution A and incubated at room temperature for 15 mins. Cells were then washed in PBSA by centrifuge at 650xg for 5min, wash discarded and cells resuspended in 100µl solution B containing 1µl IFN-γ APC and incubated at room temperature for 15 mins. This was followed by one final wash in PBSA by centrifuge at 650xg for 5min, and finally resuspended in 300µl 2% PF for FACs analysis.

2.2.4 Intranuclear staining

Ki-67, γ-H2AX and Bcl-2 staining was performed using the intranuclear Miltenyi FoxP3 Buffer Staining kit (Miltenyi Biotec, Surrey, UK). Approximately 10^6 cells were suspended in 100µl of a suitable medium in a FACS tube. If the donor was a CMV positive HLA match tetramer staining was first conducted (section 2.2.2). If they were not, or following tetramer staining cells were then surface stained with anti-CD8, anti-CD27 and anti-CD45RA at 4°C in the dark for 30mins. Cells are then washed in 1ml cold PBS by centrifuge at 650xg for 5min and resuspended in 1ml Fix/Perm solution (mix concentrate and diluent 1:3) and incubated at 4°C in the dark for 30mins. Cells are then washed in 1ml PBS by centrifuge at 650xg for 5min, wash discarded and 3ml Perm buffer added (dilute 1 in 10 in distilled water). The tubes were vortexed centrifuged at 650xg for 5min to pellet cells. This wash was repeated with 1ml Perm buffer/tube and excess liquid from the tubes blotted using tissue. Antibodies for Ki67-FITC, γ-H2AX-FITC or BCl2-FITC were then added to the relevant tubes together with isotype controls and incubated at 4°C, in the dark for 30mins. Following incubation, cells are washed in 1ml Perm buffer twice and finally resuspended in 300µl 2% PF for FACs analysis. Positive populations are identified by gating on relevant isotype controls (table 2.1).

2.3 Isolation and sorting of populations from PBMCs

2.3.1 Negative selection of $CD8^+$ T cells using MACS isolation kit.

Magnetic Cell Sorting (MACS, Miltenyi Biotec, Surrey, UK) allows the purification of cell subsets from complex cell mixtures. MACS Buffer (1 x PBS containing 0.5% BSA (5g/litre,

stored in cold room) and 2mM EDTA (4ml of 0.5M stock) was first prepared then filtered and degassed (in 50ml aliquots) before use and kept at 4°C or on ice. Degassing took approximately 10 minutes from room temp or 30 minutes from 4°C. PBMCs were extracted from whole blood (as described in 2.1.2) then washed in MACS buffer by centrifuge @1800rpm for 5mins. Subsequently, the supernatant was decanted completely and the cell pellet resuspended in 40μl buffer per 10^7 cells. 10μl biotin-Antibody cocktail was added per 10^7 cells then mixed and incubated for 10mins at 4°C. 30μl buffer per 10^7 cells and 20μl anti-biotin beads per 10^7 cells were then added, mixed well and incubated for 15mins at 4°C. Cells were then washed with 1ml MACS buffer and supernatant decanted completely. Cell pellet was then resuspended in 500μl MACS buffer per 10^8 cells (500μl if less cells). Appropriate column size was selected (MS selected for max number of labelled cells being 10^7 or LS for maximum number of labelled cells being 10^8) and placed in the magnetic field of the MACS separator. The column was prepared by washing with either 3ml (LS) or 500μl (MS) of buffer. The cell suspension was applied onto the column and effluent collected, these were the unlabelled cells, representing the enriched $CD4^+$ or $CD8^+$ T cell fraction. Finally, the column was washed with 3 x 3ml (LS) or 3 x 500μl (MS) and the total effluent collected. An aliquot was removed at this point to check cell purity. Cells retained on the column can be used as APC. In this event the column was removed from the magnet and placed in a suitable collection tube, followed by pipetting either 5ml (LS) or 1ml (MS) of buffer onto the column and immediately flushing out the cells by firmly applying the plunger supplied with the column.

2.4 Measurement of telomere length and telomerase activity

2.4.1 Telomere length via Flow-FISH

PBMCs were stained using CD4-biotin (Immunotech) and streptavidin-Cy3 (Cedarlane Laboratories) or combinations of CD8-FITC, CD45RO-FITC (BD Biosciences), CD8-Q565, CD45RA-Q605 (invitrogen) then fixed with BS3 (Pierce). Cells were washed once in hybridization buffer (70% formamide, 20 mM Tris, 150 mM NaCl, and 1% BSA) and then incubated at 82°C for 10 min with 0.75 µg/ml Cy5-conjugated telomeric (CCCTAA) peptide nucleic acid probe (Applied Biosystems). After rapid cooling on ice, the samples were hybridized for 1 h at room temperature in the dark, washed twice each in posthybridization buffer (70% formamide, 10 mM Tris, 150 mM NaCl, 0.1% BSA, and 0.1% Tween 20) and PBSA, and analyzed by flow cytometry.

Samples were analyzed with and without probe to control for differences in background fluorescence between samples. To ensure consistency of the results between experiments, two cryopreserved PBMC samples with known telomere fluorescence were used as standards. Results were obtained as median fluorescence intensity values, which could then be converted to telomere length in kilobases using a standard curve. The standard curve was constructed using 30 samples of varying telomere length analyzed both by flow-FISH and telomeric restriction fragment (TRF) analysis.

2.4.2 Measuring telomere length in CMVpp65-specific cells

To measure telomere length of CMVpp65-specific $CD8^+$ T cells, a modified form of the Flow-FISH technique (section 2.4.1) was used; phenotyping was performed using CMVpp65-specific biotin labelled pentamer (Proimmune) according to manufacturers guidelines, then a second layer staining with streptavidin-Q800 (Invitrogen) in combination with CD45RA-Q605, CD8-FITC or CD45RO-FITC. Cells were then fixed with BS3 (Pierce) and the protocol detailed in section 2.4.1, post BS3 staining, was then continued.

2.4.3 Measuring telomerase activity – the TRAP assay

Sorted cell populations (2×10^5 cells) were activated with either OKT3 (0.5µg/ml) or CMV peptide (2pg/ml) plus irradiated autologous APCs for 4 days. Following activation an aliquot of 10^4 cells were stained for Ki67 positivity. The remaining cells were centrifuged (650xg for 5min) to pellet, any medium aspirated and snap-frozen (using liquid nitrogen). Telomerase activity was determined using the telomeric repeat amplification protocol (TRAP; TRAPeze telomerase detection kit; Serologicals) as previously described (Plunkett et al., 2007). Briefly, cell extracts were obtained by treating pellets with lysis buffer. Subsequently, PCR was performed with samples adjusted to 500 $Ki67^+$ T cells per reaction (Reed et al., 2004). Telomeric elongation was performed using a [γ-P^{32}] ATP-end-labelled telomerase substrate (TS) primer. These samples were then amplified by PCR amplification, using 25 to 28 cycles of 30s at 94°C and 30s at 59°C. The PCR products were run on a 12% polyacrylamide gel (Sigma-

Aldrich) that was then exposed to an autoradiography film (Hyperfilm MP, Amersham). Telomerase activity was calculated as a ratio between the optical density of the telomeric repeat bands and one of the internal standard band.

2.5 Functional analysis

2.5.1 $CD8^+$ T cell subpopulation multiparametric flow cytometry

Multiparametric flow cytometry on total $CD8^+$ T cells was conducted at the Brighton and Sussex Medical School (BSMS) in collaboration with Professor Kern. The expression of the functional markers CD40L, IFN-γ, IL-2 and TNF-α by $CD8^+$ CD45RA/CD27 T cell subpopulations was assessed following stimulation of total PBMCs with OKT3 (0.5μg/ml) in the presence of brefeldin A for 16 hours. Boolean gating analysis was used to determine polyfunctionality of each $CD8^+$ T cell subpopulation (Harari et al., 2007).

2.5.2 Activation responses of CD45RA vs. CD45RO CMV-specific $CD8^+$ T cells

A $CD8^+$ fraction was collected by negative MACs selection (detailed in section 2.3.1). The non-CD8 fraction was retained for use as APCs; half of which were pulsed with medium containing CMVpp65 NLVPMVATV (NLV) peptide (Proimmune) for 1hr at 37C and the other half of cells with medium only, before both populations were then irradiated. Meanwhile the $CD8^+$ subset was stained with CD45RA-APC (Caltag) and CD45RO-FITC (Dako) and sorted to give CD45RA and CD45RO subpopulations of greater than 95% purity using a BD FACS Aria. 200,000 cells of each population were then placed in sterile FACs tubes and irradiated APCs added back at cell ratios of 2:1, 1:1, 1:2, and 1:4.

Additionally APCs not pulsed with NLV were added at 1:1, and an un-stimulated control (cells plus medium only) was also included. After 2hr incubation at 37°C, BFA was added and cells incubated for a further 4hrs. Following stimulation, PBMCs were then stained intracellularly with IFNγ Pe-Cy7 (BD Biosciences) as described previously in section 2.3.3.

2.5.3 Polyfunctionality of normal vs. high avidity CMV-specific CD8$^+$ T cells

Polyfunctional response of CMVpp65-specific CD8$^+$ T cells was performed by staining whole PBMCs first with either normal or mutated (null) tetramer and CD107a for 2hrs at 37°C. This was followed by the addition of pp65 peptide at concentrations of 0, 0.001, 0.01, 0.1 and 1ug/ml in the presence of Monensin and Brefeldin A for a further 4 hours at 37°C. Following stimulation, PBMCs were then stained intracellularly with IFNγ Pe-Cy7 and TNF-α APC (BD Biosciences) as described in section 2.2.3.

2.6 IL-15 experiments

2.6.1 CD45RA re-expression experiments

PBMCs were isolated (section 2.1.2) then a CD8 positive fraction was collected by magnetic bead separation (MACS) as previously described (2.2.1). One of two reversion experiments was then conducted to investigate CD45RA re-expression: 1) using four CD8$^+$ T cell subpopulation determined by CD45RA and CD27 and 2) using two CMV-specific CD8$^+$ T cell subpopulations determined by CD45RA and CD45RO. For the first experiment CD8$^+$ T cells were stained with CD45RA-APC (Caltag) and CD27-FITC (BD biosciences) and sorted using a BD FACs Aria to yield the four CD8$^+$ T cell

subpopulations: $CD45RA^{+}CD27^{+}$; $CD45RA^{+}CD27^{-}$; $CD45RA^{-}CD27^{+}$, and $CD45RA^{-}CD27^{-}$. For the second experiment $CD8^{+}$ T cells were stained with CD45RA-APC (Caltag) and sorted using a BD FACs Aria to yield a CD45RA negative fraction. Each subpopulation was individually cultured with IL-15 (10U) in a 96 well round bottom plate at a concentration of 100,000 cells per well for up to 21 days. On days 0, 1, 4, 9, 14 and 21 (depending on cell numbers) post IL-15 addition, one well of cells was harvested and viable cell numbers counted to determine cell expansion. Cells were then stained as outlined (2.2.1 and 2.2.2) for total $CD8^{+}$ T cell subpopulations (CD45RA and CD27) or CMV-specific $CD8^{+}$ T cell subpopulations (CD45RA and CD45RO).

2.6.2 Measuring IL-15 mRNA following treatment with CMV lysate or IFN-α

The mRNA level of IL-15 was measured in total PBMCs before (directly *ex-vivo*) and after 4hrs, 24hrs, 48hrs, and 72hrs of culture in the presence of the lysate from CMV-infected cells (1:10 of stock solution of 0.64mg/ml). Additionally, mRNA levels of IL-15 were also measured in total PBMCs before (directly *ex-vivo*) and after 4, 24 and 48 hours of culture in the presence of different concentration of IFN-α (Alpha 2a, 11100-1 from PBL Interferon source) ranging from 100U/ml to 1000 U/ml. Total RNA was purified with RNeasy columns (Qiagen). Reverse transcription was performed with random primers using the MuLVRT reverse transcriptase (Invitrogen) to generate cDNA. The mRNA level of IL-15 was then determined using cDNA by real-time quantitative PCR (RT-qPCR) on an ABI PRISM 7500, with TaqMan Gene Expression Master Mix according to manufacturer protocol (Applied Biosystems), with the TaqMan primers for IL-15 primers

(Hs99999039_m1). The housekeeping 18S mRNA was amplified from the same cDNA reaction mixture using the TaqMan primers for human 18S (Hs03928985_g1*). Each sample was run in triplicate and target mRNA level was expressed as a ratio to the level of 18S to control for differing levels of cDNA in each sample.

2.7 Statistical Analysis

Graph Pad Prism v5 was used to construct all graphs, dot plots and bar charts. The D'Agostino-Pearson omnibus K2 normality test was used to determine if data fitted a Gaussian (normal) distribution. Statistical significance was evaluated using the Student's t test if data followed a normal distribution and if data was also paired it was assessed using a Student's paired t test. Unpaired non-parametric data was evaluated using a Mann-Whitney U test, with a Wilcoxon matched paired test used if data points represented paired observations. All t tests performed were as two-tailed unless otherwise stated. All p-values ≤ 0.05 were regarded as significant.

3 Phenotyping $CD8^+$ and CMV-specific $CD8^+$ T cells according to age

3.1 Introduction

Longitudinal studies measuring various immunological parameters in the very old have revealed a group of biomarkers defining an immune risk phenotype (IRP) that is associated with a decreased survival rate in the very old (section 1.4.9). Cytomegalovirus (CMV) seropositivity was one of the significant factors identified. It has been proposed that this could be due to CMV infected individuals having large and persistent CMV-specific T cell expansions (TCEs), which may contribute to the inversion of the CD4/CD8 ratio and acceleration of T cell differentiation. CMV has therefore been identified as a key factor in driving immune senescence of the T cell pool (discussed in section 1.6).

3.2 Aims

The aim of this chapter was two-fold; to investigate how CMV influences the natural age-related changes seen within the CD8 compartment, and secondly, to uncover how CMV-specific TCEs may accumulate. This was achieved by phenotyping $CD8^+$ T cells and CMV-specific $CD8^+$ T cells directly *ex-vivo* from a cohort of healthy donors over a wide age range. Phenotyping determined the differentiation status of $CD8^+$ T cells by the expression of CD45RA and CD27; giving the four distinct $CD8^+$ T cell subpopulations (as described in section 1.3). The frequency of these subpopulations, their relative rate of proliferation and relative susceptibility to apoptosis was investigated in all donors and

stratified on the basis of age and CMV status, to uncover any statistically significant associations.

3.3 Results

3.3.1 Selecting suitable donors and steps for phenotypic analysis

Figure 3.1 outlines the selection process for donors. Further information can be found in the materials and methods section in chapter 2. Briefly, healthy donor volunteers were first recruited according to an exclusion criterion: all subjects were mobile, did not have any cognitive impairment, were not suffering from acute or chronic illness, and were not on medication known to affect the immune system. Fresh PBMCs from these healthy individuals were isolated and stimulated overnight with CMV lysate to identify those that were CMV positive (figure 3.2A). Those who were CMV positive were further screened to determine their suitability for CMV-specific $CD8^+$ T cell analysis using tetramers. The tetramers chosen for use in this thesis were specific for the pp65 matrix protein of CMV. CMVpp65 was chosen as it is readily targeted by the immune system and is considered the most immunodominant of the CMV epitopes (Kern et al., 2002, Moss and Khan, 2004, Wills et al., 1996). Individuals who were HLA-A*02 or HLA-B*07 positive (the most common European alleles) were therefore selected as suitable for staining with the NLVPMVATV (NLV) or TPRVTGGGAM (TPR) CMVpp65-specific tetramers respectively (figure 3.2B).

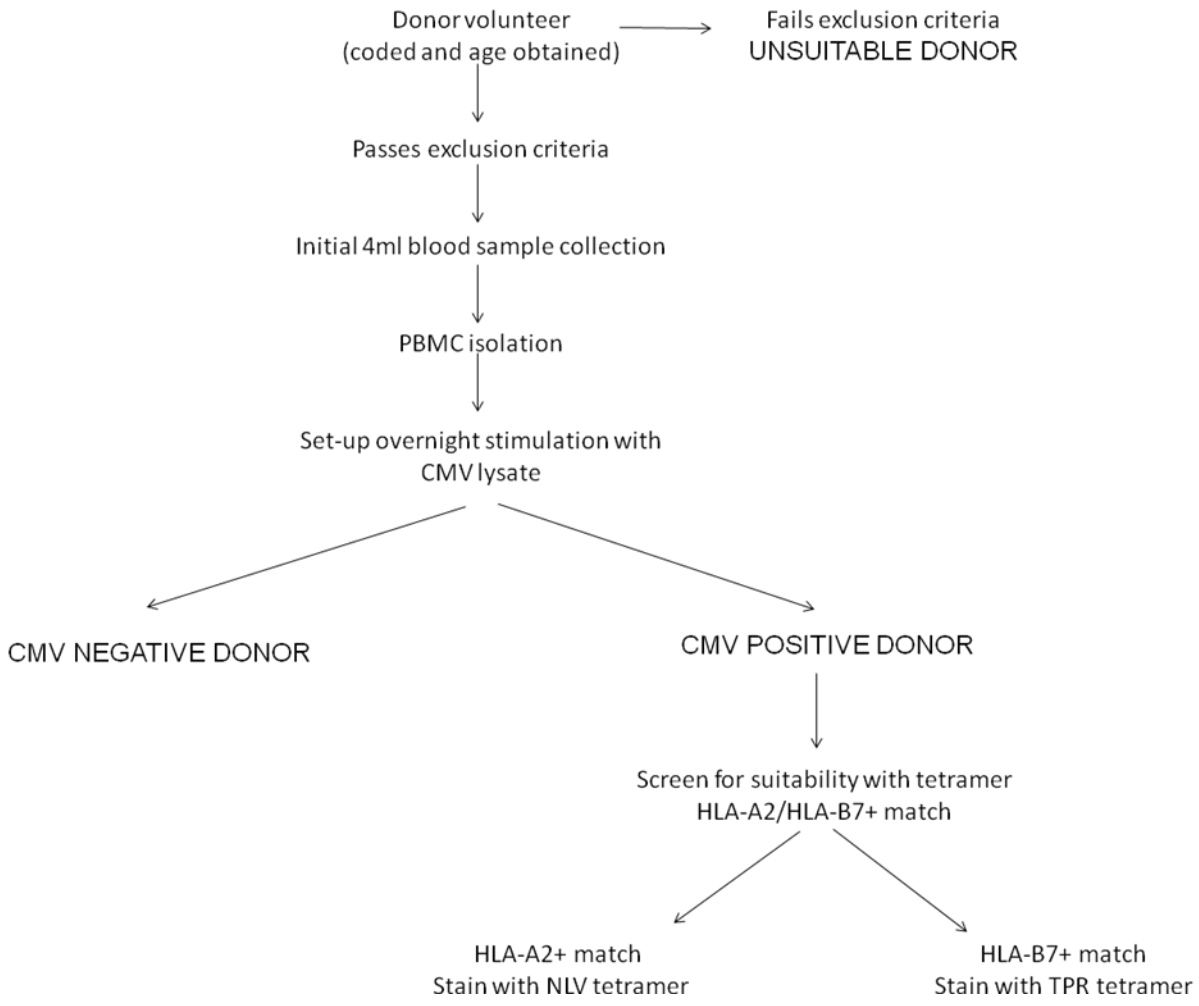
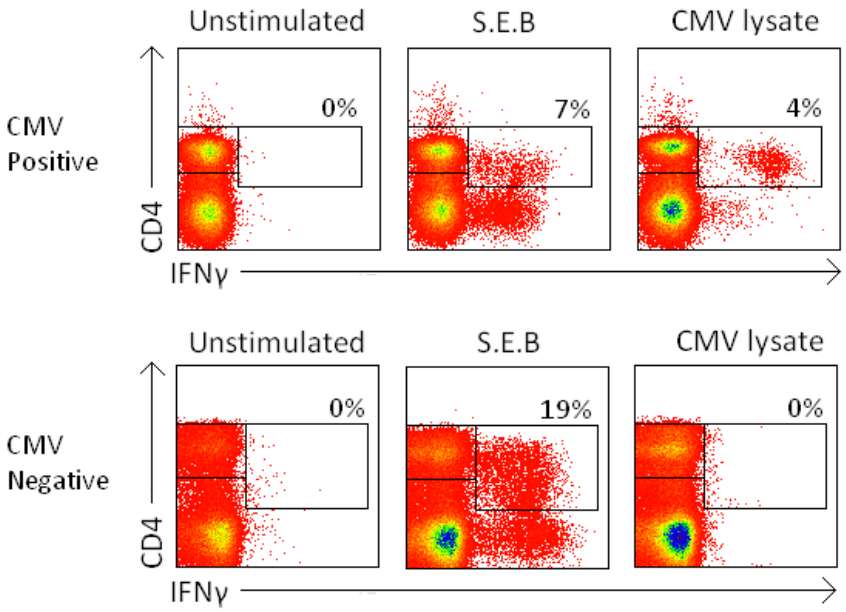


Figure 3-1. Donor selection process

Healthy donor volunteers are recruited and those not meeting the healthy criteria excluded. An initial blood sample is taken and fresh PBMCs isolated and stimulated overnight with CMV lysate, to determine CMV status. Those that are CMV positive are further screened for HLA-A*02 or HLA-B*07 match, using antibodies specific for these two antigens, in combination with CD8. HLA-A*02 or HLA-B*07 matched donors are therefore suitable for subsequent analysis using NLVPMVATV (NLV) or TPRVTGGGAM (TPR) CMVpp65-specific tetramers respectively.

(A)



(B)

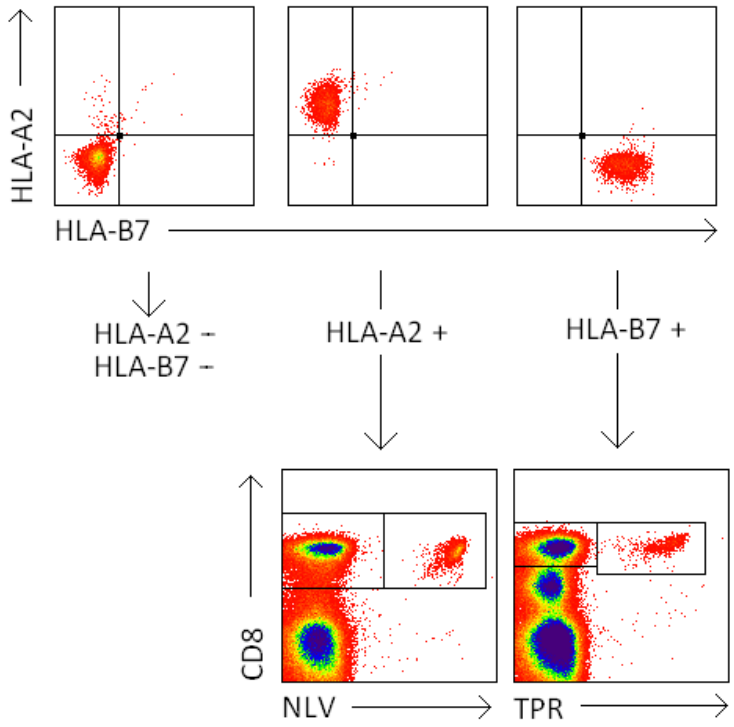


Figure 3-2. CMV positive screening and selection for tetramer analysis

(A) CMV positivity was determined by stimulating total PBMCs with either CMV lysate, an S.E.B positive control and un-stimulated (U.S) negative control. Following stimulation PBMCs were stained with CD4 and IFN-γ antibodies. A CMV positive donor (top) shows IFN-γ^+ cell numbers above background (U.S) compared with a CMV negative individual (bottom). Both donors show IFN-γ^+ cells to S.E.B. (B) individuals suitable for tetramer analysis were HLA-A*02 or HLA-B*07 matches and subsequently used for staining with NLV or TPR tetramers respectively.

3.3.2 Investigating the differentiation status of $CD8^+$ T cells

A larger number of highly differentiated $CD8^+$ T cells have been reported in older humans (Effros et al., 2005, Akbar and Fletcher, 2005) and CMV-specific $CD8^+$ T cells show a high degree of differentiation compared with most other virus-specific $CD8^+$ T cells (Appay et al., 2002). Therefore the impact of age and CMV serostatus was investigated on the $CD8^+$ T cell subpopulations in our healthy donors.

3.3.2.1 Differentiation of $CD8^+$ T cells by CD45RA and CD27 expression

Donor PBMCs were stained with antibodies to CD8, CD45RA and CD27 to identify the four $CD8^+$ T cell subpopulations as discussed in section 1.3:

$CD45RA^+CD27^+$ = Naive (T_N)
$CD45RA^-CD27^+$ = Central Memory (T_{CM})
$CD45RA^-CD27^-$ = Effector Memory (T_{EM})
$CD45RA^+CD27^-$ = Effector Memory CD45RA re-expressing (T_{EMRA})

A representative example of how these subpopulations were identified is shown in figure 3.3. Briefly, $CD8^+$ T cell subpopulations were identified first by plotting size (forward scatter, FSC) versus granularity (side scatter, SSC) to identify the live lymphocyte population (L) from monocytes (M), highly granular cells (G) and cellular debris (D). Upon gating on live lymphocytes a $CD8^{high}$ subset was then identified, separating it from $CD4^+$ T cells, B cells and natural killer (NK) cells (Trimble et al., 2000). Finally the $CD8^+$ T cell compartment was dissected via CD45RA and CD27 expression to identify the four $CD8^+$ T cell subpopulations of interest.

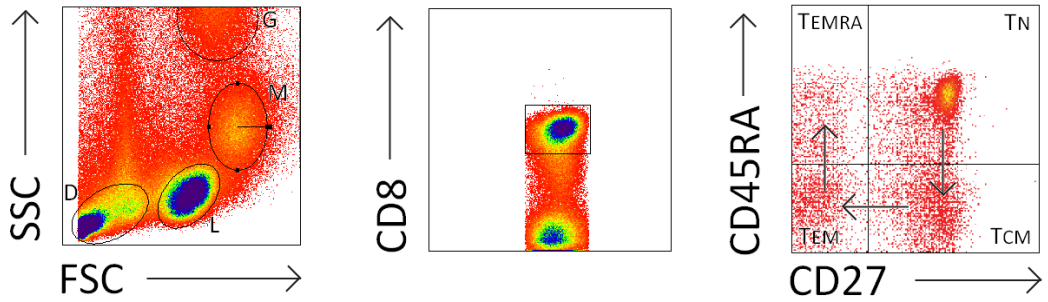


Figure 3-3. Gating method to identify CD8$^+$ T cell subpopulations from PBMCs
See text for details. Arrows displayed within the FACs plot analysis on the four subpopulations (right) denote possible differentiation pathways. Abbreviations: live lymphocytes (L); Granulocytes (G); Monocytes (M); Dead cells (D); Naive (TN); Central Memory (TCM); Effector Memory (TEM); and Effector Memory CD45RA re-expressing (TEMRA).

3.3.2.2 CD8$^+$ T cell subpopulation profiles by age group

Representative examples of healthy donor CD8$^+$ T cell subpopulation profiles in the three defined age groups: young (<35 years old); middle (35 to 65 years old) and old (>65 years old) are shown in figure 3.4. CD8$^+$ T cells in young individuals showed a predominately early differentiated profile i.e. CD45RA$^+$CD27$^+$ phenotype, compared with old individuals which showed a predominately late differentiated profile i.e. CD45RA$^+$CD27$^-$ phenotype.

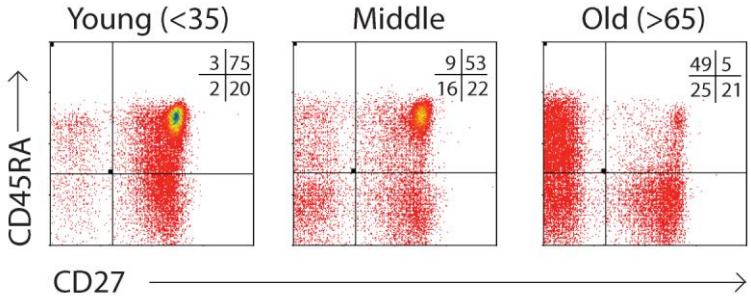


Figure 3-4. Representative density plots of CD8$^+$ T cell subpopulation profiles by age group
Density plots of healthy donor CD8 differentiation profiles as based on CD45RA and CD27 expression. Numbers in quadrants represent the percentage of the relevant subpopulation within total CD8$^+$ T cell compartment. Young individuals are defined as under 35 and old individuals as over 65years of age.

3.3.2.3 Age-related differentiation of $CD8^+$ T cells

The frequency of each subpopulation, as a percentage of the total CD8 compartment, was recorded for all donors (figures 3.5–3.8). In total, 125 healthy donors aged between 22 to 95 were analysed, with a CMV seropositivity rate of 73%. This CMV seroprevalence rate is consistent with reports (Cannon et al., 2010).

A line of best fit was generated by linear regression analysis and the correlation assessed by Pearson and Spearman rank (GraphPad Prism) for each $CD8^+$ T cell subpopulation. The $CD45RA^+CD27^+$ (Naive, T_N) subpopulation (figure 3.5A) was found to significantly decrease with age ($p<0.0001$, $R^2=0.588$) whereas there was no significant age-related change observed with the $CD45RA^-CD27^+$ (Central Memory, T_{CM}) subpopulation (figure 3.6A). However, the late differentiated $CD45RA^-CD27^-$ (Effector Memory, T_{EM}) and $CD45RA^+CD27^-$ (Effector Memory CD45RA re-expressing, T_{EMRA}) subpopulations (figures 3.7A & 3.8A respectively) significantly increased with age ($p=0.0014$, $R^2=0.798$ and $p<0.0001$, $R^2=0.350$ respectively).

3.3.2.4 CMV accelerates age-related differentiation of $CD8^+$ T cells

To investigate the influence of age and CMV seropositivity as statistically independent factors upon $CD8^+$ T cell subpopulations, data was further analysed in two ways: firstly by dividing the data into either the young or old age group and on the basis of CMV serostatus, and secondly by performing multiple linear regression analysis.

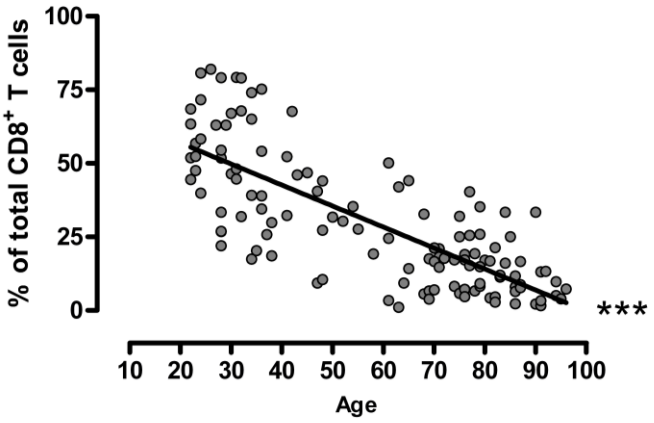
When dividing data into young (<35) or old (>65) age groups and on the basis of CMV serostatus, CMV^+ young individuals had significantly less $CD8^+$ T_N cells than CMV^- young individuals (p=0.0007, figure 3.5B). Both CMV^+ groups also had significantly more $CD8^+$ T_{EMRA} cells compared with the CMV^- groups (young p=0.001 and old p=0.002, figure 3.8B).

Multiple linear regression analysis was performed by SPSS software and revealed a highly significant relationship between the decrease in $CD8^+$ T_N cells and increase in $CD8^+$ T_{EMRA} cells with either age or CMV serostatus as independent factors (p<0.0001, figures 3.5C and 3.8C). There was also significant increases in $CD8^+$ T_{EM} cells with age or CMV serostatus (p=0.04 and p=0.007 respectively, figure 3.7C) and a significant decrease in $CD8^+$ T_{CM} cells with CMV serostatus (p=0.01, figure 3.6C), but not with age.

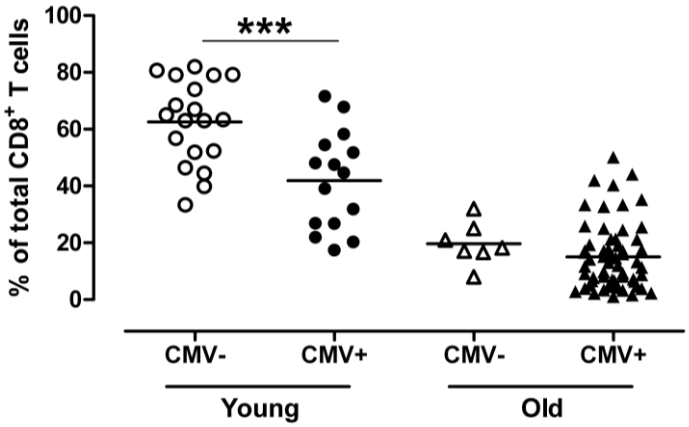
Overall, results showed both age and CMV serostatus significantly impacts the $CD8^+$ T cell compartment. However, the most significant changes were a decrease in $CD8^+$ T_N cells and an increase in the highly differentiated $CD8^+$ T_{EMRA} cells.

TN

(A)



(B)



(C)

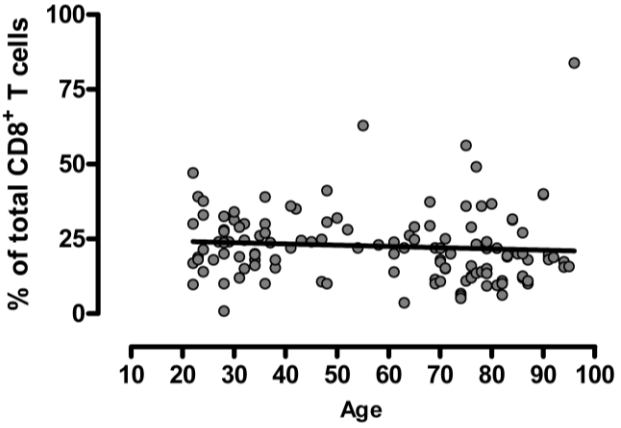
	unstandardized coefficient	P-value	95.0% CI	
			lower limit	upper limit
Age	-0.59	<0.0001	-0.7	-0.49
CMV status	-15.62	<0.0001	-21.46	-9.77

Figure 3-5. Distribution of naive (CD45RA$^+$CD27$^+$) CD8$^+$ T cells and the impact of age and CMV serostatus.

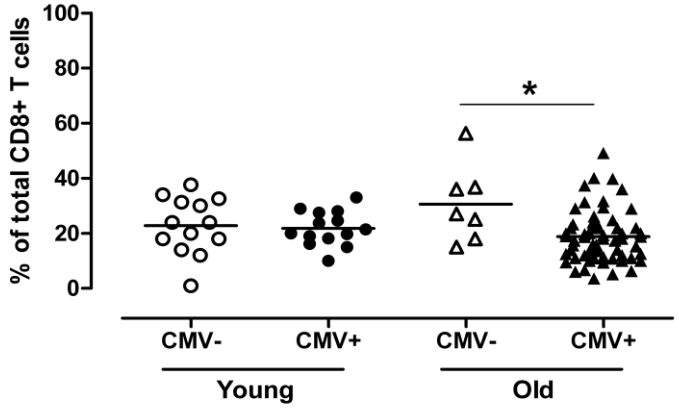
(A) Pooled data showing the distribution of Naive (CD45RA$^+$CD27$^+$) CD8$^+$ T cells defined as a percentage of the total CD8$^+$ T cell compartment within all donors (125) as stratified by age. One dot represents one donor and line of best fit shown. (B) The same data shown by age group and CMV status, donors shown: CMV-(Y)=19; CMV+(Y)=15; CMV-(O)=7; CMV-(Y)=60 (C) Table showing the un-standardized coefficient, significance and 95% confidence interval from the output of SPSS software for Naive CD8$^+$ T cells. Unit of age is equal to 1 year. *<0.05;**<0.005;***<0.0005.

TCM

(A)



(B)



(C)

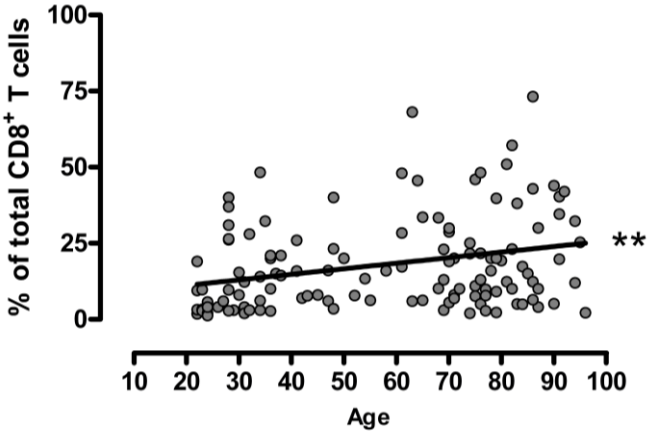
	unstandardized coefficient	P-value	95.0% CI	
			lower limit	upper limit
Age	-0.03	0.52	-0.12	0.06
CMV status	-6.18	0.01	-10.86	-1.49

Figure 3-6. Distribution of central memory ($CD45RA^{-}CD27^{+}$) $CD8^{+}$ T cells and the impact of age and CMV serostatus.

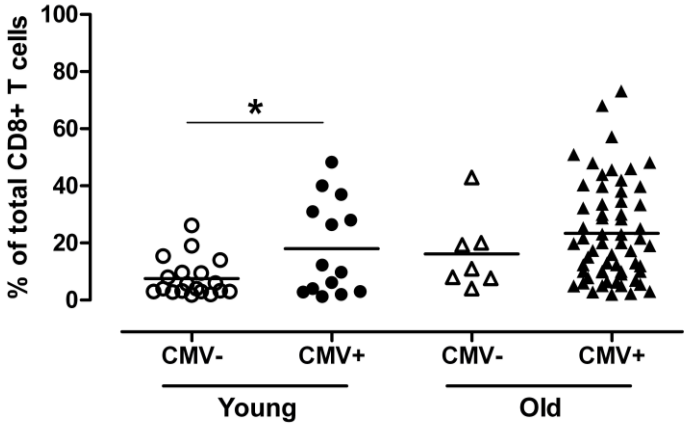
(A) Pooled data showing the distribution of central memory ($CD45RA^{+}CD27^{+}$) $CD8^{+}$ T cells defined as a percentage of the total $CD8^{+}$ T cell compartment within all donors (125) as stratified by age. One dot represents one donor and line of best fit shown. (B) The same data shown by age group and CMV status. Donors shown: CMV-(Y)=19; CMV+(Y)=15; CMV-(O)=7; CMV-(Y)=60 (C) Table showing the un-standardized coefficient, significance and 95% confidence interval from the output of SPSS software for central memory $CD8^{+}$ T cells. Unit of age is equal to 1 year. *<0.05;**<0.005;***<0.0005.

TEM

(A)



(B)



(C)

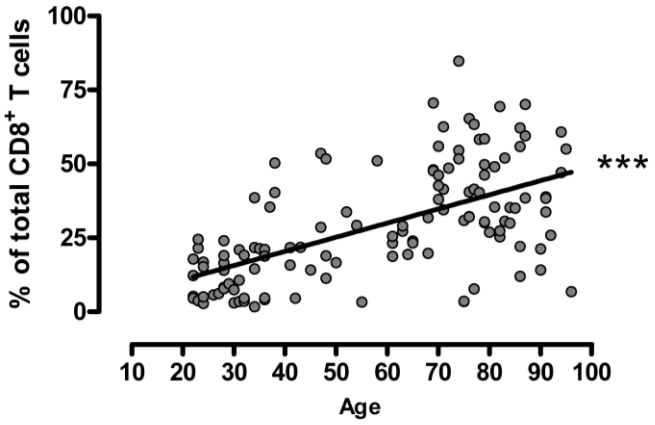
	unstandardized coefficient	P-value	95.0% CI	
			lower limit	upper limit
Age	0.125	0.04	0.005	0.24
CMV status	9.05	0.007	2.58	15.53

Figure 3-7. Distribution of effector memory ($CD45RA^-CD27^-$) $CD8^+$ T cells and the impact of age and CMV serostatus.

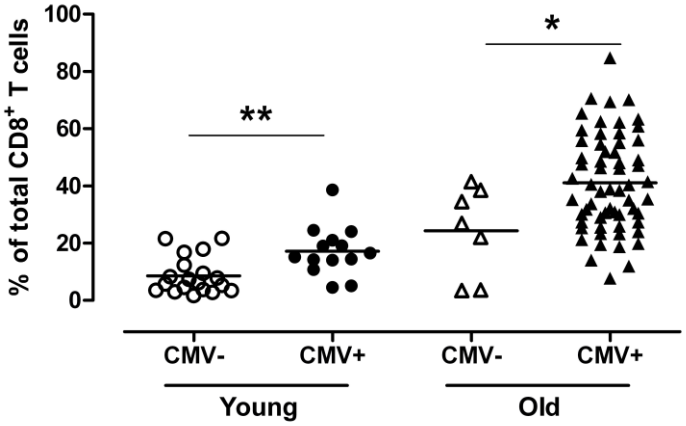
(A) Pooled data showing the distribution of effector memory ($CD45RA^-CD27^-$) $CD8^+$ T cells defined as a percentage of the total $CD8^+$ T cell compartment within all donors (125) as stratified by age. One dot represents one donor and line of best fit shown. (B) The same data shown by age group and CMV status. Donors shown: CMV-(Y)=19; CMV+(Y)=15; CMV-(O)=7; CMV-(Y)=60 (C) Table showing the un-standardized coefficient, significance and 95% confidence interval from the output of SPSS software for effector memory $CD8^+$ T cells. Unit of age is equal to 1 year. *<0.05;**<0.005;***<0.0005.

TEMRA

(A)



(B)



(C)

	unstandardized coefficient	P-value	95.0% CI	
			lower limit	upper limit
Age	0.385	<0.0001	0.27	0.5
CMV status	14.77	<0.0001	8.43	21.11

Figure 3-8. Distribution of effector memory CD45RA re-expressing ($CD45RA^+CD27^-$) $CD8^+$ T cells and the impact of age and CMV serostatus.

(A) Pooled data showing the distribution of effector memory CD45RA re-expressing ($CD45RA^+CD27^-$) $CD8^+$ T cells defined as a percentage of the total $CD8^+$ T cell compartment within all donors (125) as stratified by age. One dot represents one donor and line of best fit shown. (B) The same data shown by age group and CMV status. Donors shown: CMV-(Y)=19; CMV+(Y)=15; CMV-(O)=7; CMV-(Y)=60. (C) Table showing the un-standardized coefficient, significance and 95% confidence interval from the output of SPSS software for effector memory CD45RA re-expressing $CD8^+$ T cells. Unit of age is equal to 1 year. *<0.05;**<0.005;***<0.0005..

3.3.3 CMV-specific $CD8^+$ T cell expansion during ageing

In the last section, a significant increase in $CD8^+$ T_{EMRA} cells with both age and CMV was revealed. However, it is unclear if it is the CMV-specific $CD8^+$ T_{EMRA} cells that largely account for the expansion of this subpopulation within CMV positive individuals. Therefore, the size and phenotype of the CMV-specific $CD8^+$ T cell population was investigated.

3.3.3.1 CMV-specific $CD8^+$ T cells expands during ageing

The CMVpp65-specific $CD8^+$ T cell population has been reported to expand during ageing (Khan et al., 2002b, Koch et al., 2007). To verify this in our healthy cohort, all CMV positive donors suitable for analysis by tetramer staining were first identified (as discussed in 3.3.1). In total, 70 donors were suitable and the CMVpp65-specific compartment calculated as a percentage of their total $CD8^+$ T cell compartment. Figure 3.9A shows representative examples of the CMVpp65-specific $CD8^+$ T cell population found within old versus young CMV positive individuals. Older CMV positive individuals were found to contain larger CMVpp65-specific $CD8^+$ T cell expansions, being up to 28% of total CD8s, compared with younger CMV positive individuals. This was highlighted when data was pooled, and a significant positive correlation between donor age and size of the CMVpp65-specific compartment was revealed in total donors ($p=0.002$, $R^2=0.157$, figure 3.9B), HLA-A*02^+ donors targeting the NLV-specific epitope of CMVpp65 ($p=0.028$, $R^2=0.134$, figure 3.9B) or HLA-B*07^+ donors targeting the TPR-specific epitope of CMVpp65 ($p=0.019$, $R^2=0.227$, figure 3.9B).

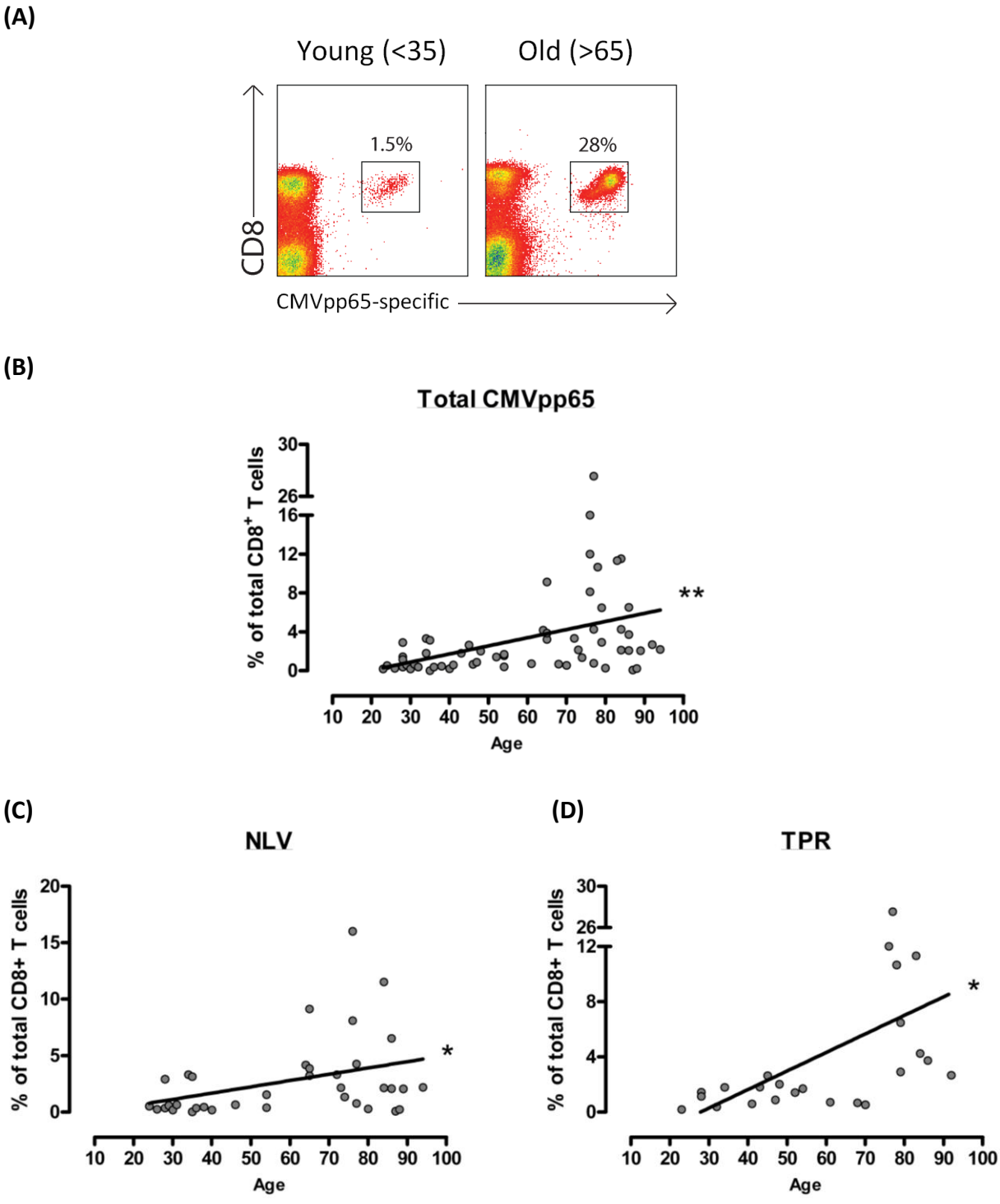


Figure 3-9. The CMVpp65-specific CD8$^+$ T cell population expands with age

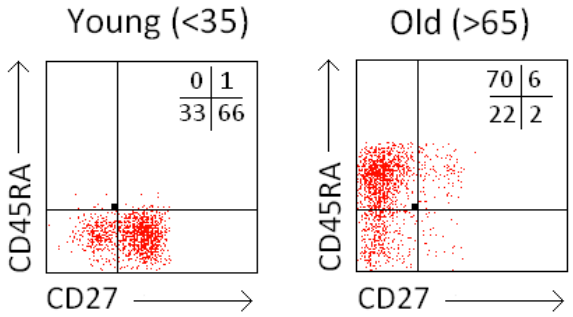
(A) Representative example of the CMVpp65-specific population identified by tetramer (NLV shown). Pooled data showing the size of the CMVpp65-specific population as a percentage of total CD8$^+$ T cells is shown for total (B) from 70 donors and separately as NLV-specific population only (C) from 36 donors or TPR-specific population only (C) from 24 donors as stratified by age. One dot represents one donor and significant correlations are represented by asterisks; *<0.05;**<0.005;***<0.0005..

3.3.3.2 CMV-specific $CD8^+$ T_{EMRA} cells dominate in older individuals

The CMVpp65-specific population was analysed for the expression of CD45RA and CD27 (figure 3.10). Older individuals frequently showed more highly differentiated $CD8^+$ CMVpp65-specific T cell populations compared with younger individuals (figure 3.10A). When data was pooled, $CD8^+$ CMVpp65-specific T_{EMRA} cells occupied a significantly higher percentage of the CMV-specific compartment in the old age group compared with the young age group (p=0.004, figure 3.10B). Conversely, $CD8^+$ CMVpp65-specific T_{CM} and T_{EM} cells occupied a significantly lower percentage of the population in the old age group compared with the young age group (p=0.002 and 0.025 respectively).

To investigate further whether this represented significant expansions of the $CD8^+$ CMV-specific T_{EMRA} cells in the old, the relative size of each CMV-specific $CD8^+$ T cell subpopulation was calculated as a percentage of total $CD8^+$ T cell subpopulation, and of total $CD8^+$ T cells, and compared by age group (figure 3.11 & 3.12 respectively). Both CMV-specific T_{EMRA} and T_N cells occupied a significantly larger proportion of total T_{EMRA} and T_N cells in older individuals compared with younger individuals (p=0.009 and p=0.0005 respectively) with no significant increase in T_{CM} or T_{EM} cells (figure 3.11). Furthermore, relative to total $CD8^+$ T cells, both CMV-specific T_{EMRA} and T_N cells were significantly higher in older individuals compared with younger individuals (p=0.0006 and p=0.008 respectively) with no significant increase in T_{CM} or T_{EM} cells (figure 3.12). Overall, results showed $CD8^+$ CMVpp65-specific T_{EMRA} cells dominated the CMVpp65-specific $CD8^+$ T cell compartment in older individuals.

(A)



(B)

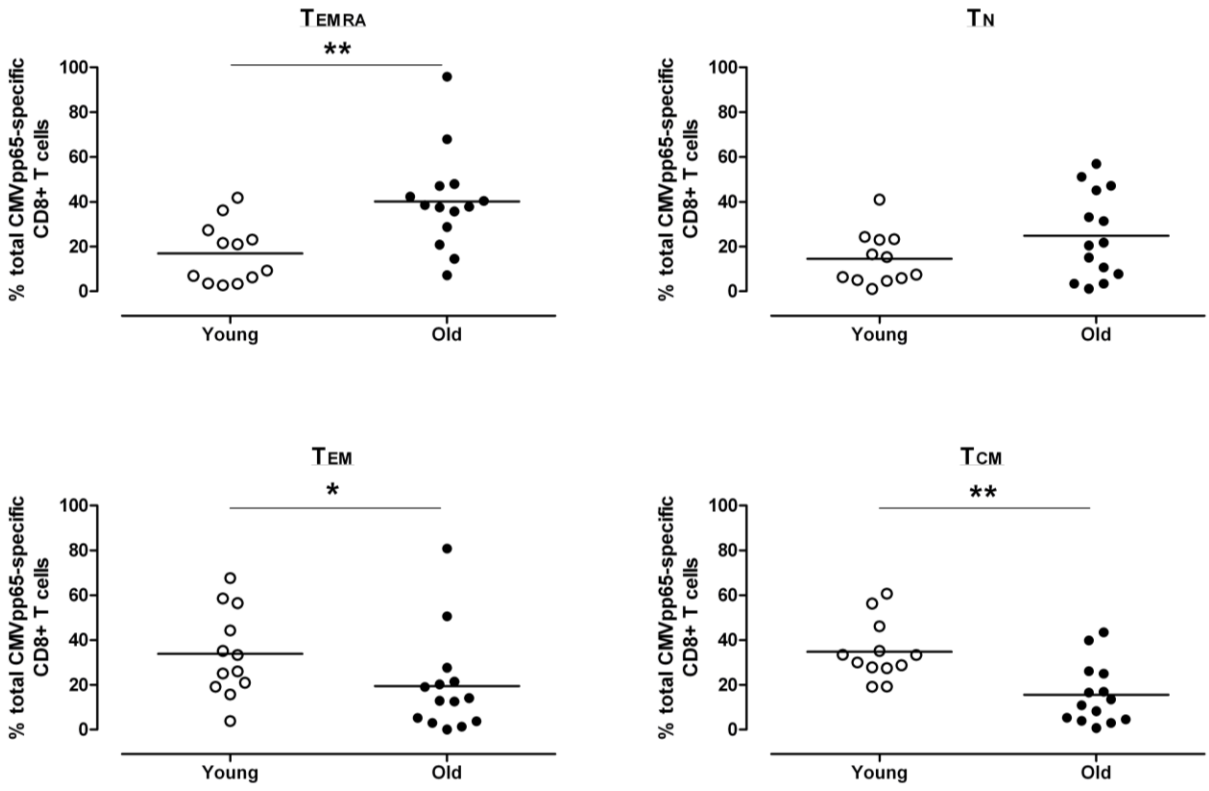


Figure 3-10. Differentiation status of CMVpp65-specific CD8$^+$ T cells in young and old donors.
(A) Representative density plots of healthy donor CMV-specific CD8$^+$ T cell differentiation on the basis of CD45RA and CD27 expression in young versus old age groups. Donor numbers shown are 12 young and 14 old. Numbers in quadrants represent percentage of the subpopulation within the total CMV-specific CD8$^+$ T cell compartment. (B) Pooled data showing the distribution of subpopulations defined as a percentage of the total CMVpp65-specific CD8$^+$ T cell compartment by age group. One dot represents one donor. Significant correlations represented by asterisks; *<0.05;**<0.005;***<0.0005.

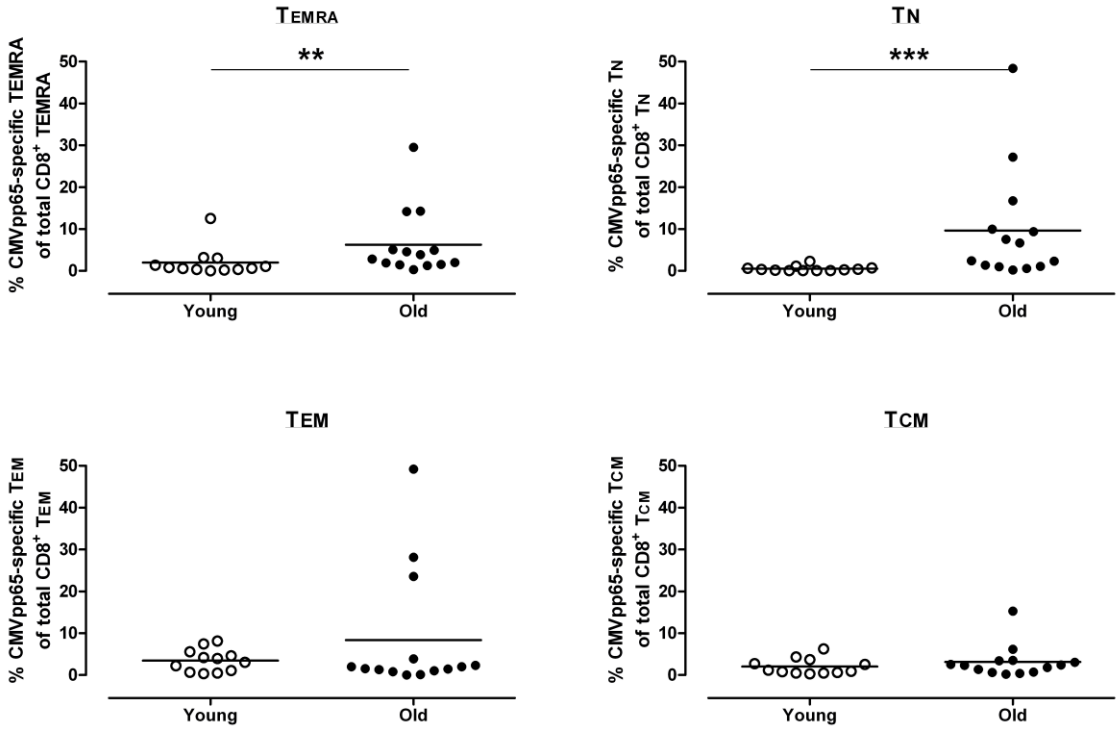


Figure 3-11. Relative size of each CMVpp65-specific CD8$^+$ T subpopulation as a percentage of total CD8+ T cell subpopulation in young and old donors.

Pooled data showing the distribution of each CMV-specific T cell subpopulation as a percentage of the total CD8$^+$ T cell subpopulation compartment by age group. Donor numbers shown are 12 young and 14 old. One dot represents one donor. Significant correlations represented by asterisks; *<0.05;**<0.005;***<0.0005.

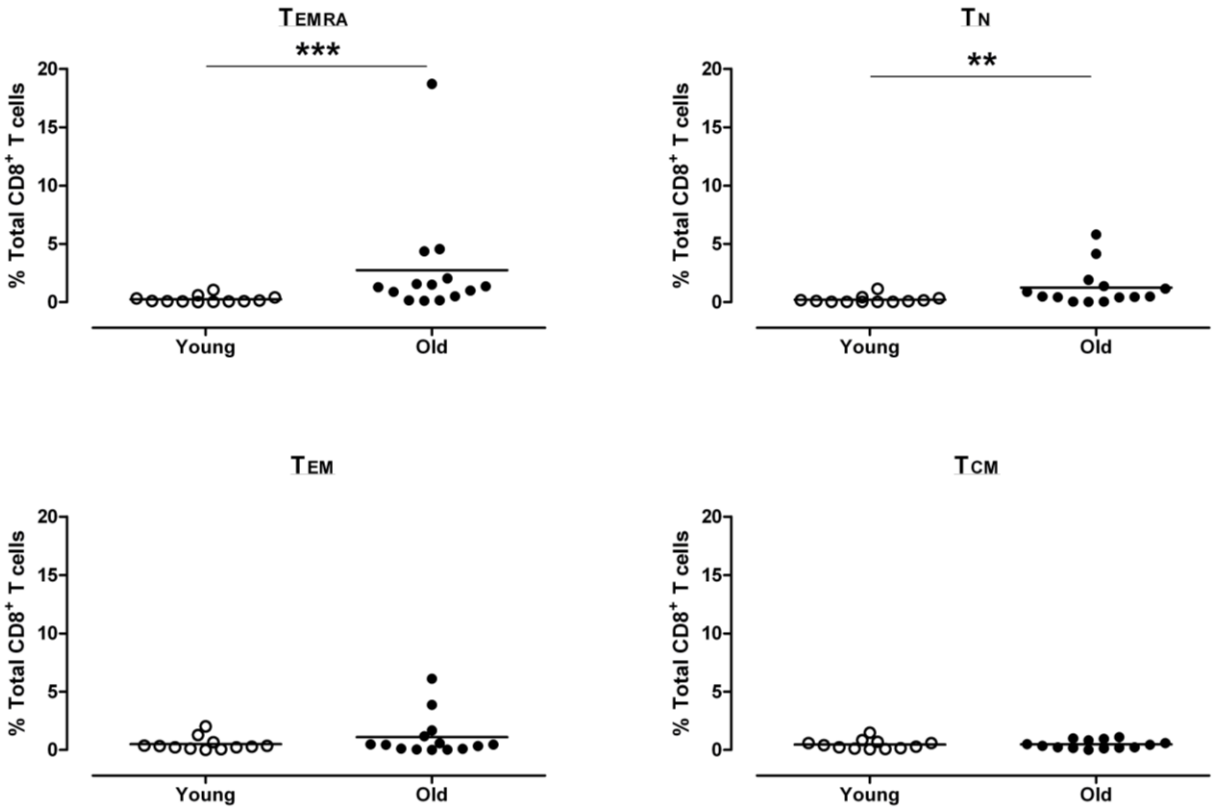


Figure 3-12. Relative size of CMVpp65-specific CD8$^+$ T subpopulations as a percentage of total CD8+ T cells in young and old donors.

Pooled data showing the distribution of subpopulations defined as a percentage of the total CD8$^+$ T cell compartment by age group. Donor numbers shown are 12 young and 14 old. One dot represents one donor. Significant correlations represented by asterisks; *<0.05;**<0.005;***<0.0005.

3.3.4 Investigating the rate of proliferation by $CD8^+$ T_{EMRA} cells

The simplest explanation for the expansion of $CD8^+$ T_{EMRA} cells would be an increase in their rate of proliferation relative to the other $CD8^+$ T cell subpopulations. Therefore the proliferative rates of $CD8^+$ and CMV-specific $CD8^+$ T cell subpopulations were investigated.

3.3.4.1 Using Ki-67 expression as a marker for proliferation

Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2 and mitosis) but is absent during cell resting (G_0) and its expression is believed to be an absolute requirement for the progression to cell division (Scholzen and Gerdes, 2000). This makes the detection of Ki-67 antigen an excellent indicator of recent proliferative activity, and has been used extensively for such purposes (Kirik et al., 2009, Endl et al., 1997, Urruticoechea et al., 2005, Velu et al., 2009, Scholzen and Gerdes, 2000, Miles et al., 2007, Roos et al., 2000, Wallace et al., 2010).

If $CD8^+$ T_{EMRA} cells, and in particular CMVpp65-specific $CD8^+$ T_{EMRA} cells, are proliferating at a high rate, this may be reflected by increased levels of Ki-67 protein present within this T_{EMRA} cells, relative to the other $CD8^+$ T cell subpopulations. Intracellular staining for Ki-67 was therefore performed on donor PBMCs directly *ex-vivo,* without stimulation, in order to provide a 'snap-shot' indicative of the proliferative activity of each $CD8^+$ T cell subpopulation *in-vivo*.

3.3.4.2 $CD8^+$ T_{EMRA} cells show low Ki-67

Both the $CD8^+$ and CMVpp65-specific $CD8^+$ T cell subpopulations were analysed for the expression of Ki-67, as shown in the representative FACs plot analysis in figure 3.13. Ki-67 positivity was analysed in total $CD8^+$ and CMVpp65-specific $CD8^+$ T cell subpopulations within 55 and 12 donors respectively (figures 3.14 & 3.15). When comparing overall Ki-67 positivity of the total $CD8^+$ T cell compartment in individuals on the basis of their age and CMV serostatus (figure 3.14A) there was no significant difference between CMV positive and CMV negative individuals in either age group. However, there were increased Ki-67 levels in $CD8^+$ T cells in the old CMV positive compared with young CMV positive age group. Additionally, no significant difference was observed for Ki-67 levels in total $CD8^+$ T cells and total CMVpp65-specific $CD8^+$ T cells within the same individuals, from a group of 12 donors (figure 3.14B). When comparing at the subpopulation level, Ki-67 levels were highest in the T_{CM} and T_{EM} subpopulations but relatively low in T_{EMRA} cells in both the $CD8^+$ (figure 3.15A) and CMVpp65-specific $CD8^+$ T cell subpopulations (figure 3.15B). Finally, further stratification of the Ki-67 data for each subpopulation by age and CMV status failed to reveal any significant associations (not shown).

Overall, results showed $CD8^+$ CMVpp65-specific T cells had low levels of Ki-67 positivity directly *ex-vivo*. Furthermore, CMV-specific $CD8^+$ T_{EMRA} cells did not show increased Ki-67 levels over other subpopulations.

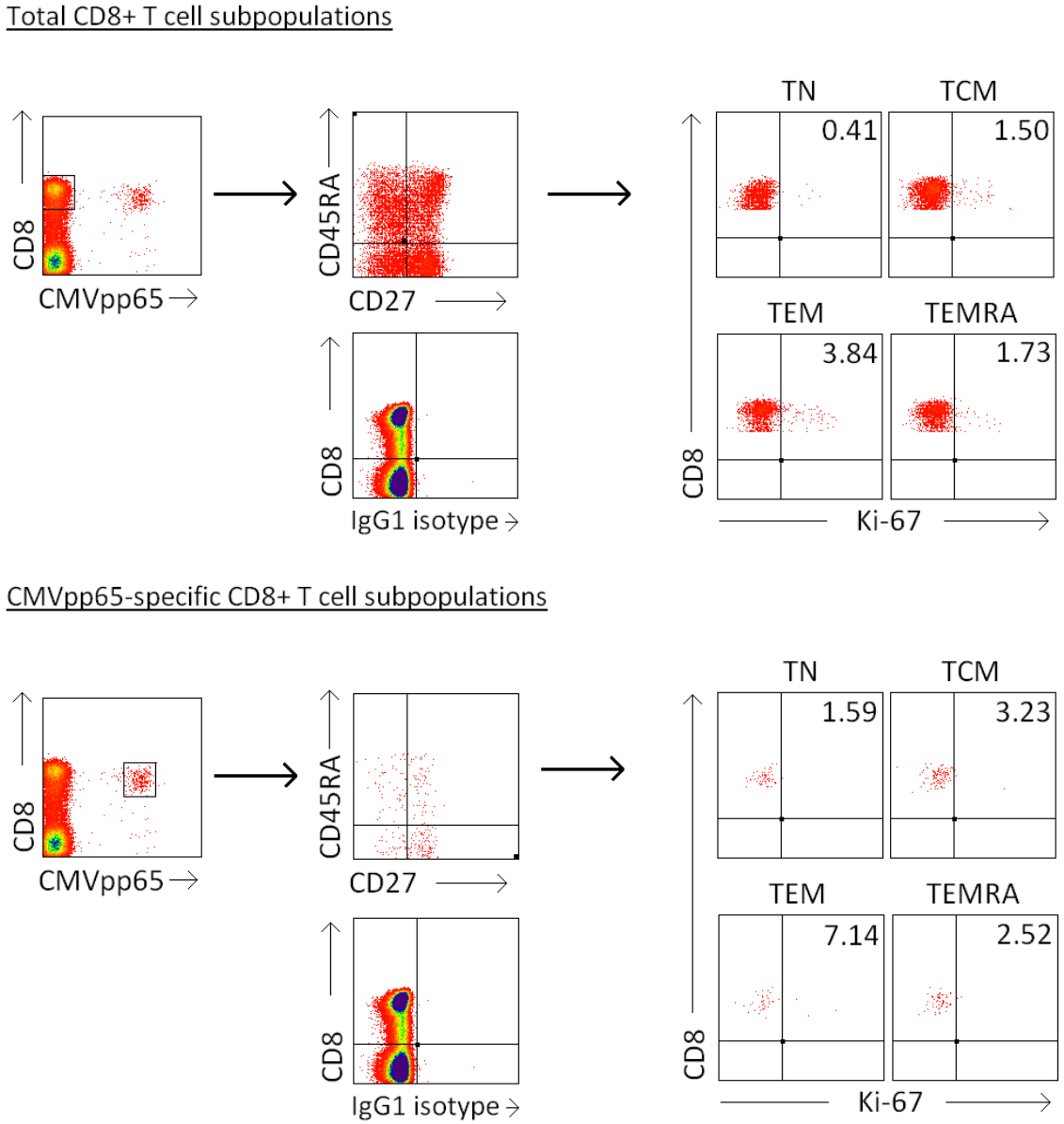
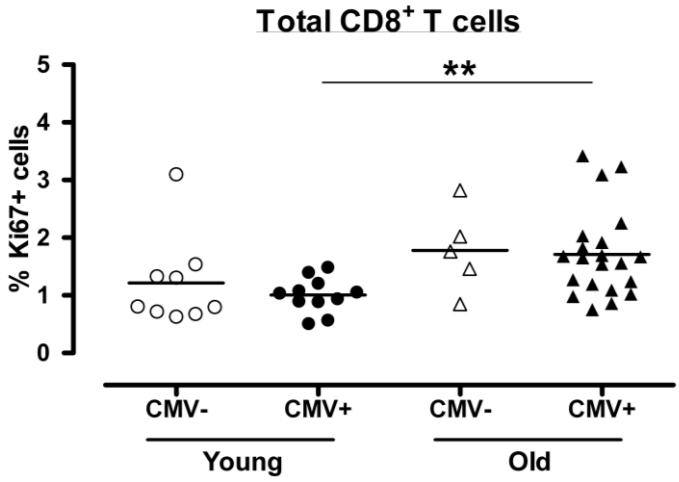


Figure 3-13. Representative example of Ki67 staining directly *ex-vivo* in one individual
Representative FACs plot analysis of Ki-67 positivity in $CD8^+$ (top) and CMVpp65-specific $CD8^+$ (bottom) T cell subpopulations within the same individual. Numbers represent percentage positivity as indicated in quadrant. Gating is determined by using manufacturer supplied Ki-67 FITC IgG1 isotype control as shown. Example shown is representative of 12 donors.

(A)



(B)

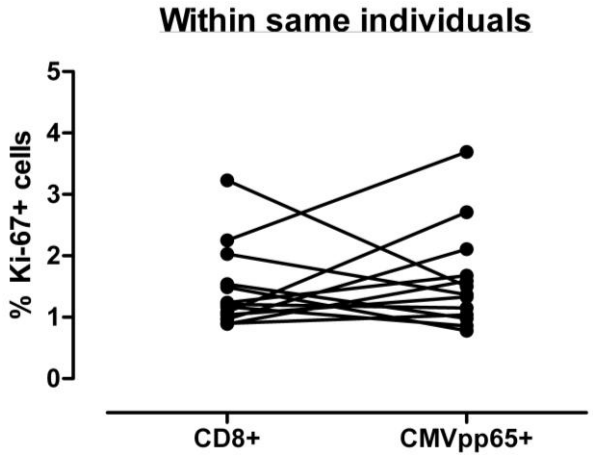
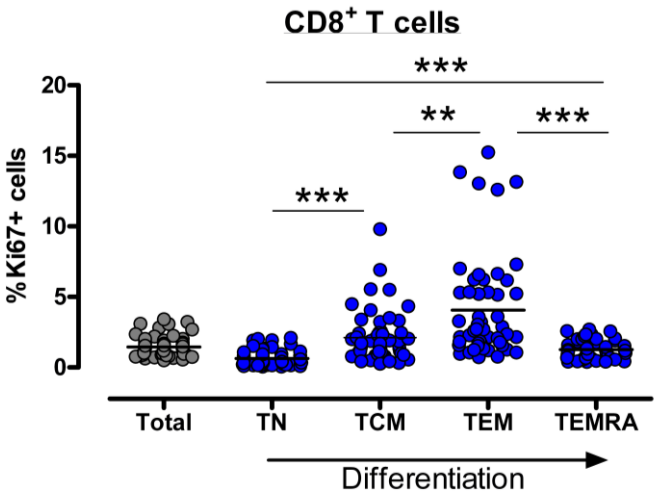


Figure 3-14. Ki-67 within total CD8$^+$ and CMVpp65-specific CD8$^+$ T cells
(A) Ki-67 positivity of total CD8$^+$ T cells by age group and CMV serostatus. Donor numbers shown: CMV-(Y)=8; CMV+(Y)=11;CMV-(O)=5;CMV+(O)=22. (B) Ki-67 positivity of total CD8$^+$ versus total CMVpp65-specific CD8$^+$ T cells within 12 of the same individuals. One dot represents one donor. *<0.05;**<0.005;***<0.0005.

(A)



(B)

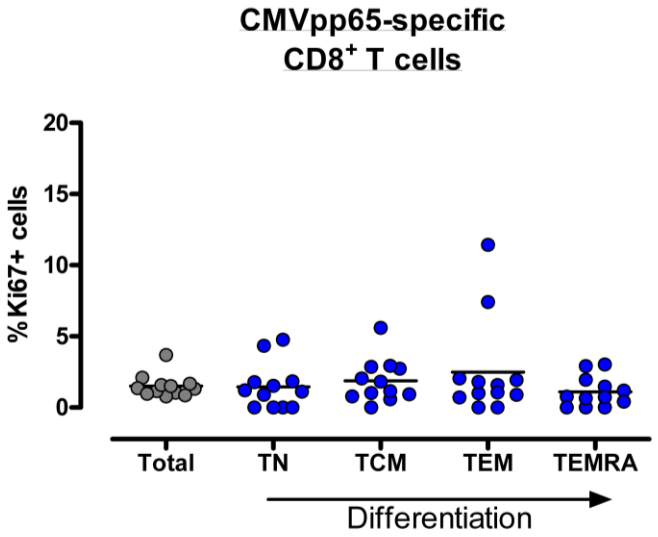


Figure 3-15. Ki-67 within CD8$^+$ and CMVpp65-specific CD8$^+$ T cell subpopulations
(A) Pooled data showing Ki-67 positivity of CD8$^+$ T cell subpopulations. 55 donors tested. (B) Pooled data showing Ki-67 positivity within each CMVpp65-specific CD8$^+$ T cell subpopulation. 12 donors tested. One dot represents one donor. *<0.05;**<0.005;***<0.0005.

3.3.5 Apoptosis resistance of $CD8^+$ and CMVpp65-specific $CD8^+$ T cells

In the last section $CD8^+$ T_{EMRA} cells showed low levels of Ki-67 protein, indicating they are not undergoing or have not recently undergone proliferation. However, this subpopulation could instead be accumulating through a resistance to apoptosis. Apoptosis is a useful way of purging the clonally expanded memory $CD8^+$ T cells following the clearance of an infection (Krammer et al., 2007), as discussed in section 1.2.4. However, the reason why CMV-specific TCEs are not deleted but instead remain a stable population is not fully understood. The next step therefore was to compare the relative susceptibility to apoptosis exhibited by each subpopulation directly *ex-vivo* to uncover if $CD8^+$ T_{EMRA} cells, in particular confer a resistance to apoptosis.

3.3.5.1 Bcl-2 expression as an indicator of susceptibility to apoptosis

Bcl-2 is an anti-apoptotic protein which has been shown to induce pro-survival properties within T cells (Adams and Cory, 1998). Abnormally high levels of Bcl-2 within cells is associated with malignancy and a resistance to apoptosis (Cotter, 2009) whereas low level Bcl-2 expression is associated with cell death and a pro-apoptotic state (Chao and Korsmeyer, 1998). There are other indicators of cell death, associated with pro-apoptotic or anti-apoptotic properties (as discussed in section 1.2.4), however the expression of Bcl-2 was chosen for analysis here as it is crucial for the survival of activated T cells (Scheel-Toellner et al., 2008) and has been used extensively elsewhere as a marker for T cell susceptibility to cell death (Aggarwal and Gupta, 1998, Doisne et

al., 2004, Libri et al., 2011, Miles et al., 2007, Salmon et al., 1994, Scheel-Toellner et al., 2008, Schluns et al., 2000, Wallace et al., 2010).

3.3.5.2 $CD8^+$ T_{EMRA} cells show little resistance to apoptosis

Intracellular staining for Bcl-2 protein was performed on donor PBMCs directly *ex-vivo.* Both the $CD8^+$ and CMVpp65-specific $CD8^+$ T cell subpopulations were analysed for the expression of Bcl-2, as shown in the representative FACs plot analysis in figure 3.15. Bcl-2 levels were analysed in total $CD8^+$ and CMVpp65-specific $CD8^+$ T cell subpopulations within 34 and 13 donors respectively (figures 3.16 & 3.17). When comparing Bcl-2 levels of the total $CD8^+$ T cell compartment in individuals on the basis of their age group and CMV serostatus (figure 3.16A) no significant differences were uncovered. However, CMVpp65-specific $CD8^+$ T cells did have significantly lower Bcl-2 levels than global $CD8^+$ T cells when compared within the same individuals ($p<0.0001$; Figures 3.16B). Bcl-2 expression was then compared in $CD8^+$ (figure 3.17A) and CMVpp65-specific $CD8^+$ (figure 3.17B) T cell subpopulations. Bcl-2 levels in $CD8^+$ T cells decreased significantly from high to low in T_N>T_{CM}>T_{EM}>T_{EMRA} subpopulations respectively. In CMVpp65-specific $CD8^+$ T cells, the T_{EM} and T_{EMRA} cells showed the lowest levels of BCl-2.

Overall, the results showed that $CD8^+$ and CMVpp65-specific $CD8^+$ T_{EMRA} cells are relatively susceptible to apoptosis compared to the other subpopulations, as based on Bcl-2 anti-apoptotic protein expression. Furthermore, CMV-specific $CD8^+$ T cells showed significantly lower Bcl-2 expression than total $CD8^+$ T cells within the same individuals.

Therefore, anti-apoptotic properties of CMV-specific $CD8^+$ T cells, in particular the T_{EMRA} cell subpopulation, are unlikely to be the cause for their persistence and expansion within the CD8 compartment.

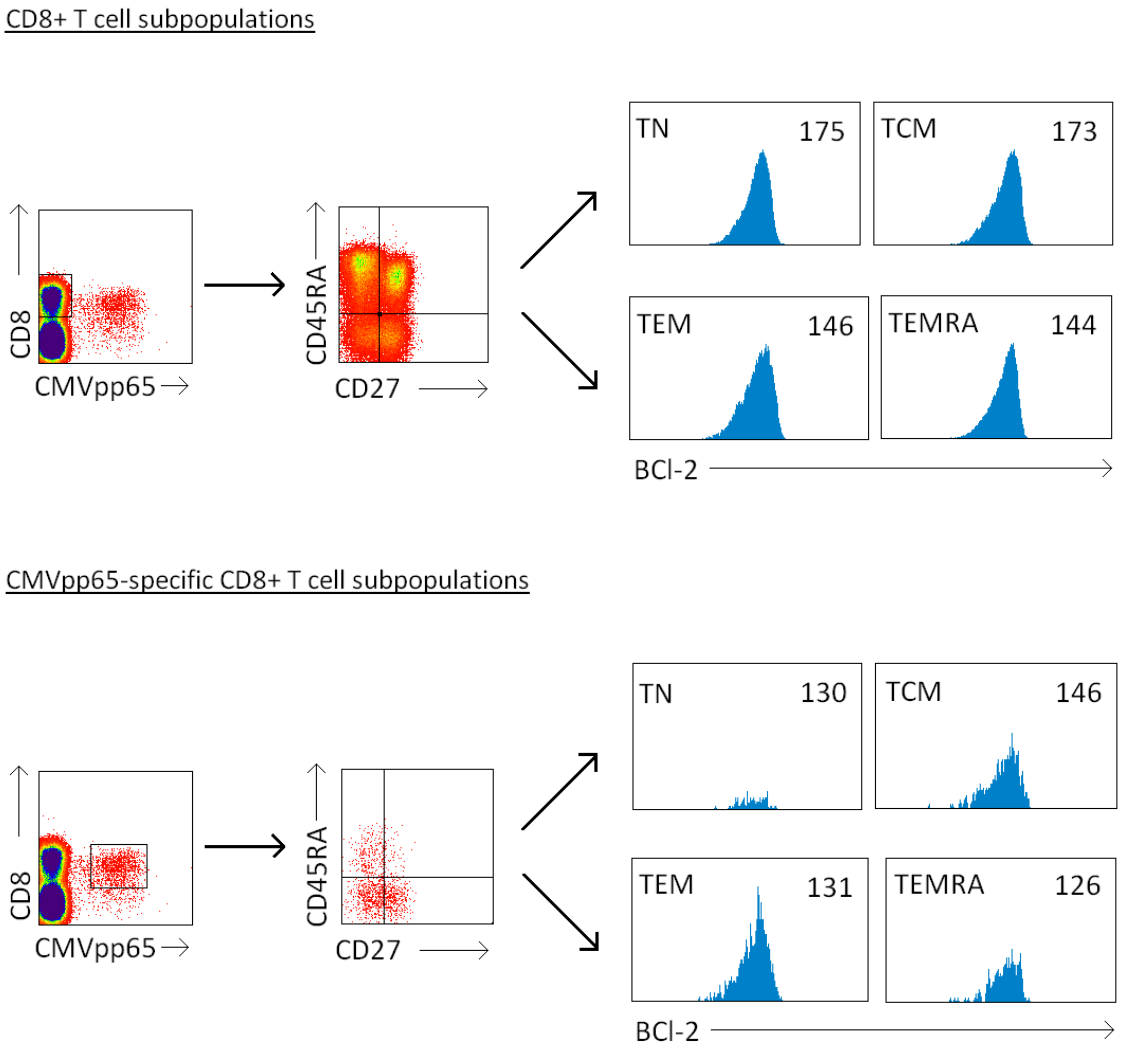
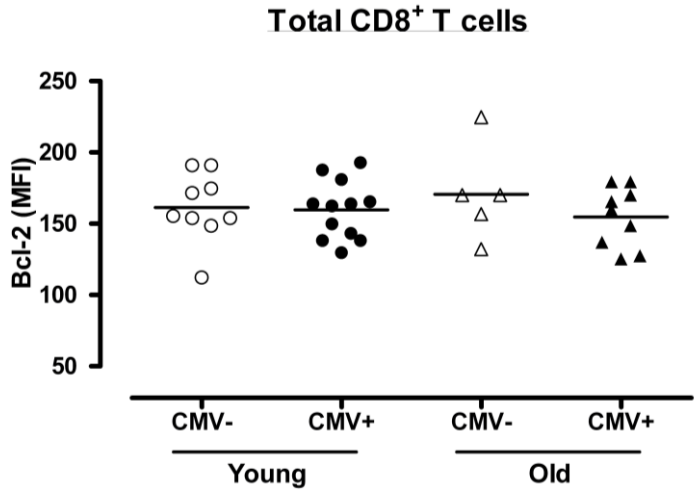


Figure 3-16. Bcl-2 staining directly *ex-vivo*
Representative FACs plot analysis of Bcl-2 staining of $CD8^+$ and CMVpp65-specific $CD8^+$ T cell subpopulations within the same individual. BCl-2 levels are recorded following the adjustment of MFI to control sample MFI levels, in order to ensure consistency between acquisitions. Control sample MFI was determined by identifying a positive population gated against an IgG1 Isotype control. Numbers represent MFI in relevant histogram. Example shown is representative of 13 donors.

(A)



(B)

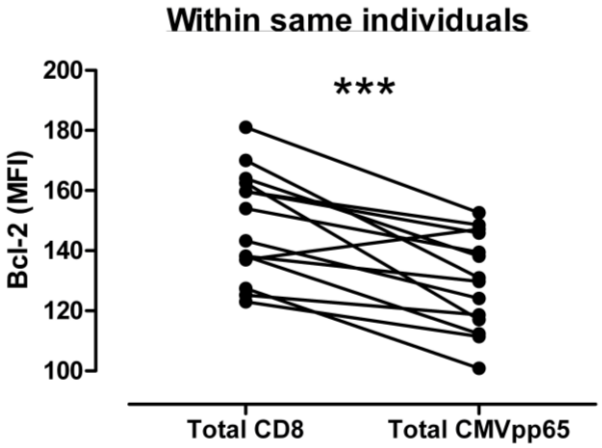
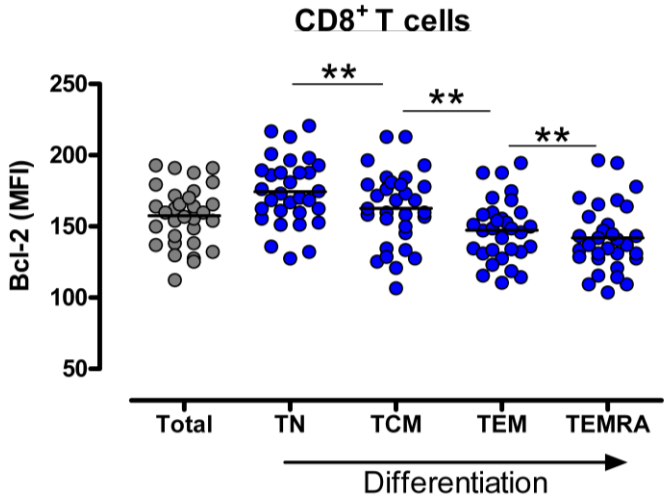


Figure 3-17. Bcl-2 levels in $CD8^+$ and CMV-specific $CD8^+$ T cells
(A) Bcl-2 levels of total $CD8^+$ T cells by age group and CMV serostatus. Donor numbers shown: CMV-(Y)=9; CMV+(Y)=12;CMV-(O)=5;CMV+(O)=9. (B) Bcl-2 levels of total $CD8^+$ versus total CMVpp65-specific $CD8^+$ T cells within 13 of the same individuals. One dot represents one donor. *<0.05;**<0.005;***<0.0005.

(A)



(B)

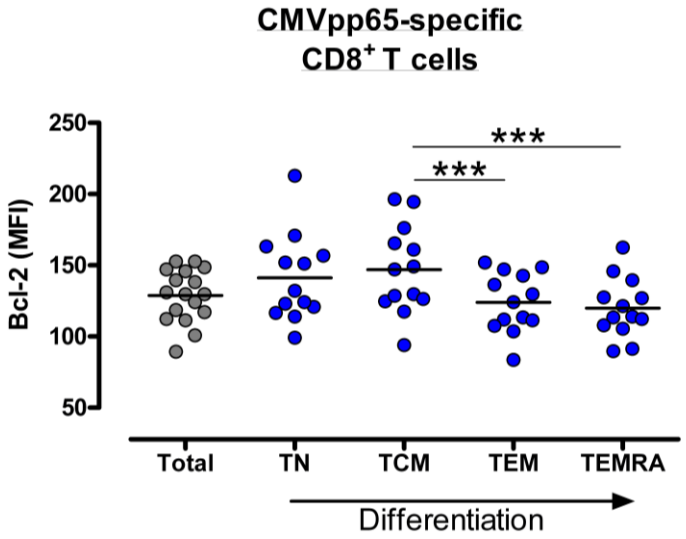


Figure 3-18. Bcl-2 expression in $CD8^+$ and $CD8^+$ CMVpp65-specific T cell subpopulations
(A) Pooled data showing Bcl-2 expression in $CD8^+$ T cell subpopulations. 34 Donors analysed. (B) Pooled data showing Bcl-2 expression in CMVpp65-specific $CD8^+$ T cell subpopulation. 13 donors analysed. One dot represents one donor. *<0.05;**<0.005;***<0.0005.

3.4 Discussion

Data presented in this chapter highlights the impact of CMV infection upon global CD8 populations within healthy individuals. CMV infection was found to exaggerate the natural age-related decrease in naive $CD8^+$ T cells and increase in $CD8^+$ T_{EMRA} cells. This has recently been confirmed elsewhere (Chidrawar et al., 2009). Most importantly, results showed that as the CMV-specific $CD8^+$ T cell pool expands with age, it becomes increasingly dominated by CMV-specific $CD8^+$ T_{EMRA} cells. Therefore the aim of this chapter, to investigate how CMV-specific TCEs appear, became focused around this accumulating CMV-specific T_{EMRA} subpopulation.

To induce and maintain such a large population of CMV-specific T cells, chronic stimulation by antigen is thought to be necessary, suggesting CMV could be reactivating continuously (van Leeuwen et al., 2002). However, Ki-67 analysis indicated CMV-specific $CD8^+$ T cells, and CMV-specific $CD8^+$ T_{EMRA} cells in particular, show low level proliferation, not an increase, as would be expected and commensurate with their accumulation. Ki-67 is indicative of current or recent proliferation by cells based on the presence of Ki-67 protein, so therefore has its limitations. Another way to investigate proliferation would be by using the Carboxyfluorescein succinimidyl ester (CFSE) assay, which directly identifies dividing cells and can be used in combination with flow cytometry (Parish, 1999). However, results presented here are consistent with observations that during active CMV infection, CMV-specific $CD8^+$ T cells exhibit high Ki-67 positivity, whereas in convalescence Ki-67 levels are found to be comparable to global $CD8^+$ T cells, despite

the CMV-specific population persisting (Miles et al., 2007). The authors also found it was during convalescence that the CMV-specific CD45RA$^+$ memory population appeared. Furthermore, others investigating CMV-specific CD8$^+$ T cell turnover during convalescence, using *in-vivo* labelling with deuterated glucose, shown CMVpp65-specific CD8$^+$ T cells have lower uptake of deuterated glucose than background CD8s (Wallace et al., 2010), indicative of reduced proliferation despite the population being observed to persist within donors over the time-points sampled. One possibility therefore is that following an initial burst of proliferation, effector CMV-specific CD8$^+$ T cells that are CD45RO$^+$ during primary infection cease dividing and undergo a switch back to the CD45RA$^+$ phenotype.

Once generated, are the CMV-specific CD8$^+$ TCEs therefore persisting through a resistance to apoptosis? The argument for the requirement of antigen to maintain memory cells is contradicted by findings that virus-specific CD8$^+$ T cells can persist indefinitely after being adoptively transferred to a virus-free host (Hou et al., 1994, Lau et al., 1994). CMV-specific TCEs and in particular CMV-specific CD8$^+$ T_{EMRA} cells may therefore persist through a resistance to apoptosis rather than induction by CMV antigen. This was tested by investigating the expression of anti-apoptotic molecule Bcl-2. There were no significant differences in Bcl-2 levels in CD8$^+$ T cells found on the basis of age and CMV in our cohort, despite reports on the contrary that Bcl-2 levels of CD8$^+$ T cells are lower in older individuals (Aggarwal and Gupta, 1998). It has been shown previously that EBV-specific CD8$^+$ T_{EMRA} cells show a resistance to apoptosis through

increased Bcl-2 expression upon resolution of infectious mononucleosis (Dunne et al., 2002). However, the relative levels of apoptosis resistance exhibited by CMV-specific $CD8^+$ T cells in this study were significantly lower compared with the global CD8 compartment. Furthermore, $CD8^+$ T_{EMRA} cells exhibited the lowest Bcl-2 levels of all CMV-specific $CD8^+$ T cell subpopulations. These findings therefore do not support an increased resistance to apoptosis as a cause for their accumulation. However, the expression of other anti-apoptotic molecules besides Bcl-2 or a decrease in expression of pro-apoptotic molecules must be investigated to rule this out completely.

One issue that arises when analyzing CD45RA/CD27 subpopulations of CMV-specific $CD8^+$ T cells is whether the $CD45RA^+CD27^+$ 'T_N' phenotype is actually a true naïve subpopulation. For example, the mean percentage of $CD45RA^+CD27^+$ T_N cells within the CMVpp65-specific compartment was found to be approximately 20% (figure 3.10B), which would be high for an un-primed naïve CMVpp65-specific T cell population. Secondly, the high Bcl-2 levels seen in total $CD45RA^+CD27^+$ $CD8^+$ T cells were not matched by $CD45RA^+CD27^+$ CMVpp65-specific $CD8^+$ T cells. Furthermore, this population has lower CD27 expression compared with total $CD45RA^+CD27^+$ $CD8^+$ T cells (not shown) in addition to low CCR7 and high LFA-1 expression (Faint et al., 2001). Taken together, this suggests $CD45RA^+CD27^+$ CMVpp65-specific $CD8^+$ T cells are a primed memory population undergoing CD45RA re-expression. To a degree, this fits with observations during primary acute CMV infections that CMV-specific $CD45RO^+$ memory $CD8^+$ T cells dominate whereas in convalescence a significant $CD45RA^+$ memory population appears

(Kuijpers et al., 2003, van de Berg et al., 2008, Wills et al., 2002). Indeed, results presented in this chapter showed CMV-specific 'T_N' cells were found to increase in numbers commensurate with T_{EMRA} cells, which may indicate these are a transitionary 'pseudo naive' CD45RA re-expressing memory population.

However, CMVpp65-specific $CD8^+$ T_{EMRA} cells were not sufficiently large enough to solely account for total $CD8^+$ T_{EMRA} cells, although the proportion of CMV-specific T_{EMRA} and T_N cells out of total $CD8^+$ T_{EMRA} and T_N cells were significantly larger in older individuals. This means the shrinking T_N population in older individuals likely contain a significant population of primed memory cells, including those specific for CMV. Furthermore, the finding that CMV seropositivity alone increases T_{EMRA} cell numbers with age but CMV-specific T_{EMRA} cells in the old still occupy a significantly higher percentage of total T_{EMRA} cells further highlights that CMV-specific T_{EMRA} cells come to dominate in older individuals. Still, $CD8^+$ T_{EMRA} cells specific for other CMV proteins and other viral proteins are likely to contribute to the global increase in the $CD8^+$ T_{EMRA} population. For example,, CMV IE-1-specific $CD8^+$ T cells can occupy up to 40% of the CD8 compartment and can show a high degree of CD45RA expression (Khan et al., 2010, Khan et al., 2002a).

In conclusion, this chapter has confirmed a relationship between CMV, ageing and the changes within the CD8 compartment. The most prominent finding is that $CD8^+$ T_{EMRA} cells accumulate with age despite showing low levels of Ki-67 and Bcl-2. This suggests that $CD8^+$ T_{EMRA} cells may instead be generated from other $CD8^+$ memory T cells which

have ceased to proliferate. This non-proliferative state can result from the exhaustion or senescence of the memory T cells that represent different pathways for T cell fate (section 1.4.8). This will therefore be explored further in the coming chapters, which will focus on how senescent $CD8^+$ T_{EMRA} cells are at the telomeric level. Furthermore, it will be investigated whether CMV positivity can impact negatively on the overall telomere length of an individual, as a result of the accumulation of these highly differentiated $CD8^+$ T cells.

4 CMV infection and telomere length attrition during ageing

4.1 Introduction

Telomeres are instrumental in determining the lifespan of cells and can trigger a state of senescence when critically short (sections 1.1.2 & 1.4.8). Telomere length is therefore indicative of how close T cells are to senescence. Indeed, the telomere length of a T cell is predictive of its replicative capacity *in-vitro* (Plunkett et al., 2005). Naïve T cells have longer telomeres than memory T cells, and older individuals have shorter telomere lengths compared to the young (Akbar et al., 2004). However, there is a large variation between individuals of the same age (Rufer et al., 1999). This could be due to a variety of factors, including inter-individual genetic differences, cumulative oxidative damage, and chronic inflammatory environments (Valdes et al., 2005, Epel et al., 2004).

The impact of antigenic burden throughout life may also be a factor that determines telomere length variation. However, this has not been extensively investigated. Repeated stimulation can eventually lead to loss of telomerase activity in highly differentiated $CD8^{+}CD27^{-}CD28^{-}$ memory T cells, which is linked to decreased Akt phosphorlyaltion (Plunkett et al., 2007, Henson et al., 2009). Chronic virus-specific $CD8^{+}$ T cells also shown progressive telomere shortening (Plunkett et al., 2001), suggesting these memory cells are limited in their capacity to be maintained through proliferation.

4.2 Aims

In the previous chapter, CMV serostatus was shown to have a significant impact on the CD8 compartment of healthy individuals, by accelerating the loss of T_N and accumulation of highly differentiated $CD8^+$ and CMV-specific $CD8^+$ T_{EMRA} cells. This may disrupt the 'immune space' by competitively squeezing out other T cells (Akbar and Fletcher, 2005, Franceschi et al., 2000, Nikolich-Zugich et al., 2004, Pawelec et al., 2005, Vasto et al., 2007). Furthermore, this may serve to simultaneously lower the overall telomere length of the T cell compartment, as a result of the accumulation of highly differentiated cells with shorter telomeres. This is important, as short telomeres have been associated with an increased risk of susceptibility to death by infection and various other conditions (as discussed in 1.1.2).

The aim of this chapter therefore, was to determine whether CMV infection and CMV-specific TCEs can impact telomere lengths in healthy individuals. Telomere lengths of lymphocyte subsets within our healthy cohort were measured to determine the significance of CMV serostatus on natural age-related telomere length attrition. The telomere lengths of $CD8^+$ and CMV-specific $CD8^+$ T_{EMRA} cells were also investigated in relation to the other subpopulations, to determine the effect their accumulation has on the overall telomere length of individuals. Furthermore, defining the telomere length of these $CD8^+$ T_{EMRA} cells may help explain why they persist within the CD8 compartment, despite having a low proliferative and anti-apoptotic phenotype.

4.3 Results

4.3.1 Measuring telomere lengths

Telomere lengths can be measured using a variety of methods (reviewed in(Baird, 2005). The technique used throughout this thesis is called flow cytometric fluorescence in-situ hybridization (flow-FISH) which was adapted in our lab from an original protocol by the Rufer group (Rufer et al., 1998, Faint et al., 2001, Reed et al., 2004). Flow-FISH uses peptide nucleic acid (PNA) probes, which specifically bind the hexameric repeats of telomeres during a hybridisation step. When these PNA probes are fluorescently labelled they therefore equate intensity of the signal with telomere length. When this procedure is coupled with flow cytometry, flow-FISH has the advantage over other techniques in its ability to analyse telomere lengths within single cell populations.

Figure 4.1 outlines the analysis of telomere length using the flow-FISH technique. Further details can be found in materials and methods (section 2.4). A standard curve was generated previously in our lab by plotting the telomere length MFI against actual telomere length, as determined by telomeric restriction fragment (TRF) analysis, for healthy individuals over a wide age range (Fletcher et al., 2005, Reed et al., 2004). Therefore, telomere MFI readings, once converted using this standard curve, equated to estimations of real telomere lengths.

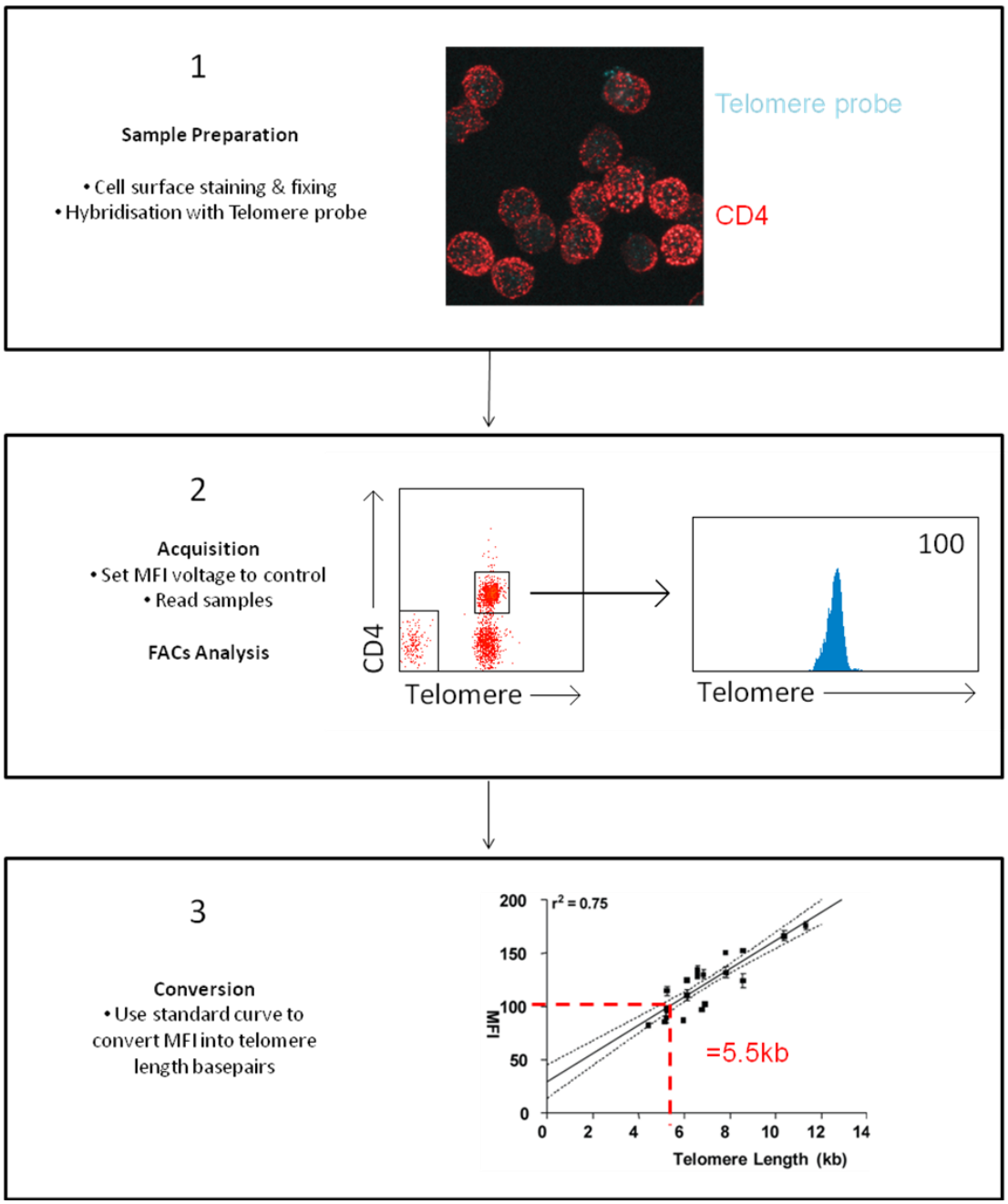


Figure 4-1. The Flow-FISH procedure

The flow-FISH procedure is shown for $CD4^+$ T cell telomere length analysis as an example. The process is broken down into 3 steps: 1. sample preparation - isolated PBMCs are cell surface stained with CD4 then fixed with BS3. They are then hybridised with the telomere probe using a heat step process. Confocal microscopy image shows $CD4^+$ T cells (red) hybridised with a quantitative telomere PNA probe (blue), the intensity of which is equal to telomere length; 2. Acquisition and analysis – first the telomere MFI is adjusted to the control i.e. sample with known telomere length MFI. Once properly adjusted and compensated the other samples are acquired in triplicate. The mean MFI of the triplicates is calculated i.e. 100 following FACs analysis. 3. Conversion - finally the mean MFI values are converted using a standard curve to estimate real telomere length, in this case MFI of 100 equates to 5.5kb telomere length.

4.3.2 CMV accelerates age-associated telomere attrition in T cells

As discussed in section 1.6, evidence suggests it is the CD8 compartment that is most affected by CMV infection largely due to the CMV-specific $CD8^+$ TCEs it induces. The first step therefore was to investigate the natural age-related attrition of telomeres within $CD8^+$ T cells compared with the other lymphocyte subsets and to analyse any significant impact CMV may have on the rate of telomere attrition. Flow-FISH was therefore conducted on healthy donor PBMCs on the four cell subsets: total lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, and the remaining $CD4^-CD8^-$ lymphocytes (predominately B cells).

4.3.2.1 Telomere lengths in total lymphocytes

The pooled data for telomere lengths in total lymphocytes is shown in figure 4.2. In total 110 donors were analysed, 47% of which were CMV positive. The rate of total lymphocyte telomere attrition was found to be significantly correlated with age in both CMV positive ($p<0.0001$, $r^2=0.615$) and CMV negative individuals ($p<0.0001$, $r^2=0.416$) as depicted in figure 4.2A. However, the base pair per year (bpy) loss in CMV positive individuals was greater, equating to 61_{bpy} compared with CMV negative individuals at 46_{bpy}. When data is further separated into old or young age groups and on the basis of CMV positivity (figure 4.2B); significantly shorter telomeres were found in CMV positive old individuals versus CMV negative old individuals ($p=0.005$). However, there was no significant difference between young CMV positive versus young CMV negative individuals.

4.3.2.2 Telomere lengths in $CD4^-CD8^-$ lymphocytes

The pooled data for telomere lengths of $CD4^-CD8^-$ lymphocytes is shown in figure 4.3. In total 58 donors were analysed, of which 62% were CMV positive. The rate of $CD4^-CD8^-$ telomere attrition was found to be significantly correlated with age in both CMV positive ($p=0.0002$, $r^2=0.505$) and CMV negative individuals ($p=0.0015$, $r^2=0.260$) as depicted in figure 4.3A. The base pair per year loss in CMV positive and CMV negative individuals was similar however at 46_{bpy} and 43_{bpy} respectively. When data is further separated into old or young age groups and on the basis of CMV positivity (figure 4.3B) no significant association was uncovered.

4.3.2.3 Telomere lengths in $CD4^+$T cells

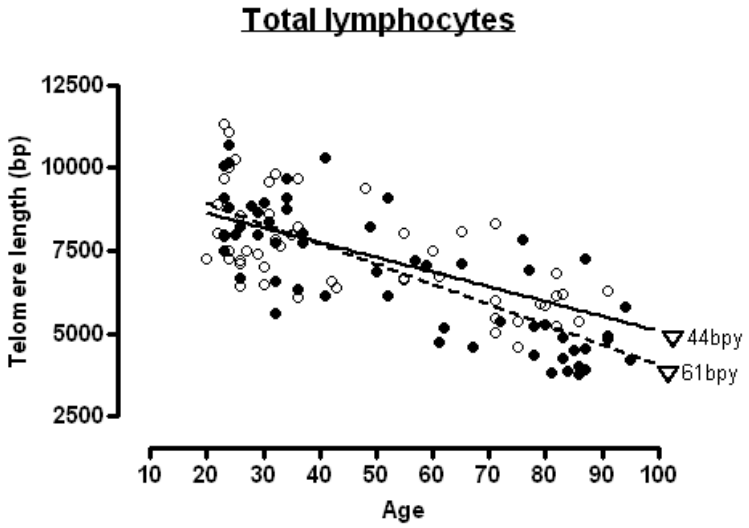
The pooled data for telomere lengths of $CD4^+$ T cells is shown in figure 4.4. In total 110 donors were analysed, 47% of which were CMV positive. The rate of $CD4^+$ T cell telomere attrition was found to be significantly correlated with age in both CMV positive ($p<0.0001$, $r^2=0.582$) and CMV negative individuals ($p<0.0001$, $r^2=0.410$) as shown in figure 4.4A. The base pair per year loss in CMV positive individuals was greater, equating to 63_{bpy} versus 48_{bpy} in CMV negative individuals. Furthermore, when divided by age group and CMV positivity (figure 4.4B) $CD4^+$ T cell telomeres were significantly shorter in CMV positive old versus CMV negative old individuals ($p=0.011$). There was no significant difference in telomere lengths between young CMV positive versus young CMV negative individuals

4.3.2.4 Telomere lengths in $CD8^+$T cells

The pooled data for telomere lengths of $CD8^+$ T cells is shown in figure 4.5. In total 69 donors were analysed, of which 57% were CMV positive. Telomere attrition was found to be most pronounced in $CD8^+$ T cells relative to the other subsets analysed. Base pair loss was significantly correlated with age among the populations studied with both CMV positive ($p<0.0001$, $r^2=0.784$) and CMV negative individuals ($p<0.0001$, $r^2=0.587$) showing significant attrition with age (figure 4.5A). However, the base pair per year loss in CMV positive individuals was the most marked of all populations, equating to 94_{bpy} versus 77_{bpy} in CMV negative individuals. When the data was further divided into old or young categories and on the basis of CMV positivity (figure 4.5B) $CD8^+$ T cells exhibit significantly shorter telomeres in CMV positive old versus CMV negative old ($p=0.0004$).

Overall, results demonstrated the most significant telomere attrition was seen in the $CD8^+$ T cell compartment. Furthermore, CMV positive individuals were associated with an accelerated rate of telomere shortening. The large CMV-specific TCEs which occupy the CD8 compartment in CMV seropositive individuals may lower the overall telomere length of the CD8 compartment as a whole, thus providing an explanation for this result. This could also account for the lack of significance in telomere length in the young age group on the basis of CMV positivity, as CMV-specific TCEs are smaller in CMV positive young versus CMV positive old individuals. Therefore, to investigate the impact of CMV on telomere lengths further it was necessary to look at telomere lengths within $CD8^+$ and CMV-specific $CD8^+$ T cell subpopulations.

(A)



(B)

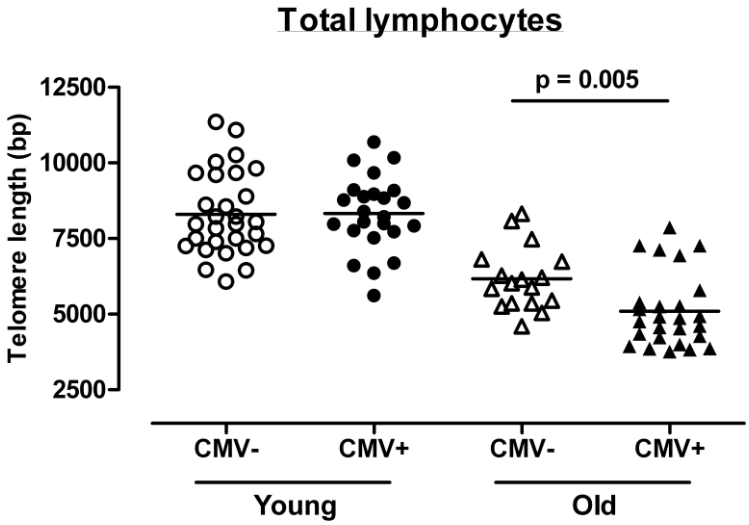
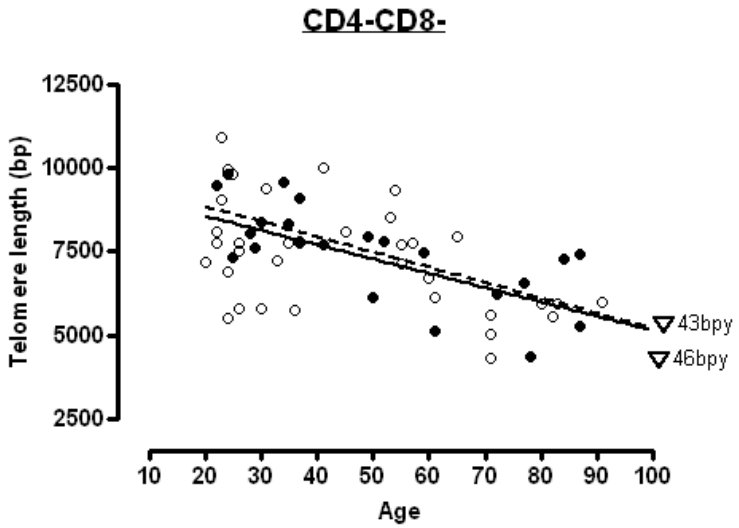


Figure 4-2. The impact of age and CMV serostatus on telomere lengths in total lymphocytes
(A) Telomere lengths of total lymphocytes in 110 donors stratified by age and CMV status: open circles represent CMV negative individuals with the correlation shown by a solid line; closed circles represent CMV positive individuals with the correlation shown by a dashed line. The gradient is representative of base pair loss per year (bpy). (B) Donor telomere lengths stratified according to CMV status and age group; young (<35) and old (>65). Donor numbers: CMV-(Y)=29; CMV+(Y)=24; CMV-(O)=17; CMV+(O)=26. Significant differences only displayed by p values.

(A)



(B)

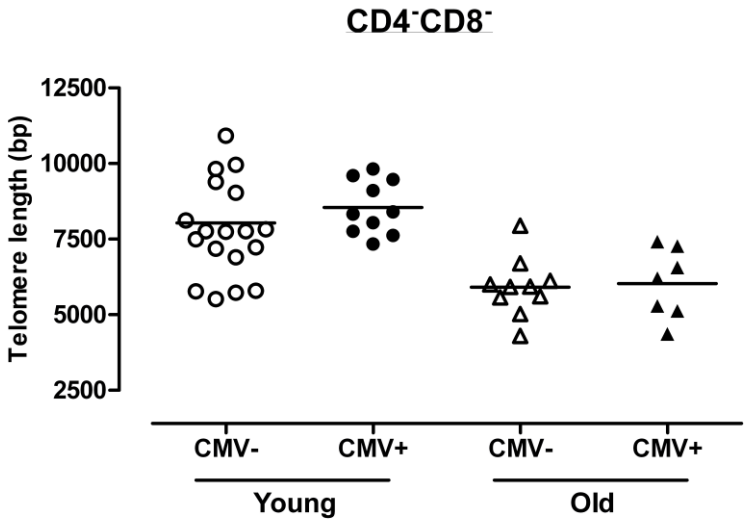
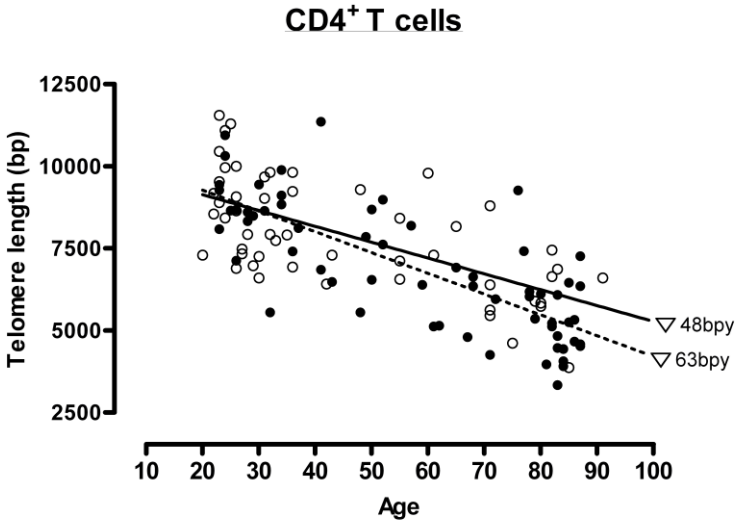


Figure 4-3. The impact of age and CMV serostatus on telomere lengths in CD4$^-$CD8$^-$ lymphocytes
(A) Telomere lengths of CD4$^-$CD8$^-$ lymphocytes in 58 donors stratified by age and CMV status: open circles represent CMV negative individuals with the correlation shown by a solid line; closed circles represent CMV positive individuals with the correlation shown by a dashed line. The gradient is representative of base pair loss per year (bpy). (B) Donor telomere lengths stratified according to CMV status and age group; young (<35) and old (>65). Donor numbers: CMV-(Y)=19; CMV+(Y)=10; CMV-(O)=10; CMV+(O)=7. Significant differences only displayed by p values.

(A)



(B)

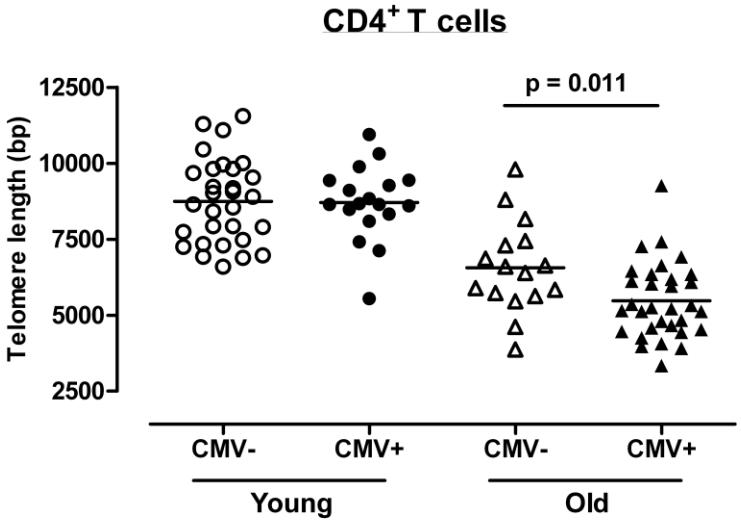
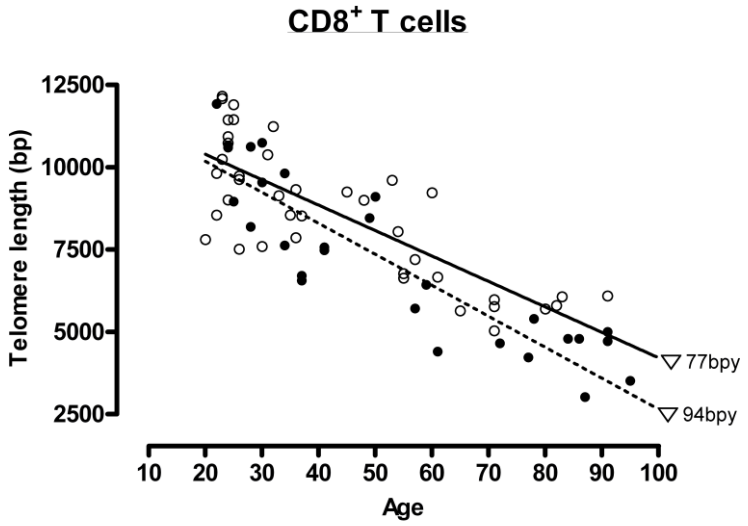


Figure 4-4. The impact of age and CMV serostatus on telomere lengths in CD4$^+$ T cells
(A) Telomere lengths of CD4$^+$ T cells in 110 donors stratified by age and CMV status: open circles represent CMV negative individuals with the correlation shown by a solid line; closed circles represent CMV positive individuals with the correlation shown by a dashed line. The gradient is representative of base pair loss per year (bpy). (B) Donor telomere lengths stratified according to CMV status and age group; young (<35) and old (>65). Donor numbers: CMV-(Y)=30; CMV+(Y)=18; CMV-(O)=16; CMV+(O)=32. Significant differences only displayed by p values.

(A)



(B)

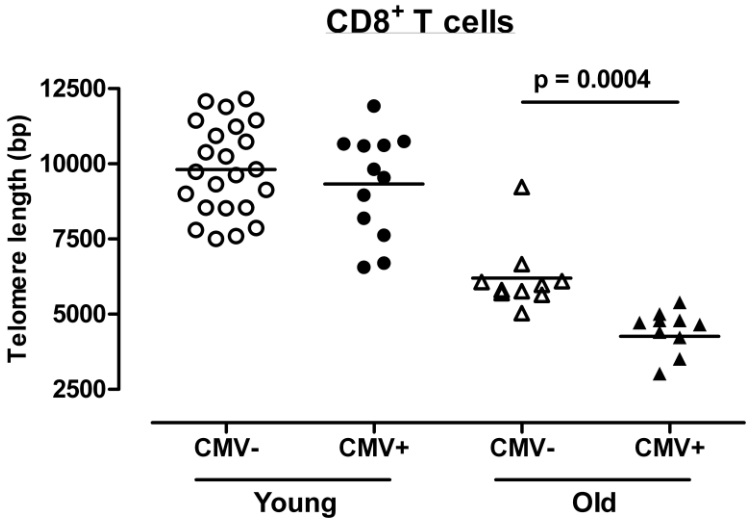


Figure 4-5. The impact of age and CMV serostatus on telomere lengths in CD4$^+$ T cells
(A) Telomere lengths of CD8+ T cells in 69 donors stratified by age and CMV status: open circles represent CMV negative individuals with the correlation shown by a solid line; closed circles represent CMV positive individuals with the correlation shown by a dashed line. The gradient is representative of base pair loss per year (bpy). (B) Donor telomere lengths stratified according to CMV status and age group; young (<35) and old (>65). Donor numbers: CMV-(Y)=23; CMV+(Y)=12; CMV-(O)=10; CMV+(O)=11. Significant differences only displayed by p values.

4.3.3 Using Quantum dot technology in the Flow-FISH assay

Investigating telomere lengths in $CD8^+$ T cell subpopulations and CMV-specific $CD8^+$ T cell populations can be difficult using flow-FISH as the range of flurochromes that can be used to define populations are limited due to the denaturing effect of the telomere probe hybridization step (requiring a temperature of 82°C for 10mins). To overcome this problem it is often necessary to sort populations beforehand, requiring more blood, obtained fresh on the day, followed by the expensive and time consuming step of cell sorting. However, if more heat stable flurochromes are available it should be possible to expand the panel for use in flow-FISH and obviate the need for cell sorting and larger numbers of cells being required for analysis. To address this, I investigated the availability of more robust (heat-stable) antibody labels. Quantum dot (Qdot) labelled antibodies (from Invitrogen) have a novel design giving them improved structural stability over original flurochromes. I subsequently chose to test these in the flow-FISH assay.

4.3.3.1 Qdots show superior heat stability during flow-FISH

The first step was to test a selection of Qdots against original fluorochromes for thermal stability during the hybridisation step. Fluorescence intensities were recorded before and after treatment of the fluorochromes FITC, PE, PerCP, APC and Cy3 compared with those of Qdots 605, 705 and 800 after treatment as depicted in the histograms of figure 4.6. Qdots were found to withstand the heat treatment very well compared with the traditional fluorochromes, sustaining original pre-treatment fluorescence levels.

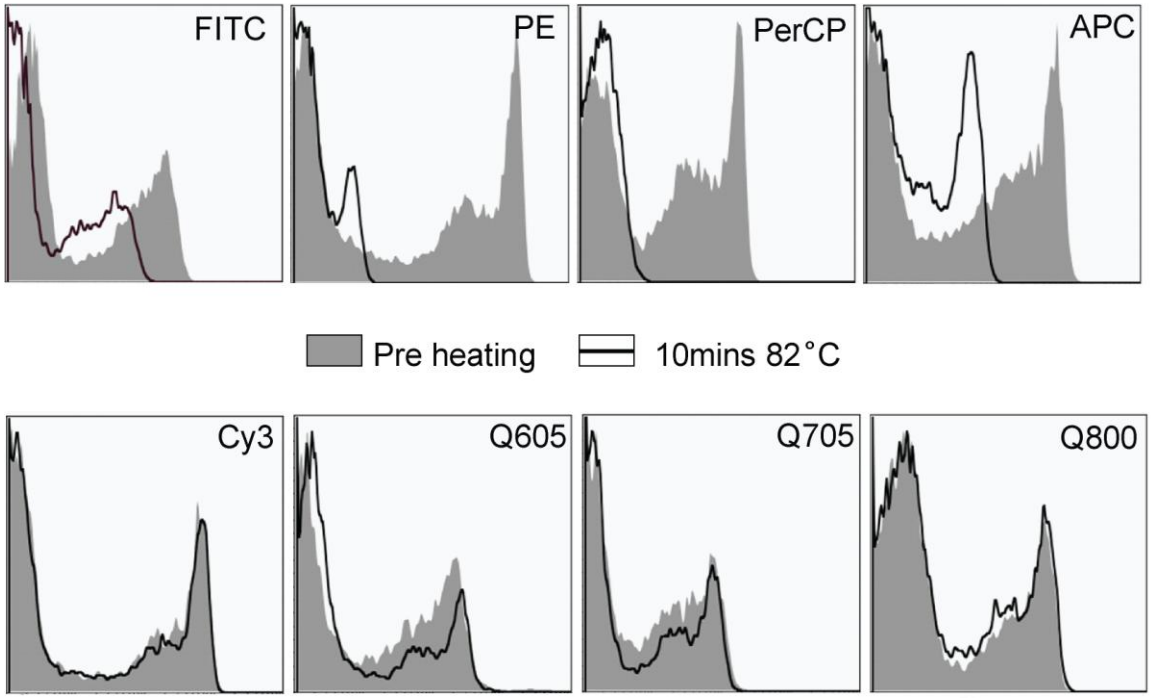
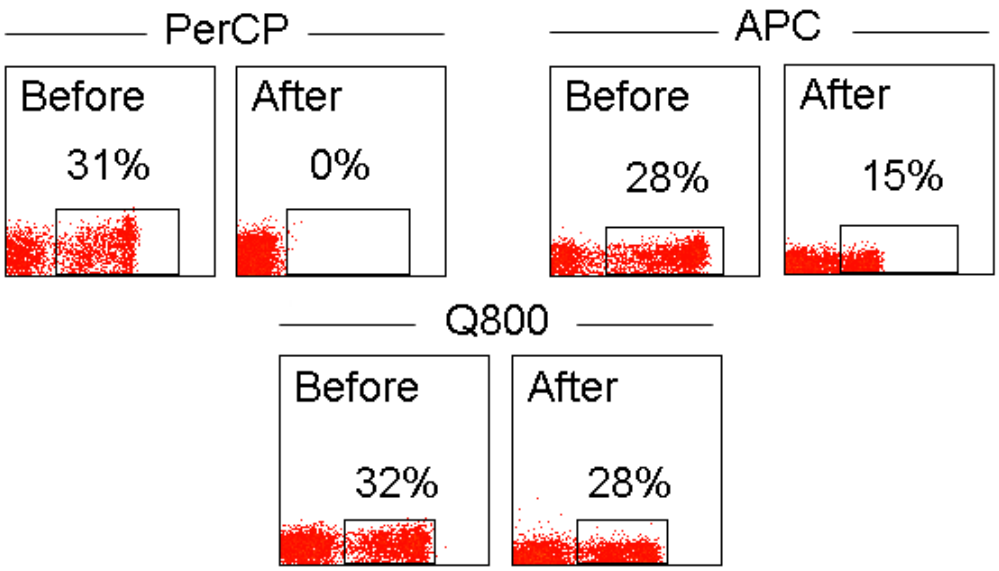


Figure 4-6. Histograms depicting original fluorochromes versus Qdots
Comparative analysis of flurochromes FITC, PE, PerCP, APC and Cy3 versus Qdots Q605, 705 and 800 pre-heating (shaded histogram) and after hybridisation step (open histogram). Representative example of 3 experiments.

Qdots could retain flurorescence after the heat hybridisation step, however, it is not just a drop in fluorescence intensity that may result from heat-treatment during the hybridisation step, but also a loss in percentage of identifiable population. To illustrate this point, we need to look at the identification of positively stained populations during FACs plot analysis (figure 4.7A). Here, it is shown that some fluorochomes, for example PerCP, denature significantly during hybridisation and result in no PerCP positive population being identifiable after treatment. Other fluorochromes, however, for example APC, partially survive the heat hybridisation step but this results in a considerable portion of the population identified by APC being lost upon analysis i.e. 15% vs. 28% as illustrated in the FACs plots example. This can be particularly problematic in the analysis of telomere lengths in small cell numbers e.g. virus-specific $CD8^+$ T cell populations. No such loss of population occurs when using Qdots however, as illustrated by identification with Q800 in the example.

The percentage of population retained was therefore calculated in this way for each fluorochome versus Qdot label before and after heat treatment and data consolidated and summarised in the bar chart of figure 4.7B. Here, the mean of the three experiments as a percentage of the original MFI retained (relative to 100% pre-treatment) is compared in the panel. As can be seen, Qdots show superior heat stability which results in the original population identified pre-treatment still being retained post-treatment.

(A)



(B)

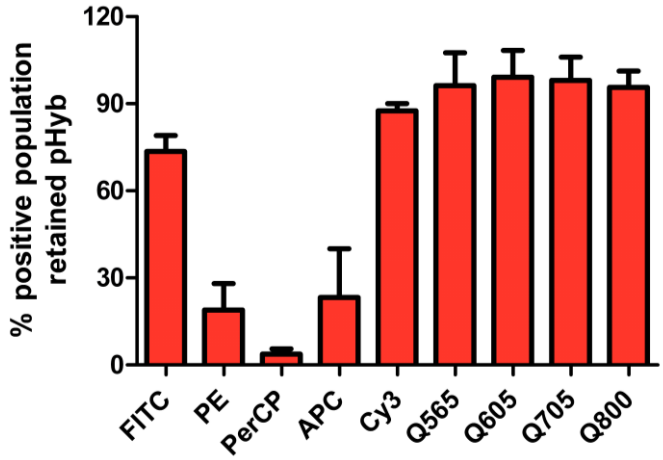


Figure 4-7. The use of Quantum dot technology in the flow-FISH protocol
(A) Comparison of FACs plot analysis used to determine positive populations using PerCP, APC or Qdot 800. (B) Bar charts represent 3 individual experiments showing mean and SD of percentage of original signal retained.

Qdots were shown to have superior signal retention over the original flurochromes, making them an ideal addition to the flow-FISH assay. This meant the original fluorochrome panel could be expanded from three colours to five or more colours (based on a BD LSRI) as summarised in table 4.1:

Original Flow-FISH Panel	New Flow-FISH Panel
FITC	Q565
Cy3	FITC
Cy5	Q605
	Q800
	Cy5

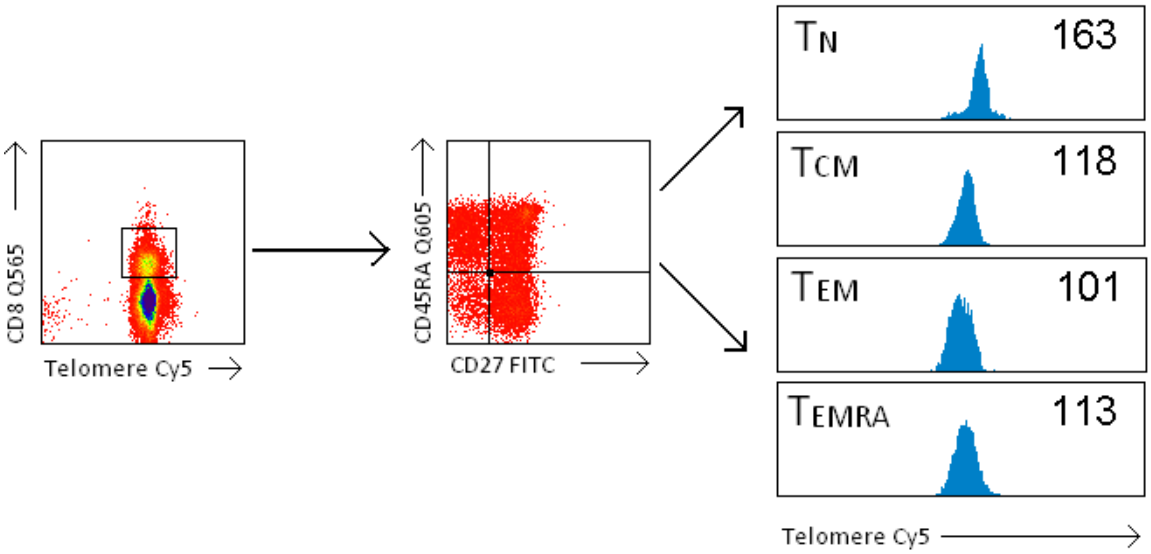
Table 4-1. Original vs. New Flow-FISH set-up on the BD LSR I

4.3.4 Telomere lengths within $CD8^+$ T cell subpopulations

4.3.4.1 Telomere lengths within $CD8^+$ T cell subpopulations

In section 4.3.2, it was shown that $CD8^+$ T cells were most affected by age associated telomere loss, and CMV positivity increased this rate of attrition. Since CMV may cause the accumulation of late differentiated $CD8^+$ T cells which have shorter telomeres, the expanded flow-FISH protocol was used to analyse telomere lengths within $CD8^+$ T cell subpopulations and stratify according to donor age and CMV serostatus. Previously fresh blood was needed and sorted prior to telomere analysis of populations requiring more than 2 colour stain, however, using Qdots in the flow-FISH protocol it was now possible to use frozen PBMCs for direct determination of telomere length analysis. It total, 26 frozen donor PBMCs were analysed in this way as shown in figure 4.8.

(A)



(B)

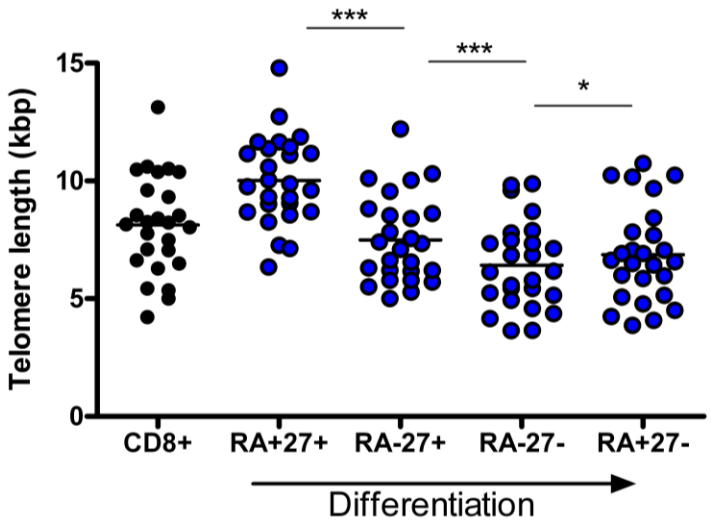


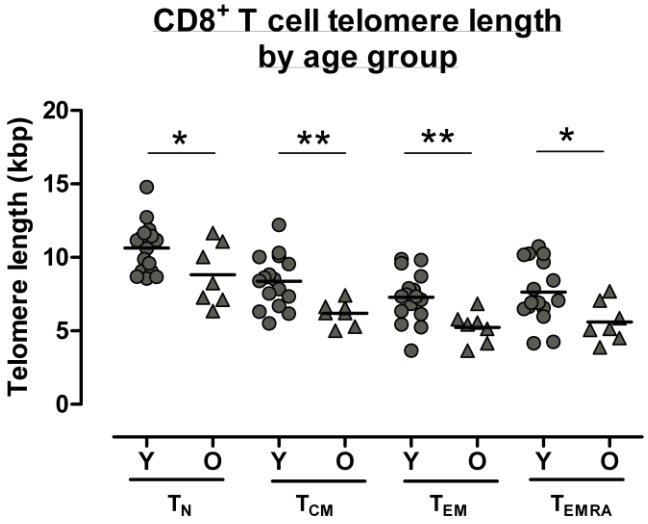
Figure 4-8. Using Qdots to investigate telomere lengths within CD8$^+$ T cell subpopulations
(A) Representative FACs plot analysis showing 4-colour flow-FISH using two Qdots (565, 605) and two regular flurochromes (Cy5 and FITC) in combination. (B) Pooled data of CD8$^+$ T cell subpopulation telomere lengths. 26 donors shown. Significant differences displayed by * values. *<0.05;**<0.005;***<0.0005.

Figure 4.8A shows a representative example of the implemented of Qdots CD8-Q565 and CD45RA-Q605 in the flow-FISH protocol to analyse telomere lengths in $CD8^+$ T cell subpopulations. Figure 4.8B shows total $CD8^+$ T cell subpopulation telomere lengths in 26 donors. Telomere lengths were observed to shorten significantly, traversing from $CD8^+$ T_N to T_{CM} ($p<0.0001$) then from T_{CM} to T_{EM} ($p<0.0001$) but then increase in length from T_{EM} to $CD8^+$ T_{EMRA} cells ($p=0.018$). This implied that $CD8^+$ T_{EMRA} cells are not the most prone to telomere-dependent senescence of the memory subpopulations.

4.3.4.2 Impact of CMV on $CD8^+$ T cell subpopulation telomere lengths

Data was further stratified on the basis of age or CMV serostatus (Figure 4.9). When telomere lengths are stratified according to age (figure 4.9A) those in the older age group had significantly shorter telomeres in each subpopulation compared with the young (T_N Y vs. O $p=0.040$; T_{CM} Y vs. O $p=0.006$; T_{EM} Y vs. O $p=0.008$; T_{EMRA} Y vs. O $p=0.028$;). When telomere lengths are stratified according to CMV serostatus (figure 4.9B) although telomere lengths are shorter in CMV positive individuals, only the $CD8^+$ T_{EMRA} subpopulation is significantly shorter in CMV positive compared with CMV negative individuals ($p=0.011$). Results show the compounding effect of age and CMV on $CD8^+$ T cell subpopulation telomere lengths. This indicates the accumulation of highly differentiated $CD8^+$ T cells may contribute to the overall lowering of telomere length in total $CD8^+$ T cells, but also shows that within these subpopulations there is also significant shortening by age.

(A)



(B)

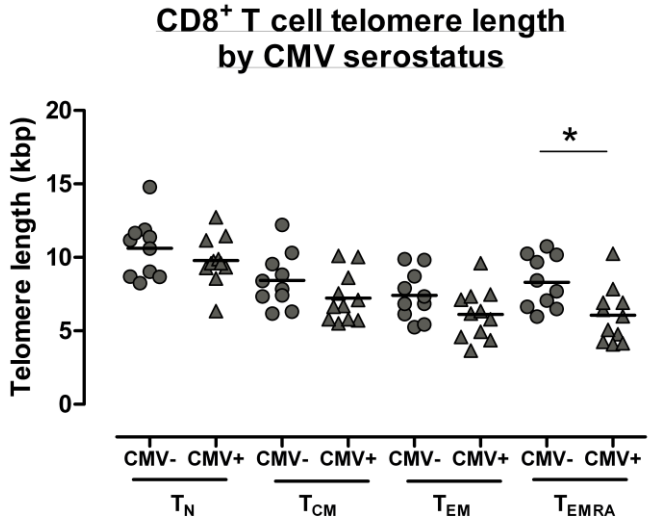


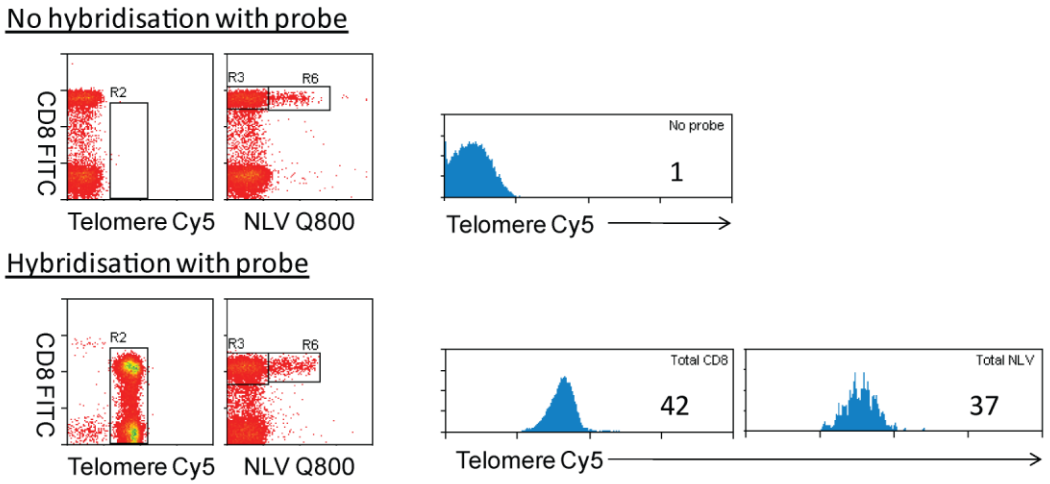
Figure 4-9. Telomere lengths of CD8$^+$ T cell subpopulations grouped by age and CMV status
(A) Pooled data showing CD8$^+$ T cell subpopulation telomere lengths by age group. 16 young and 7 old donors shown. (B) Pooled data showing CD8$^+$ T cell subpopulation telomere lengths by CMV status. 10 CMV negative and 11 CMV positive shown. Significant differences displayed by * values. *<0.05;**<0.005;***<0.0005.

4.3.5 Telomere lengths within CMV-specific $CD8^+$ T cells

4.3.5.1 Telomere lengths within CMV-specific $CD8^+$ T cells

With such large CMV-specific TCEs appearing with ageing, the telomere lengths of this population need to be investigated. Previously it has been problematic to determine telomere lengths of virus-specific $CD8^+$ T cells using tetramers in flow-FISH, as the fluorochromes with which tetramers are engineered can be denatured during the heat hybridisation step. Sorting CMV-specific populations is theoretically possible, but difficult, as the populations can be small, especially in younger donors. To overcome this problem, Qdot streptavidin conjugates were tested in combination with unlabelled biotinylated pentamer (supplied by Proimmune) in the flow-FISH assay. Pentamers were chosen over of tetramers purely for practical reasons: pentamers were available fully formed and unlabelled, whereas tetramers were tagged with PE as part of their tetramerisation step with streptavidin-PE and therefore Qdot streptavidin conjugates could not be used. A representative example of how Qdots and pentamers were used to identify CMV-specific $CD8^+$ T cell telomere lengths is shown in figure 4.10A. FACs plot analysis illustrates staining before (top) and after (bottom) the telomere probe heat hybridisation step. The CMV-specific population of 0.96% of $CD8^+$ T cells was successfully retained following the hybridisation step (0.9%) and telomere lengths successfully determined, as depicted by the relevant histograms. This was extended to a 5 colour flow-FISH assay using CD8-Q565, Pentamer (NLV) – Q800, CD45RA-Q605 and CD45RO-APC and telomere probe-Cy5 to determine telomere lengths in total CD8, total CMV-specific and $CD45RA^+$ and $CD45RO^+$ CMV-specific populations (figure 4.10B).

(A)



(B)

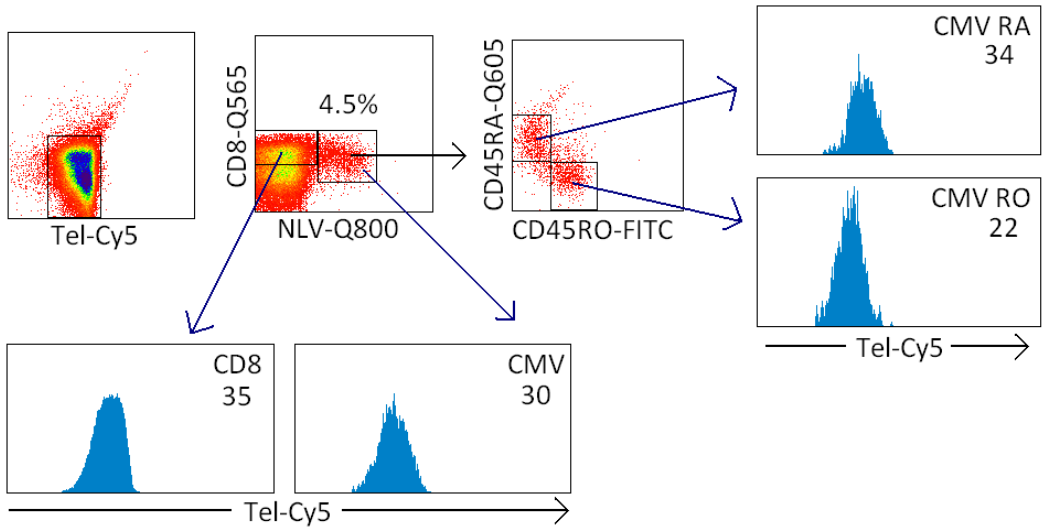


Figure 4-10. Determining telomere lengths of CMV-specific CD8$^+$ T cell subpopulations
(A) Stability of tetramer staining before and after heat hybridisation step of the flow-FISH protocol as illustrated by FACs plot analysis. (B) The implementation of Qdots to perform 5 colour flow-FISH to determine telomere lengths within CMV-specific CD45RA and CD45RO memory populations. Values within histograms represent telomere length MFI of the relevant population.

Two subpopulations, CD45RA and CD45RO, as opposed to the four CD45RA/CD27 subpopulations, were investigated to maximise the total number of CD45RA re-expressing memory CMV-specific $CD8^+$ T cells. This was predominately due to young individuals in particular often showing smaller CMV-specific TCEs. Furthermore, as the CMV-specific compartment was shown to become increasingly dominated by $CD8^+$ T_{EMRA} and 'T_N' cells during ageing, the aim was to measure telomere lengths in all CD45RA re-expressing memory versus all the CD45RO expressing memory CMVpp65-specific $CD8^+$ T cells. This was to determine if CD45RA re-expression on $CD8^+$ CMV-specific T cells was accompanied by shorter telomeres, commensurate with their accumulation.

Figure 4.11 shows an analysis conducted on 6 young and 6 old HLA matched individuals. Significant longer telomere lengths were found in the CD45RA versus CD45RO CMVpp65-specific memory $CD8^+$ T cells in both young and old age groups (both p=0.03). Furthermore, the telomere lengths in total CMVpp65-specific $CD8^+$ T cell populations were significantly shorter in older subjects than in younger individuals (p=0.008). Results show that both $CD8^+$ T_{EMRA} cells and CMV-specific $CD8^+$ $CD45RA^+$ T cells do not have the shortest telomeres of all memory subpopulations. This also indicates they are not the most prone to telomere-dependent senescence and may attribute to their persistence, potentially being maintained in a telomere-independent manner. However, $CD8^+$ T_{EMRA} cell telomeres are still relatively short and likely to contribute to the impact of CMV on shortening telomeres through their accumulation within the CD8 compartment of CMV positive individuals.

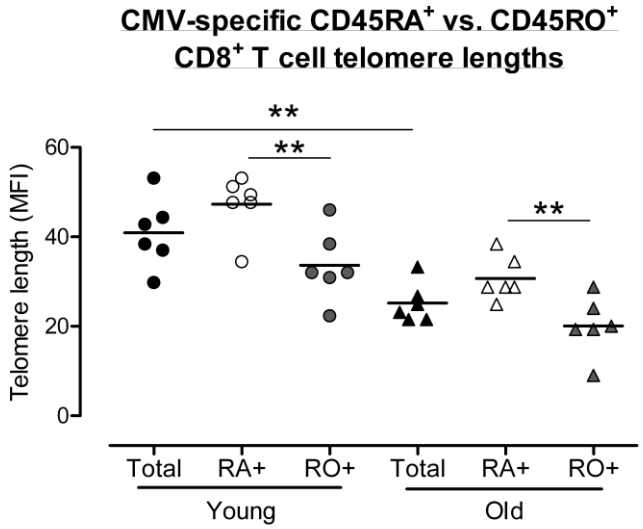


Figure 4-11. Telomere lengths of $CD8^+$ CMVpp65-specific CD45RA and CD45RO memory subpopulations
Total CMVpp65-specific and CMVpp65-specific CD45RA or CD45RO memory telomere lengths within young or old age groups. 6 young and 6 old donors shown. Significant differences displayed by *values. *<0.05;**<0.005;***<0.0005.

4.4 Discussion

Mounting evidence highlights the detrimental association between relatively short telomeres and disease, as discussed in the introduction of this chapter. However, the effect of antigenic burden upon telomere lengths during healthy ageing is less well documented. Such a study has been presented in this chapter, concluding that CMV can significantly impact telomere lengths in healthy individuals.

The results showed age-related telomere attrition to be most prominent in T cells, in particular $CD8^+$ T cells, but not $CD4^-CD8^-$ (mainly B cell) subsets. Furthermore, CMV positivity significantly affected the rate of telomere shortening, by increasing the average rate of base pair attrition per year and being associated with shorter telomere lengths in CMV positive old versus CMV negative old individuals. These findings fit to some degree with a recent study of patients with coronary heart disease (Spyridopoulos et al., 2009) which showed that CMV positive patients had significantly shorter $CD8^+$ T cell telomeres than CMV negative patients, while no other cell type was affected by CMV status. But the present study serves to highlight how healthy individuals may also be affected by common persistent viral infections such as CMV.

Indeed, the fact that CMV can lower an individual's telomere length may negatively impact their predisposition to death by infection and a variety of diseases (Samani et al., 2001, Calado and Young, 2009, Brouilette et al., 2007, Brouilette et al., 2003, Cawthon et al., 2003). The loss of virus-specific T cells through telomere-dependent senescence

could also compromise the individual's immunity to that virus. This is further highlighted by low telomerase activity, the enzyme which adds back telomere repeats, being found in T cells specific for CMV and other chronic viral infections such as HIV, HCV and HBV (Bestilny et al., 2000, Fletcher et al., 2005, Satra et al., 2005). This suggests a common mechanism whereby persistent antigenic stimulation results in increased T cell turnover and telomere shortening within virus-specific T cells.

An increase in $CD8^+$ T_{EMRA} cells with age and CMV was shown in the previous chapter; however results show these cells did not show the shortest telomere lengths of the memory subpopulations. This was especially prominent in the CMV-specific $CD8^+$ compartment, wherein the $CD45RA^+$ memory population had significantly longer telomeres than the $CD45RO^+$ memory subpopulation. Indeed, this fits with similar observations seen with EBV-specific CD45RA re-expressing memory $CD8^+$ T cells, which were shown to have longer telomeres than their $CD45RO^+$ counterparts (Dunne et al., 2002). This suggests that the effect of CMV infection in accelerating telomere attrition and reducing overall CD8 T cell telomere length may largely be due to the $CD45RO^+$ subpopulations. However, it must be noted that all memory populations have shorter telomere lengths compared with naive so any increase in memory $CD8^+$ T cells at the cost of naive $CD8^+$ T cells will serve to shorten the overall telomere length. We have since published this in collaboration with another group, showing highly differentiated T cells correlate with shorter telomeres (van de Berg et al., 2010). This is further highlighted by the fact that despite T_{EMRA} cells accumulating in the old CMV-specific

compartment, the CMV-specific compartment overall showed significantly shorter telomere lengths than in the young CMV-specific compartment, suggesting this population is driven closer to senescence during ageing.

The next chapter will focus on how T_{EMRA} cells may be generated *in-vivo*. To address this, the proliferative potential and telomerase activity of the $CD8^+$ T cells subpopulations will be compared *in-vitro*. Furthermore, whether T_{EMRA} cells can be maintained homeostatically by cytokine will also be investigated.

5 The generation of CMV-specific $CD8^+$ T cell expansions during ageing

5.1 Introduction

In the present study $CD8^+$ T_{EMRA} cells have been shown to accumulate during ageing and are present at a significantly higher frequency in CMV positive individuals. This is despite $CD8^+$ T_{EMRA} cells being highly differentiated and showing signs of low turnover and high susceptibility to apoptosis when analysed directly *ex-vivo* which suggests they may be close to senescence. However, it has also been shown that $CD8^+$ T_{EMRA} cells have relatively long telomeres for a memory $CD8^+$ T cell subpopulation, indicating they have residual capacity for replication which may explain their persistence and accumulation within the $CD8^+$ T cell compartment. It is therefore possible that $CD8^+$ T_{EMRA} cells may be close to senescence through telomere-independent pathways. Indeed, cellular senescence can occur by telomere-independent mechanisms as the result of DNA damage caused by reactive oxygen species, ionizing radiation, chromatin perturbation and activation of p53 and stress pathways (Campisi and d'Adda di Fagagna, 2007, Passos and Von Zglinicki, 2006). This initiates a DNA damage response similar to telomere-dependent senescence (d'Adda di Fagagna, 2008).

It is also unclear whether CMV-specific $CD8^+$ T_{EMRA} cells are maintained by periodic CMV reactivation or generated in the absence of antigen by homeostatic signals. Indeed, the two are not mutually exclusive, as T_{EMRA} cells could be generated from previously

antigen driven memory subpopulations which later undergo homeostatic CD45RA re-expression. Evidence to support this hypothesis comes from studies with IL-15, a cytokine that is essential for $CD8^+$ T cell memory survival (Tough et al., 2000). IL-15 was shown to generate $CD8^+$ T_{EMRA} cells from $CD8^+$ T_{CM} cells (Geginat et al., 2003) and also cause CD45RA re-expression on $CD45RO^+$ EBV-specific $CD8^+$ memory T cells (Dunne et al., 2005) in the absence of antigen *in-vitro.*

As T_{EMRA} cells are found predominately in older individuals and are also increased in those who are CMV positive, this suggests ageing and/or chronic CMV infection may cause changes in the cytokine milieu which favours their generation. Indeed, there is evidence to support this hypothesis. Previous data has shown that CMV stimulated plasmacytoid dendritic cells can produce high levels of IFN-α, which can lead to down-regulation of co-stimulatory molecules and inhibition of telomerase activity in activated $CD4^+$ T cells (Fletcher et al., 2005). This is significant as the antiviral effects of IFN-α can also lead to the secondary production of cytokines such as IL-2 and IL-15 (Tough et al., 1996). In fact, acute viral infections i.e. HBV, Dengue virus, and Influenza virus were shown to trigger bystander activation and expansion of CMV-specific $CD8^+$ T cells through IL-15 (Sandalova et al., 2010). Furthermore, elevated levels of pro-inflammatory cytokines including IFN-α and IL-15 have been reported during ageing (Gangemi et al., 2005, Zhang et al., 2002, De Martinis et al., 2005, Franceschi et al., 2007). Therefore, chronic inflammation during ageing may lead to increased circulating IL-15 levels which drive the appearance of $CD8^+$ T_{EMRA} cells.

5.2 Aims

The aim of this chapter is to determine how $CD8^+$ T_{EMRA} cells are generated. Low proliferative and low anti-apoptotic levels coupled with relatively long telomeres for a memory subpopulation suggests they could be approaching senescence by telomere-independent pathways. To address this, the potential of $CD8^+$ T_{EMRA} cells to proliferate efficiently in response to activation will be investigated. Specifically, the level of proliferation and telomerase activity exhibited following activation will be determined in $CD8^+$ T_{EMRA} cells and compared with the other $CD8^+$ T cell subpopulations. Senescence-related DNA damage, whether resulting from telomere dependent or telomere independent DNA damage can be identified by molecules such as the histone protein γH2AX (Passos et al., 2010). Therefore the levels of γH2AX within $CD8^+$ T cell subpopulations will also be determined.

As discussed earlier, evidence suggests a role for IL-15 cytokine in the generation of $CD8^+$ T_{EMRA} cells. Therefore the proliferative and phenotypic changes of CMV-specific $CD8^+$ T cells in response to IL-15 will be investigated. Additionally, whether CMV can cause increased IL-15 levels *in-vitro* will be assessed in order to question the mechanism whereby chronic CMV infection may influence the appearance of more $CD8^+$ T_{EMRA} cells through bystander proliferation *in-vivo*.

5.3 Results

5.3.1 Proliferative responses of $CD8^+$ T cell subpopulations to stimulus

In chapter 3, CMV-specific $CD8^+$ T_{EMRA} cells showed low levels of proliferation on the basis of Ki-67 expression compared with other memory $CD8^+$ T cell subpopulations when sampled directly *ex-vivo*. One possibility for the low proliferative capacity may be that $CD8^+$ T_{EMRA} cells are resting memory T cells that have not seen antigen recently. Alternatively, this may indicate $CD8^+$ T_{EMRA} cells are a senescent population that are un-responsive to antigen. The first step therefore was to investigate if $CD8^+$ T_{EMRA} cells can proliferate efficiently when reactivated.

5.3.1.1 $CD8^+$ T_{EMRA} cells show poor proliferative responses

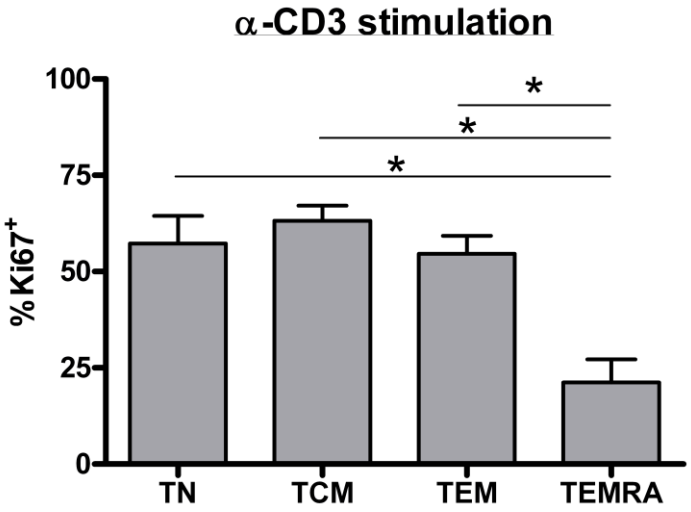
The proliferative responses of $CD8^+$ T cell subpopulations to a broad stimulus were investigated. Donor PBMCs were first negatively selected for $CD8^+$ T cells then further sorted by CD45RA and CD27 to give the four $CD8^+$ T cell subpopulations of interest. Each subpopulation was then separately seeded at 100,000 cells and stimulated with anti-CD3 by placing in OKT3 coated wells for a period of 4 days. The proliferative response (Ki-67 positivity) on day 4 was recorded for each $CD8^+$ T cell subpopulation (figure 5.1A) as determined by the mean responses of 7 donors tested. $CD8^+$ T_{EMRA} cells showed significantly lower Ki-67 levels (with a mean of 21%) compared with other $CD8^+$ T cell subpopulations (means all above 50%) indicating $CD8^+$ T_{EMRA} cells have poor proliferative responses to a broad activation *in-vitro*.

5.3.1.2 CMV-specific $CD8^+$ T_{EMRA} cells show poor proliferative responses

Next, the proliferative responses of $CD8^+$ CMVpp65-specific T cell subpopulations to CMVpp65 peptide were investigated. Donor PBMCs from a CMV positive individual were first selected for $CD8^+$ T cells by negative magnetic bead selection and then further sorted on the basis of CD45RA and CD27 to give the four $CD8^+$ T cell subpopulations of interest. Each subpopulation was then separately seeded at 100,000 cells per well and stimulated with CMVpp65 peptide for 4 days. The proliferative response (Ki-67 positivity) was subsequently recorded for each $CD8^+$ T cell subpopulation (figure 5.1B) as based on the mean responses of 5 individuals tested. CMV-specific $CD8^+$ T_N cells showed the highest Ki-67 levels (mean of 24%) with T_{CM} showing the second highest response (mean of 13%) and both T_{EM} and T_{EMRA} subpopulations showing the lowest responses (mean of 10%). Results showed CMV-specific $CD8^+$ T cells have a relatively poor response to CMVpp65-specific activation *in-vitro*, with CMV-specific $CD8^+$ T_{EMRA} cells showing among the lowest Ki-67 levels in response to activation of all subpopulations.

Overall results showed that $CD8^+$ and CMV-specific $CD8^+$ T_{EMRA} cells show poor proliferative responses to activation. This is consistent with Ki-67 positivity observed directly ex-vivo in chapter 3 and provides further support that $CD8^+$ T_{EMRA} cells may be close to senescence.

(A)



(B)

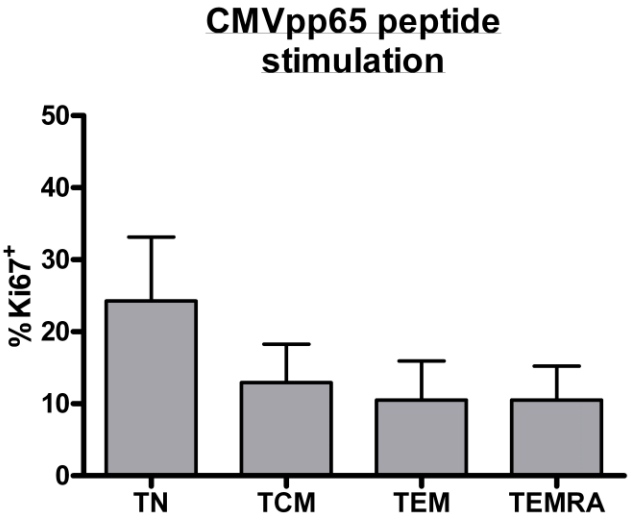


Figure 5-1. Proliferative responses of sorted CD8$^+$ and CMV-specific CD8$^+$ T cell subpopulations to stimulation

CD8$^+$ T cell subpopulations were sorted at >98% purity then stimulated separately in (A) OKT3 coated wells for 4 days at which Ki67 levels were recorded (mean of 7 experiments) or in (B) CMVpp65 peptide containing medium for 4 days at which Ki67 levels were recorded (mean of 5 experiments). Significant differences displayed by *values. *<0.05;**<0.005;***<0.0005.

5.3.2 Telomerase activity of $CD8^+$ and CMV-specific $CD8^+$ T cells

The attrition of telomere length can be suppressed by the enzyme telomerase, which adds back telomeric repeats (Akbar and Vukmanovic-Stejic, 2007). However, it has been shown that repeated stimulation can eventually lead to the loss of telomerase activity in highly differentiated $CD8^+CD27^-CD28^-$ memory T cells (Plunkett et al., 2007, Henson et al., 2009). In the previous chapter $CD8^+$ T_{EMRA} cells and CMV-specific $CD8^+$ $CD45RA^+$ memory cells were shown to have significantly longer telomeres than $CD8^+$ T_{EM} cells or CMV-specific $CD8^+$ $CD45RO^+$ memory T cells respectively. This may indicate $CD8^+$ T_{EMRA} cells have relatively high telomerase activity that maintains the telomere length within these cells. Therefore, the activity of telomerase was compared in $CD8^+$ and CMV-specific $CD8^+$ T cell subpopulations.

5.3.2.1 Measuring telomerase activity using the TRAP assay

The analysis of telomerase activity was conducted using the telomeric repeat amplification protocol (TRAP) assay, as described in materials and methods (section 2.4.3). Briefly, cell extracts from stimulated cells were isolated and PCR was performed with samples adjusted to 500 $Ki67^+$ T cells per reaction (Reed et al., 2004). Telomeric elongation was performed using a [γ-P^{32}] ATP-end-labelled telomerase substrate (TS) primer and samples amplified by PCR. The PCR products were then run on a polyacrylamide gel which was subsequently exposed to an autoradiography film. Telomerase activity was then calculated as a ratio between the mean optical densities of the telomeric repeat bands and the internal standard control band.

5.3.2.2 $CD8^+$ T_{EMRA} cells show low level telomerase activity

Telomerase activity was measured in sorted $CD8^+$ T cell subpopulations following stimulation with anti-CD3 and irradiated autologous APCs. A representative example of relative telomerase activity in one donor is shown by the autoradiography of a TRAP assay acrylamide gel (figure 5.2A). $CD8^+$ T_{EMRA} cells were found to have the lowest levels of telomerase activity of all $CD8^+$ T cell subpopulations. Telomerase activity was calculated as mean telomerase activity per subpopulation from the consolidation of 5 experiments (figure 5.2B).

5.3.2.3 CMV-specific $CD8^+$ T_{EMRA} cells show low level telomerase activity

Telomerase activity was measured in sorted $CD8^+$ T cell subpopulations from CMV positive individuals following stimulation with CMVpp65 peptide (2pg/ml) plus irradiated autologous APCs. A representative example of relative telomerase activity in one donor is illustrated by the autoradiography of a TRAP assay acrylamide gel in figure 5.2C. CMV-specific $CD8^+$ T_{EMRA} cells were found to have the lowest levels of telomerase activity of all $CD8^+$ T cell subpopulations, as highlighted by the mean telomerase activity per subpopulation determined from 3 experiments (figure 5.2D).

Results showed that telomerase activity in both $CD8^+$ and CMVpp65-specific $CD8^+$ T_{EMRA} cells were the lowest of all subpopulations, equating to a restriction in their ability to be maintained through subsequent division and indicative of a senescent subpopulation.

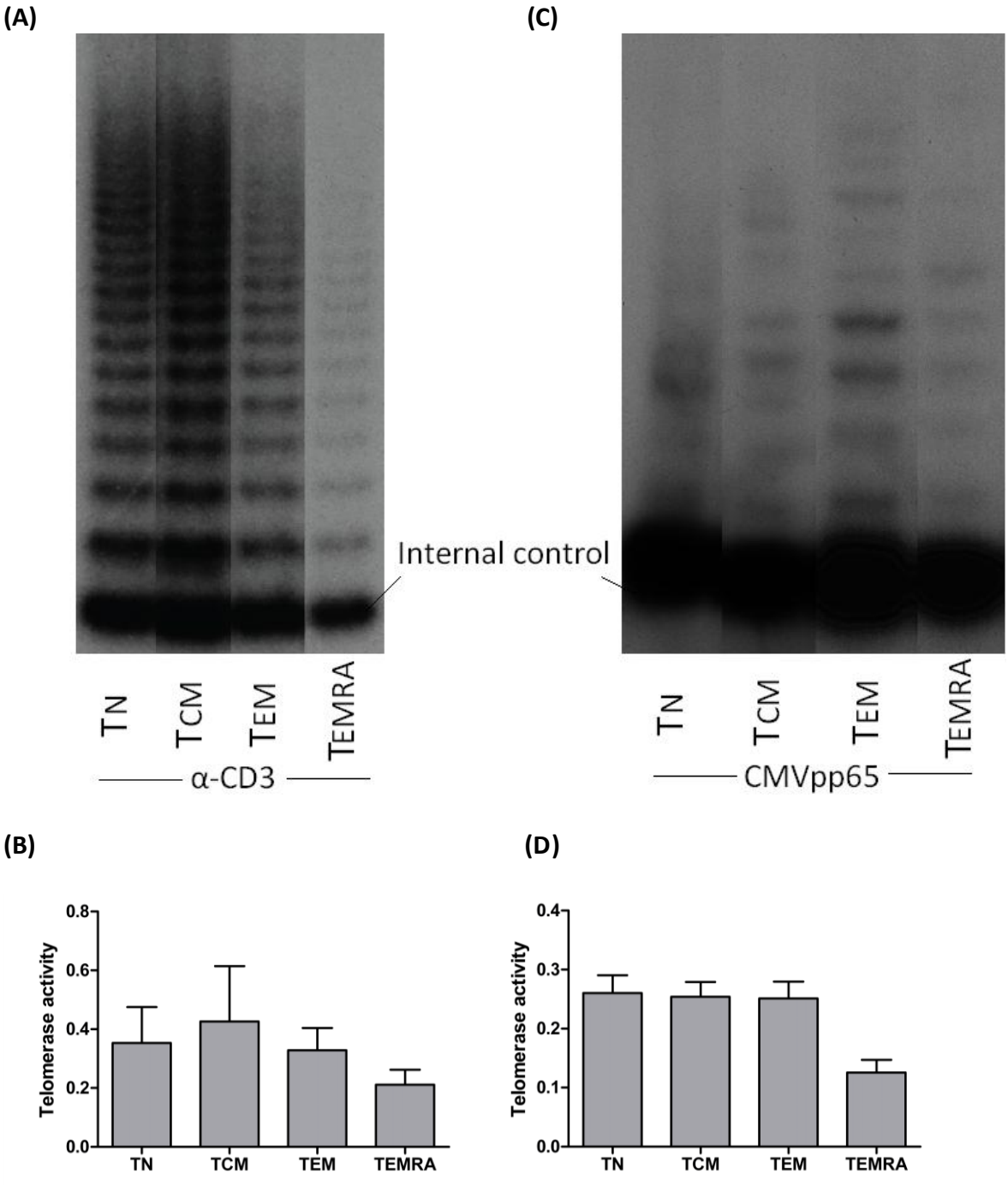


Figure 5-2. Telomerase levels in CD8$^+$ and CMV-specific CD8$^+$ T cell subpopulations

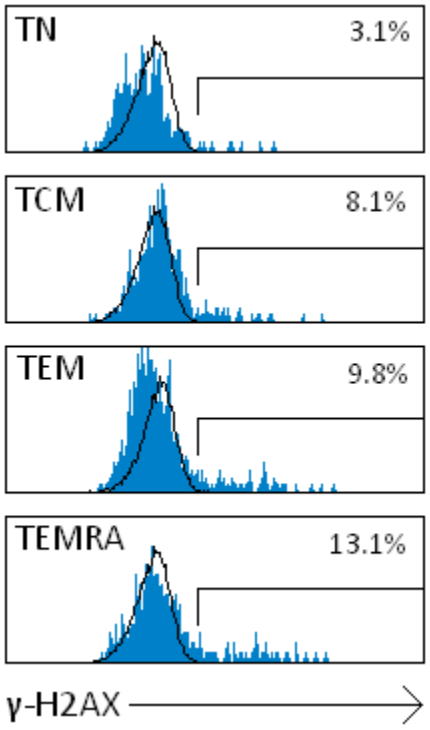
Telomerase activity was determined using the TRAP assay. (A) Sorted CD8$^+$ T cell subpopulations were stimulated with OKT3 and irradiated APCs for 4 days. Autoradiography of a TRAP assay 12% acrylamide gel from a representative example is shown. (B) Bar chart shows mean relative telomerase levels from five experiments. (C) Sorted CD8$^+$ T cell subpopulations from CMV positive individuals were stimulated with CMVpp65 peptide and irradiated APCs for 4 days. Autoradiography of a TRAP assay 12% acrylamide gel from a representative example is shown. (D) Bar chart shows mean relative telomerase levels from three experiments.

5.3.2.4 $CD8^+$ T_{EMRA} cells show evidence of high DNA damage

The low levels of telomerase activity found in $CD8^+$ and CMV-specific $CD8^+$ T_{EMRA} cells indicate they are highly differentiated cells which have undergone repeated cellular proliferation and are close to senescence (Fletcher et al., 2005, Weng et al., 1996, Plunkett et al., 2007). However, the previous chapter showed $CD8^+$ T_{EMRA} cells exhibit relatively long telomere lengths for a memory subpopulation and most significantly CMV-specific $CD8^+$ $CD45RA^+$ memory T cells have significantly longer telomeres than CMV-specific $CD8^+$ $CD45RO^+$ memory T cells. This dichotomy suggests that rather than being lost through repeated stimulation, telomerase may be shut off or inhibited in $CD8^+$ T_{EMRA} cells. As discussed in the introduction of this chapter, cellular senescence can also occur by telomere-independent mechanisms as the result of DNA damage. One such indicator of DNA damage is γ-H2AX (Passos et al., 2010).

The levels of γ-H2AX were analysed in the four $CD8^+$ T cell subpopulations directly *ex-vivo* (figure 5.3). $CD8^+$ T_{EMRA} cells exhibited significantly higher levels of γ-H2AX compared with the other subpopulations. This supports the hypothesis that $CD8^+$ T_{EMRA} cells may be close to telomere-independent senescence through the accumulation of DNA damage. These are preliminary results and are not sufficient in numbers to determine the influence of age or CMV serostatus as independent factors. Furthermore, γ-H2AX levels on CMV-specific $CD8^+$ T cell subpopulations require investigation.

(A)



(B)

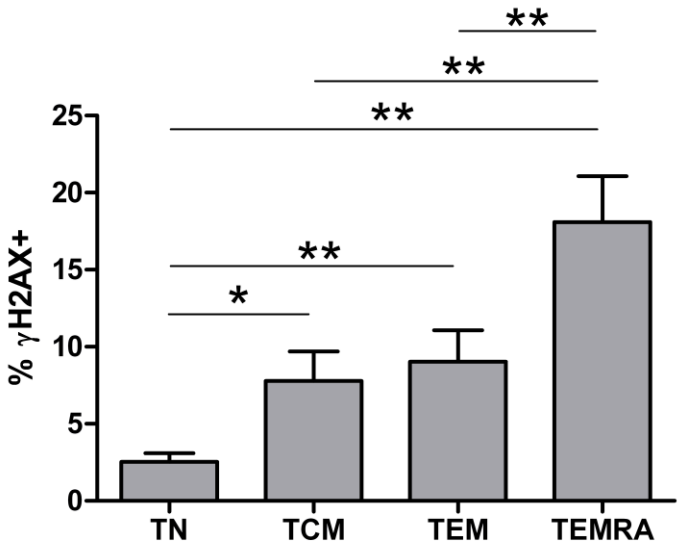


Figure 5-3. DNA damage levels as indicated by γ-H2AX expression on CD8+ T cell subpopulations
(A) Representative histograms showing γ-H2AX levels in each subpopulation. Filled blue histogram represents sample and empty histogram IgG1-488 isotype control. (B) Pooled data from 8 individuals showing γ-H2AX levels of $CD8^+$ T cell subpopulations. Standard error bars shown. 8 young individuals tested (4 CMV+/4 CMV-). *<0.05;**<0.005;***<0.0005.

5.3.3 Proliferative and phenotypic responses to IL-15 cytokine

It has been shown previously that IL-15 can cause CD45RA re-expression on CD45RA negative $CD8^+$ T cell memory populations in the absence of antigenic stimulation *in-vitro* (Dunne et al., 2005, Geginat et al., 2003). This suggests a mechanism by which $CD8^+$ T_{EMRA} cells may be generated *in-vivo*.

The potential of IL-15 to cause CD45RA re-expression on CD45RA negative $CD8^+$ and CMV-specific $CD8^+$ T cells was therefore investigated. Figure 5.4 outlines the two experimental approaches used to investigate this. In the first experiment (figure 5.4A) the proliferative and phenotypic responses of the four $CD8^+$ T cell subpopulations, as identified by CD45RA and CD27 expression, were sorted then cultured individually with one shot of 10U IL-15 cytokine. In the second experiment (figure 5.4B) the CD45RA negative fraction only was sorted from $CD8^+$ T cells taken from a CMV positive individual then cultured with one shot of 10U IL-15 cytokine. In the second experiment the CMV-specific population was identified by tetramer to analyse CMV-specific $CD8^+$ T cell proliferation and CD45RA re-expression during IL-15 treatment.

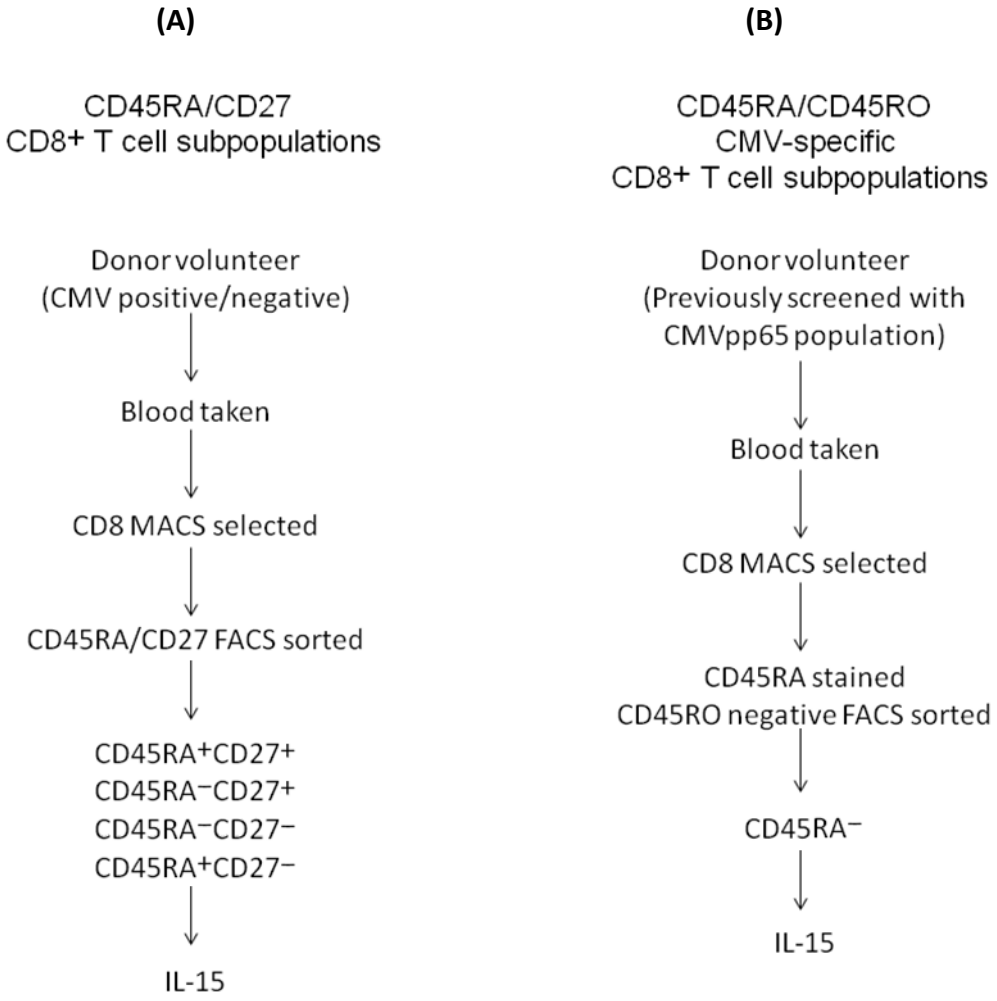


Figure 5-4. Schematic of IL-15 reversion experiments
IL-15 reversion experiments were conducted in one of two ways; as depicted by (A) for the four $CD8^+$ T cell subpopulations and (B) for one CD45RA negative subpopulation.

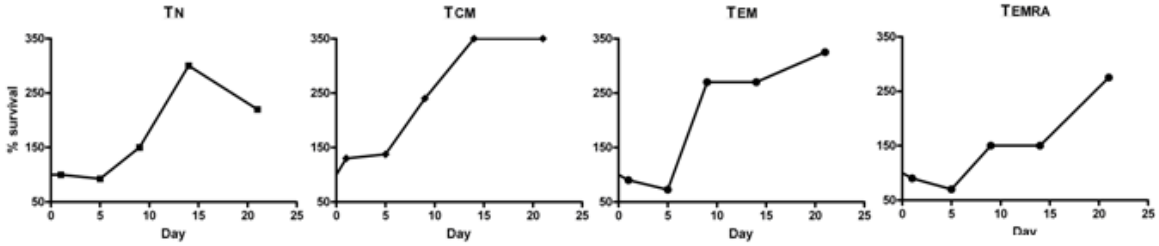
5.3.3.1 IL-15 maintains the survival and proliferation of $CD8^+$ T cells

The ability of IL-15 to maintain the survival of memory $CD8^+$ T subpopulations in the absence of antigen was tested. The four $CD8^+$ T cell subpopulations were sorted and individually cultured with IL-15. The number of cells recovered at time-points indicated were recorded and expressed as a percentage of the initial seeded numbers (figure 5.5A). $CD8^+$ T_{CM} and T_{EM} cells proliferated efficiently in response to IL-15 treatment, reaching close to 350% of the original seeded cell numbers. Although $CD8^+$ T_{EMRA} cells initially proliferated slowly, by day 21 cell numbers were relatively high, at approx 260% of seeded numbers. $CD8^+$ T_N cells were less responsive to IL-15 and numbers by day 21 were comparably lower than the other subpopulations. When not treated with IL-15, cell death was observed to occur within 4 days (not shown). Results confirm that IL-15 can maintain the proliferation of memory $CD8^+$ T cells in the absence of antigen.

5.3.3.2 IL-15 causes CD45RA re-expression

The expression of CD45RA and CD27 was also determined during IL-15 treatment. Samples of cells were taken at days 0, 1, 9, 14, and 21 then stained with antibodies to CD8, CD45RA and CD27. CD45RA re-expression was found to increase commensurate with days of IL-15 treatment on both $CD8^+$ T_{CM} and $CD8^+$ T_{EM} cells, resulting in approximately 60% and 45% $CD45RA^+$ re-expression on these subpopulations respectively (figure 5.5B).

(A)



(B)

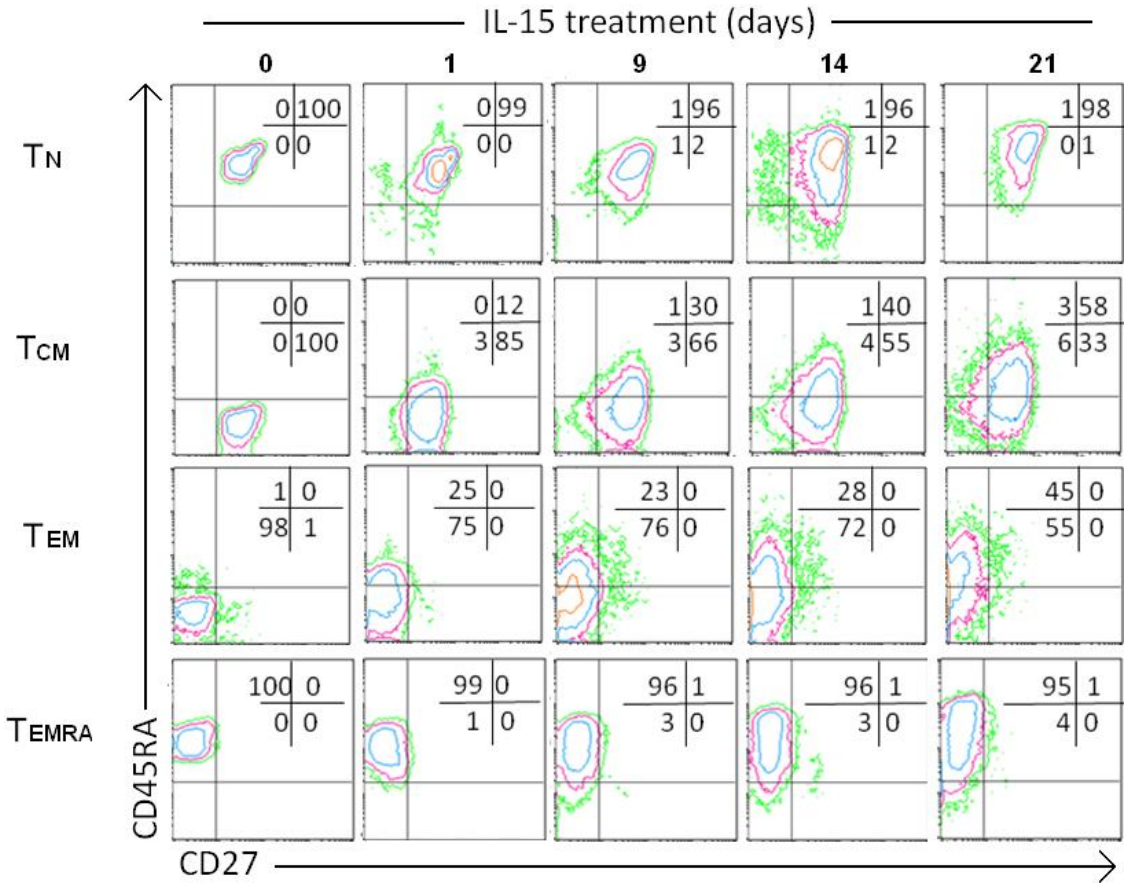


Figure 5-5. Proliferative and phenotypic responses of CD8$^+$ T cell subpopulations to IL-15 treatment
CD8$^+$ T cell subpopulations were sorted on the basis of CD45RA and CD27 then cultured individually with IL-15 (10U). (A) The percentage survival of CD8$^+$ T cell subpopulation nubers with time (in days) post IL-15 treatment as determined by viable cell counting on days 0, 1, 9, 14 and 21. Percentage cell survival calculated based on original seeded numbers at day 0 (set to 100%). (B) FACs plot analysis showing CD45RA/CD27 phenotype of each CD8$^+$ T cell subpopulation on days 0, 1, 9, 14 and 21. Numbers in quadrants represent percentages of total live cells. Experiment shown is representative of two.

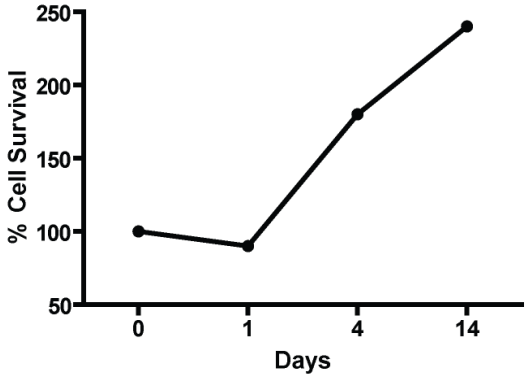
5.3.3.3 IL-15 causes CMV-specific T cells to proliferate

Next, the response of CMV-specific $CD8^+$ T cell responses to IL-15 was investigated. Sorted CD45RA negative $CD8^+$ T cells were obtained from CMV positive individuals who were HLA matched for analysis of the CMVpp65-specific $CD8^+$ T cell population. CD45RA negative $CD8^+$ T cells were seeded at 100,000 cells per well and cultured over 14 days with 10U of IL-15. At time-points 0, 1, 4 and 14 one well was harvested, viable cell numbers counted and total cells stained with CMVpp65-specific tetramer together with CD8, CD45RA and CD45RO antibodies. IL-15 was shown to cause the expansion of this CD45RA negative subpopulation to approximately 250% of its original seeded number by day 14 (figure 5.6A). This indicated that IL-15 can drive the expansion of CMV-specific $CD8^+$ T cells efficiently in the absence of antigen.

5.3.3.4 IL-15 causes CMV-specific T cell CD45RA re-expression

Commensurate with the expansion of this population was the re-expression of CD45RA on CD45RA negative CMVpp65-specific $CD8^+$ T cells. This is shown in the representative example FACs plot analysis of one individual (figure 5.6B). Here, the percentage of CD45RA re-expression is displayed in the relevant quadrant of total $CD8^+$ T cells (top panel) and CMV-specific $CD8^+$ T cells (bottom panel). Both of these populations showed increased expression of CD45RA by day 14 compared with initial levels at day 0, equating to 47% and 39% CD45RA positive population on total CD45RA negative $CD8^+$ and CMV-specific $CD8^+$ T cells respectively. Results of three experiments confirm CD45RA re-expression in response to IL-15 treatment in-vitro.

(A)



(B)

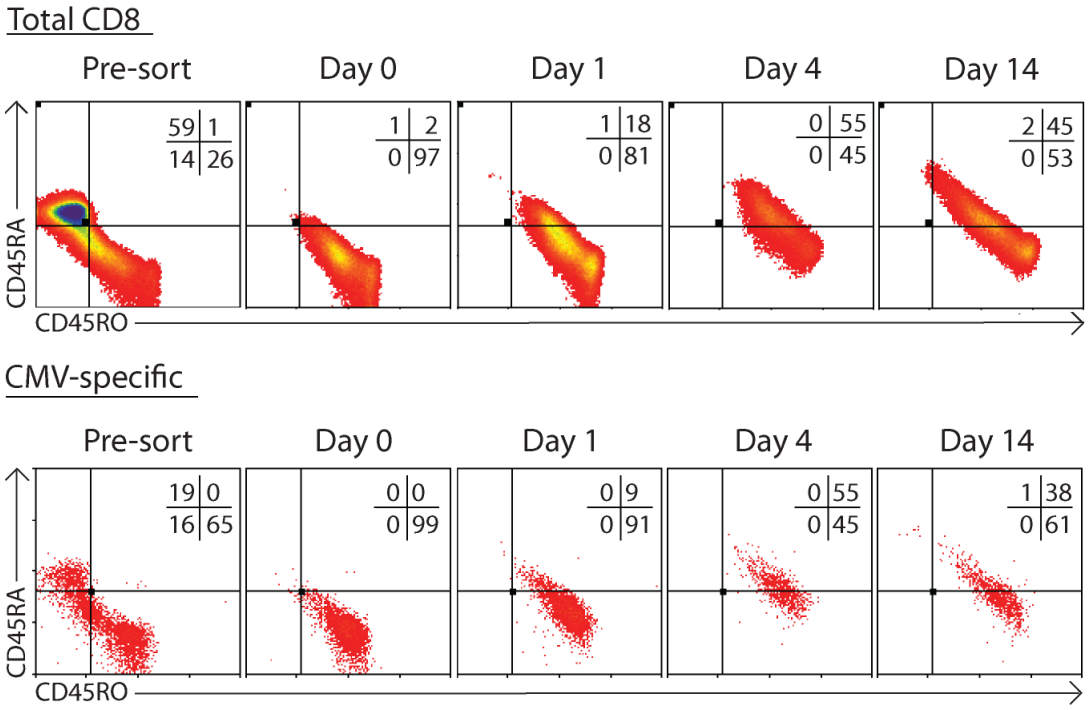


Figure 5-6. Homeostatic proliferation and CD45RA re-expression on $CD8^+$ and CMVpp65-specific $CD8^+$ CD45RA negative T cells during IL-15 treatment

(A) The percentage recovery of $CD8^+$ CD45RA- T cells with time (in days) during IL-15 treatment, as determined using viable cell count from 100% at day 0 then subsequently at days 1, 4, and 14. (B) FACs plot analysis showing CD45RA re-expression on the sorted CD45RA negative population at days 0 to 14. Numbers represent percentages in quadrants. Experiment shown is representative of three.

5.3.3.5 CMV lysate and IFN-α increases production of IL-15

Previous data has shown that CMV lysate stimulated plasmacytoid dendritic cells (pAPCs) can produce high levels of IFN-α (Fletcher et al., 2005). IFN-α may also cause the secondary production of IL-15 (Tough et al., 1996), which has been implicated in the generation of CD45RA re-expressing memory subpopulations, as shown in the previous section and elsewhere (Dunne et al., 2005, Geginat et al., 2003, Sandalova et al., 2010).

Therefore, the potential of CMV to cause the production of IL-15 was investigated. IL-15 mRNA levels were measured by RT-PCR in total PBMCs from CMV positive individuals, following the treatment of cells with either IFN-α or CMV lysate. In each case two experiments were conducted. For the first experiment, treating PBMCs with IFN-α for 4, 24 and 48 hours (figure 5.7A) showed a 4 hour treatment yielded maximal production of IL-15 mRNA, with a 4.8 fold increase at a concentration of 500U/ml compared with no IFN-α treatment. This 4 hour treatment was then repeated on a different donor (figure 5.7B) and a 3 fold increase in IL-15 mRNA was observed at a peak concentration of 1000U/ml. Both results demonstrated that treatment with IFN-α resulted in increased production of IL-15 mRNA from baseline levels (i.e. no IFN-α treatment). When PBMCs were treated with CMV lysate for 24, 48 and 72hrs (figure 5.7C) a 24hr treatment yielded maximal production of IL-15 mRNA, with a 2 fold increase compared with no CMV lysate treatment. This 24 hour treatment was then repeated on a different donor (figure 5.7D) and a 3.5 fold increase in IL-15 mRNA levels was observed. Results showed that CMV lysate can lead to increased levels of IL-15 mRNA in total PBMCs.

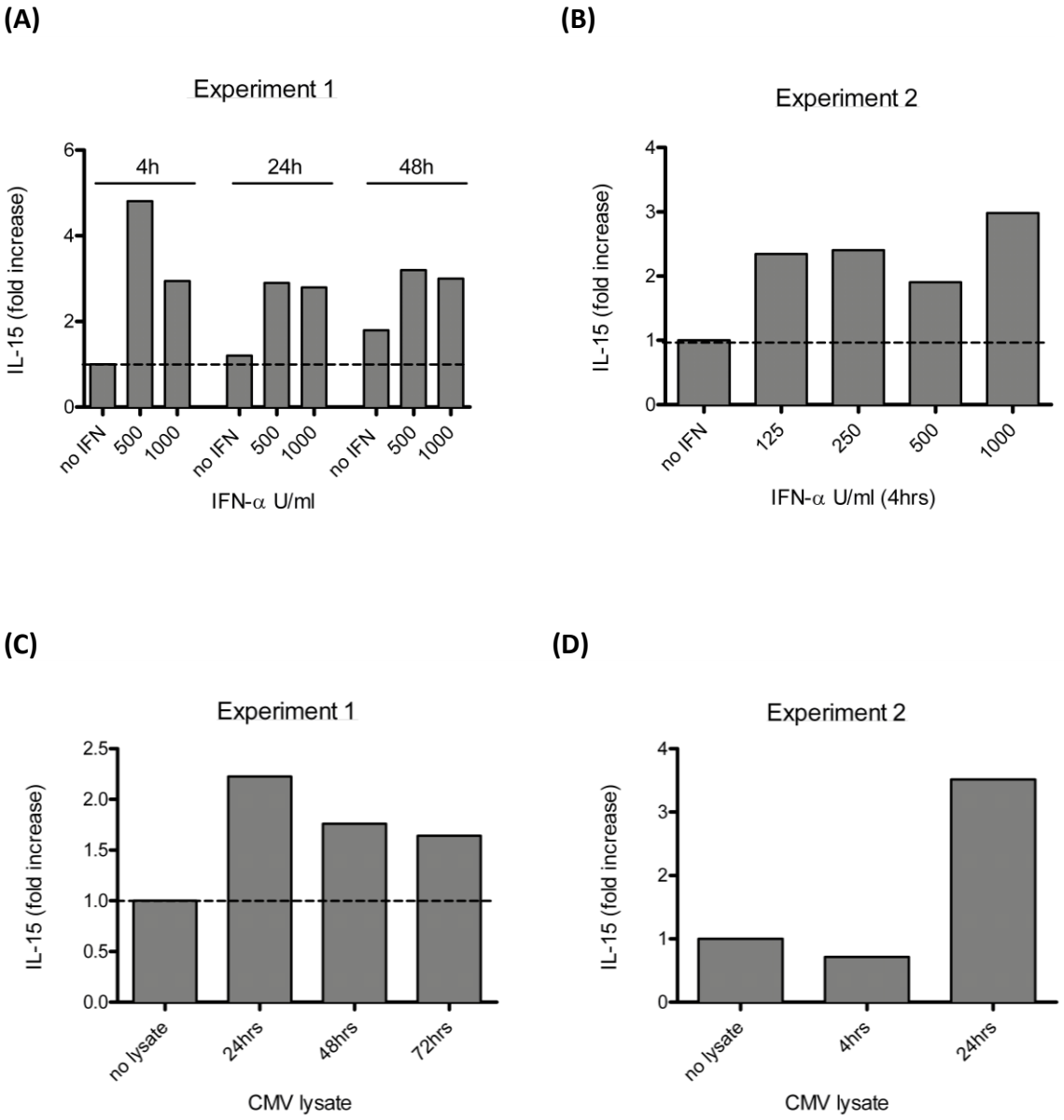


Figure 5-7. IL-15 production following the stimulation of total PBMCs with either IFN-α or CMV lysate
The production of IL-15 mRNA was determined by RT-PCR following the treatment of total PBMCs with either IFN-α (A&B) or CMV lysate (C&D) for times and concentrations indicated.

5.4 Discussion

The aim of this chapter was to investigate how $CD8^+$ T_{EMRA} cells may be generated. In conclusion, results suggest that this subpopulation may be accumulating through homeostatic-driven proliferation from previously generated $CD8^+$ T_{CM} or $CD8^+$ T_{EM} subpopulations. This is based on the low level proliferative responses and low telomerase activity exhibited by $CD8^+$ T_{EMRA} cells upon stimulation, and observations IL-15 caused CD45RA re-expression on CD45RA negative $CD8^+$ and CMV-specific $CD8^+$ T cells.

Low levels of telomerase activity found in $CD8^+$ and CMV-specific $CD8^+$ T_{EMRA} cells indicates they are highly differentiated cells which have undergone repeated cellular proliferation, and are close to senescence (Fletcher et al., 2005, Weng et al., 1996, Plunkett et al., 2007). Indeed, this is consistent with findings of low Ki-67 and Bcl-2 levels in $CD8^+$ and CMV-specific $CD8^+$ T_{EMRA} cells directly *ex-vivo* (chapter 3). Furthermore, despite having relatively long telomeres for a memory subpopulation, $CD8^+$ T_{EMRA} cells showed high levels of DNA damage in the form of γ-H2AX histone protein expression. Overall, results suggest that $CD8^+$ T_{EMRA} cells may be close to senescence through telomere-independent mechanisms. Indeed, ongoing work is investigating p38, a mitogen activated protein kinase involved in stress related responses which can trigger apoptosis. Preliminary work (not shown) indicates that the inhibition of p38 through BIRB 796, an inhibitor of p38 signaling (Bagley et al., 2007) can enhance telomerase activity.

Work presented in this chapter also showed IL-15 can maintain the homeostatic proliferation of CMV-specific $CD8^+$ memory T cells. This is in-line with reports that CD27/CD28 negative memory cells can be maintained efficiently by IL-15 cytokine (Chiu et al., 2006). Most importantly, IL-15 caused CD45RA re-expression on CMV-specific $CD45RO^+$ $CD8^+$ T cells, supporting similar findings with EBV-specific $CD8^+$ T cells (Dunne et al., 2005) and total $CD8^+$ T_{CM} cells (Geginat et al., 2003). This identifies a key role for IL-15 in the generation of $CD8^+$ T_{EMRA} cells.

It is possible the persistence of CMV and other viruses may lead to increased levels of IL-15 *in-vivo* and subsequently drive the generation of $CD8^+$ T_{EMRA} cells. IL-15 is produced by dendritic cells, monocytes and endothelial cells during and following viral infections (Oppenheimer-Marks et al., 1998, Waldmann, 2006). Furthermore, acute viral infections i.e. HBV, Dengue virus, and Influenza virus have been shown to trigger bystander activation and expansion of CMV-specific and EBV-specific $CD8^+$ T cells through IL-15 (Sandalova et al., 2010). It must also be remembered that $CD8^+$ T_{EMRA} cells are not just seen in CMV positive individuals but also in CMV negative individuals, but at significantly lower numbers. CMV negative individuals will be infected with other chronic viruses; on average a healthy individual is infected with 8-12 chronic viruses (Virgin et al., 2009). Therefore, it is possible that other viruses may also be causing bystander proliferation and generation of $CD8^+$ T_{EMRA} cells through IL-15 or related cytokines. Previous work has shown that CMV lysate can produce high levels of IFN-α through plasmacytoid dendritic cells (Fletcher et al., 2005). Results presented in this chapter show CMV lysate can also

increase IL-15 production *in-vitro*, although the main source is yet to be determined, as total PBMCs were stimulated and individual cell types not separated. However, taken together, evidence suggests that chronic infection may lead to increased IL-15 levels.

Another possibility is that CMV-specific $CD8^+$ T cells are more amenable to the effects of IL-15, and therefore more likely to undergo CD45RA reversion. One possibility is that CMV-specific $CD8^+$ T cells have increased IL-15 receptor expression levels. The IL-15 receptor is part of the common cytokine-receptor γ-chain family, sharing the γ-chain (CD132) with IL-2, IL-4, IL-7, IL-9 and IL-21, but is distinguished separately by the expression of IL-15Rα and IL-15Rβ (Waldmann, 2006, Khaled and Durum, 2002). The mechanism of signalling through IL-15 is still poorly understood however, as IL-15Rα can be found both directly on T cells or trans-presented to T cells by DCs or monocytes (Khaled and Durum, 2002, Ma et al., 2006, Waldmann, 2006, Geginat et al., 2003, Sauce et al., 2006). Therefore, T cells expressing higher levels of IL-15Rα or other constituents of the receptor (e.g. IL-15Rβ or CD132) may confer a higher affinity for soluble IL-15. Indeed, IL-15 is a limiting factor, and can determine the number of each type of lymphocyte with competitors dying through neglect (Khaled and Durum, 2002). Therefore, determining expression of IL-15R may be important in this context. Interestingly, age-related $CD8^+$ T cell clonal expansions in mice show higher IL-15Rα compared with other $CD8^+$ T cells (Messaoudi et al., 2006). In humans, CMV-specific $CD8^+$ T cells retain high IL-15Rα expression following acute infection compared with EBV-specific $CD8^+$ T cells following acute infection (Sauce et al., 2006). With such

complex mechanism of action, measuring IL-15Rα levels on T cells has proved difficult and variable, with reports of very low or absent expression on T cells (Geginat et al., 2003, Sauce et al., 2006). Furthermore, attempts to determine IL-15Rα levels on $CD8^+$ and CMV-specific $CD8^+$ T cell subpopulations in this study have unfortunately been unsuccessful.

The key findings of this chapter therefore are that $CD8^+$ T_{EMRA} cells may be generated from the previously proliferating memory subpopulations. CMV also has the potential to increase IL-15 *in-vitro* which may point to a role in the generation of $CD8^+$ T_{EMRA} cells *in-vivo*. Longer telomeres coupled with low proliferative capability, low telomerase activity and low Bcl-2 levels indicates they may be a population which is close to senescence through telomere-independent mechanisms. The next chapter will therefore investigate if $CD8^+$ T_{EMRA} cells are also close to exhaustion i.e. whether or not they can exhibit efficient functional responses upon activation.

6 The functional quality of CMV-specific $CD8^+$ T cells during ageing

6.1 Introduction

Dealing with a chronic viral infection presents a unique challenge to T cells, as they must control the infection over one's lifetime in the face of possible T cell exhaustion. Therefore the importance of maintaining a quality immune response during a persistent infection is becoming increasingly recognised: bad prognoses with chronic infections such as Hepatitis C and HIV are associated with exhausted virus-specific T cells whereas favourable prognoses are associated with virus-specific T cells exhibiting broad polyfunctional responses upon activation (Betts et al., 2006, Effros, 2004, Golden-Mason et al., 2008, Jones et al., 2008, Kaufmann and Walker, 2008, Cellerai et al., 2011). Indeed, it is considered the more effector functions that constitute a T cell response the more protective the response will be (Makedonas and Betts, 2006, Seder et al., 2008). In the context of vaccination, poor polyfunctional responses to flu vaccine equate with poor protection, a problem frequently encountered when vaccinating the old (Kovaiou et al., 2007, McElhaney and Dutz, 2008, Seder et al., 2008, Weinberger et al., 2008).

In addition to a broad polyfunctional response, the avidity of a virus-specific $CD8^+$ T cell for antigen is also becoming increasingly recognised as an important factor in the control of infection. However whether high or low avidity T cells are most beneficial during a chronic infection is debateable. To highlight this, two studies investigating the

relationship between avidity of $CD8^+$ T cells and disease progression in HIV (Almeida et al., 2007, Harari et al., 2007) both found a broad polyfunctional response was a signature of virus-specific $CD8^+$ T cells in HIV non-progressors but one group identified these 'superior' cells as highly avid (Almeida et al., 2007) whereas the other group identified them as low avidity cells (Harari et al., 2007). Indeed, the hypothesis is plausible either way, as high avidity T cells will recognise viral antigen more efficiently than low avidity T cells but the former may become functionally exhausted as a result of more frequent activation during chronic infection.

Does the accumulation of CMV-specific TCEs with ageing suggest a lack of control of chronic CMV infection later in life? The large numbers of CMV-specific $CD8^+$ T cells in the old have been reported as being dysfunctional when compared with those in the young (Almanzar et al., 2005, Ouyang et al., 2003b) however there is little evidence of CMV reactivation in the old suggesting the virus is kept under control. One hypothesis is that $CD8^+$ T cells that are most optimal against CMV but become exhausted, while the less optimal $CD8^+$ T cells are consequently recruited, albeit in greater numbers, to control CMV. Evidence supporting this comes from observations of how healthy individuals control symptomatic primary CMV infections. In one study (Lidehall et al., 2005), by relating the number of CMV-specific $CD8^+$ T cells found within donors to their total IFN-γ production recorded upon CMV stimulation, results showed individuals controlled infection by either mounting small highly efficient clones or compensating less efficient clones with increased cell numbers. In another study, by comparing CMV and EBV

responses *in-vitro* it was also shown that older individuals (>60) produced significantly lower IFNγ/virus-specific T cell ratios than those in the 40-60 age group (Khan et al., 2004).

6.2 Aims

It has been proposed that $CD8^+$ T_{EMRA} cells may be an end-stage population as they show a high level of differentiation (Appay et al., 2002) and exhibit poor proliferative potential upon stimulation (Geginat et al., 2003). Indeed, previous chapters have confirmed that $CD8^+$ T_{EMRA} cells show low proliferation directly *ex-vivo* and in response to activation. Furthermore, results have shown that $CD8^+$ T_{EMRA} cells may be close to telomere-independent senescence as they show a high susceptibility to apoptosis, low telomerase activity and high level DNA damage in the form of γH2AX expression.

The aim of this chapter therefore was to question whether these accumulating CMV-specific $CD8^+$ T_{EMRA} cells are also close to exhaustion, representing a less efficient population controlling CMV infection by a means of quantity over quality, or if they have maintained an ability to respond functionally upon activation. In order to address this question, the polyfunctional responses of $CD8^+$ T_{EMRA} cells to activation were compared with other $CD8^+$ T cell subpopulations in both the global $CD8^+$ and CMV-specific $CD8^+$ T cell compartments. Furthermore, the avidity for antigen was investigated to determine whether this may impact the functional quality of the $CD8^+$ T cell response and also whether donor age or $CD8^+$ T cell phenotype influences this response.

6.3 Results

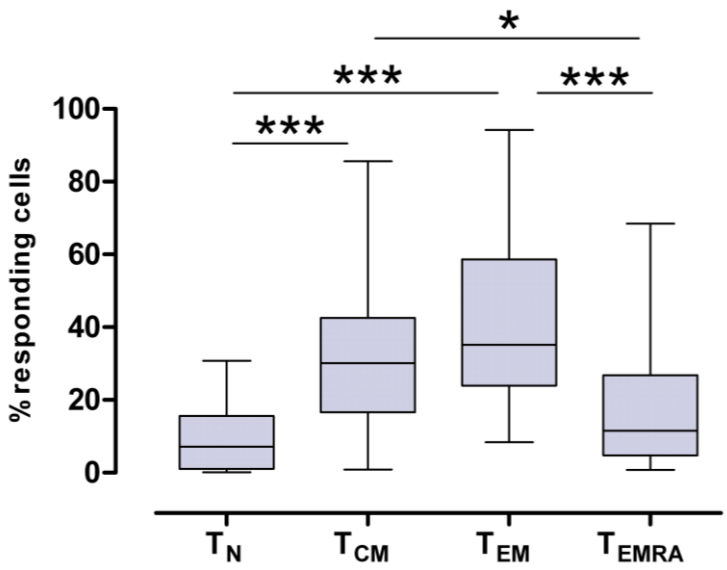
6.3.1 $CD8^+$ T cell subpopulation functional responses to activation

Multiple functional markers are currently being used to identify quality T cells i.e. polyfunctional T cells which confer enhanced viral control (Casazza et al., 2006, Kirchner et al., 2008, Betts et al., 2006). It was therefore the aim to investigate the polyfunctional responses exhibited by $CD8^+$ T cell subpopulations upon activation.

6.3.1.1 $CD8^+$ T_{EMRA} cells show low frequency responses to activation

Polyfunctional responses were measured by recording the simultaneous production of CD40L, IFN-γ, IL-2, and TNF-α of $CD8^+$ T cell subpopulations following an anti-CD3 stimulus. PBMCs from 27 young (18 CMV positive) and 12 old (9 CMV positive) donors were used for analysis. The frequency of responding cells i.e. cells responding with at least one cytokine was recorded for all cells as a percentage of the total subpopulation (figure 6.1A). $CD8^+$ T_{EMRA} cells had significantly lower numbers of responding cells compared with $CD8^+$ T_{EM} cells ($p<0.0001$) and $CD8^+$ T_{CM} cells ($p=0.01$). However, when analysing the polyfunctional profile of those responding cells (figure 6.1B) no significant difference in polyfunctionality was uncovered between the $CD8^+$ T cell subpopulations. Furthermore, when data was divided on the basis of age and CMV seropositivity (not shown) no further significant differences were uncovered on the basis of their polyfunctional responses to activation.

(A)



(B)

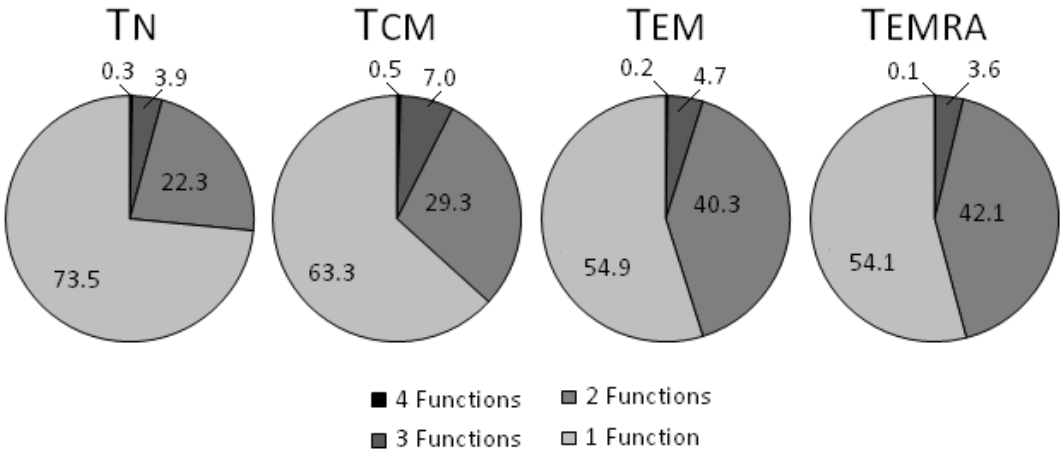


Figure 6-1. Analysing polyfunctional responses of CD8$^+$ T cell subpopulations to activation
Total PBMCs were stimulated by OKT3 and functional outputs determined by CD8$^+$ T cell subpopulation. (A) Responding cells determined by production of at least one function by subpopulation. (B) Corresponding polyfunctional profile of responding cells represented by CD40L, IL-2, IFN-γ, and TNF-α readouts in any combinations of 4, 3, 2, or 1 function expressed. Numbers shown are the mean percentages from 27 young (18 CMV positive) and 12 old (9 CMV positive) donors. Significant correlations represented by asterisks; *<0.01;**<0.001;***<0.0001.

6.3.2 CMV-specific CD45RA vs. CD45RO functional responses

In the previous section, $CD8^+$ T_{EMRA} cells showed low frequency responses to α-CD3 stimulus compared with $CD8^+$ T_{CM} and T_{EM} cells. Next, the CMV-specific response to activation was compared between CMV-specific CD45RA vs. CMV-specific CD45RO memory $CD8^+$ T cells. IFN-γ production following CMVpp65 peptide pulsed APCs stimulation was used to determine the percentage of CMV reacting cells, as IFN-γ has been shown to be the best indicator of activation (Kirchner et al., 2008). The outline for this experiment is shown in figure 6.2.

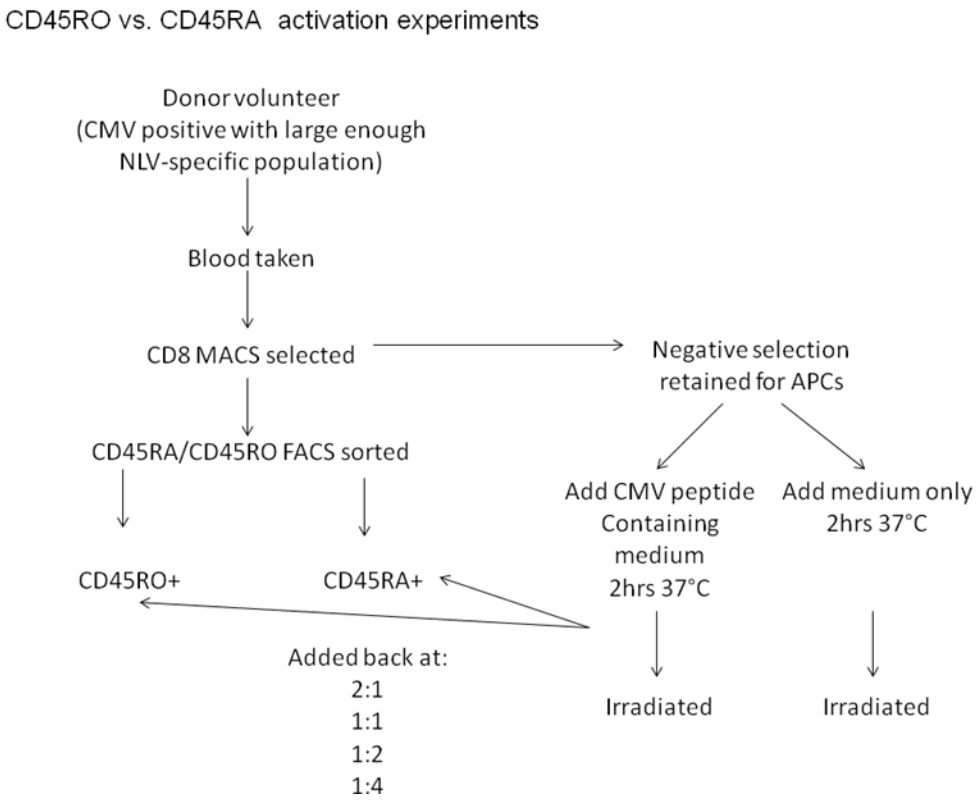


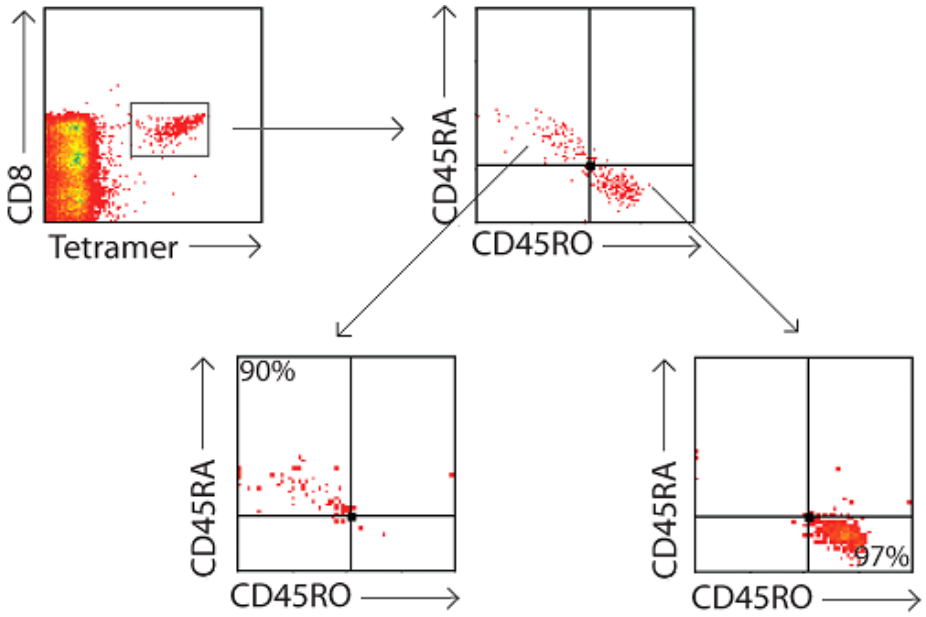
Figure 6-2. Outline for CMV-specific CD45RA vs. CD45RO $CD8^+$ T cell activation experiments

6.3.2.1 CMV-specific CD45RA$^+$ T cells show low frequency responses

CD8$^+$CD45RA$^+$ and CD8$^+$CD45RO$^+$ pure populations were sorted from HLA-matched individuals with identifiable CD45RA and CD45RO CMVpp65-specific subpopulations (representative example figure 6.3A). These subpopulations were then separately stimulated with either non-pulsed or CMVpp65-pulsed irradiated autologous APCs at various cell to cell ratios. An un-stimulated control containing medium only was also included. Focusing on the response of a representative individual (figure 6.3B); stimulation by non-specific APCs resulted in no IFN-γ production by either CMV-specific CD45RA$^+$ or CD45RO$^+$ CD8$^+$ T cell subpopulation when compared to the un-stimulated control. When stimulated with CMVpp65-pulsed APCs, IFN-γ was produced in a dose-responsive manner commensurate with increased APCs cell numbers added. The response of CMV-specific CD45RO$^+$ cells peaked at ≈30% IFN-γ$^+$ at a ratio of 1:2 cells to CMV-specific APCs, with no additive effect at a ratio of 1:4. In comparison, CMV-specific CD45RA$^+$ cells in the same individual showed lower IFN-γ production under the same conditions peaking at just ≈13% IFN-γ$^+$ at the highest ratio of 1:4 cells.

The results suggest the CMV-specific CD8$^+$ CD45RA$^+$ memory T cells show a lower functional response to activation with cognate CMV peptide compared with CD8$^+$ CMV-specific CD45RO$^+$ memory T cells.

(A)



(B)

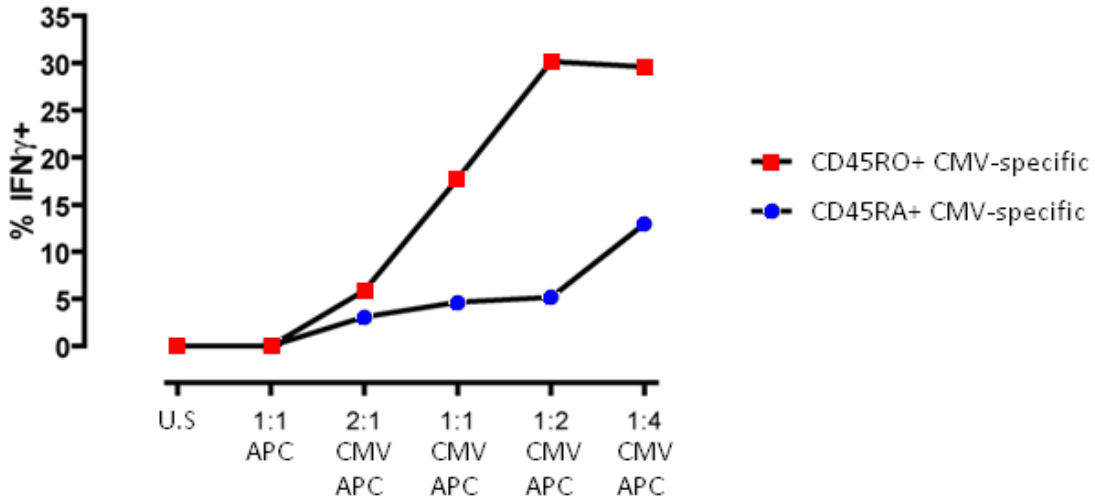


Figure 6-3. Functional response of CMV-specific CD45RA vs. CD45RO memory CD8$^+$ T cell populations
(A) FACs plots illustrating CMV-specific CD45RA$^+$ and CD45RO$^+$ CD8$^+$ T cell memory populations post sorting via FACs Aria. (B) These CMV-specific CD45RA$^+$ and CD45RO$^+$ CD8$^+$ T cell memory populations were stimulated separately with un-pulsed APCs or increasing ratios of NLV-pulsed APCs and IFN-γ response recorded. U.S. represents the un-stimulated control (containing no APCs). This is a representative of two experiments.

6.3.3 Investigating CMV-specific CD8$^+$ T cell avidity for antigen

Lower functional responses to activation may be related to T cell avidity. The Virus-specific T cell population consists a heterogeneous mixture of T cells bearing high or low affinity TCR for peptide (Villacres et al., 2003). As discussed in the introduction of this chapter, it is unclear whether high or low avidity T cells are most optimal at controlling chronic infection. Therefore, the identification of high avidity CD8$^+$ T cells within the CMV-specific population was investigated on the basis of donor age and phenotype.

6.3.3.1 Using mutated 'CD8null' tetramers to identify high avidity T cells

As discussed in chapter 1 (section 1.2.6), high avidity virus-specific CD8$^+$ T cells can be identified using mutated (CD8null) tetramers. A high avidity population can therefore be identified and compared to the normal population binding regular un-mutated tetramer, as illustrated when staining using these two tetramers within the same donor (figure 6.4).

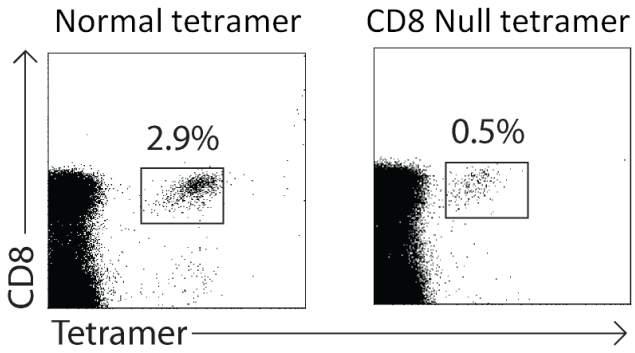


Figure 6-4. Staining with normal versus mutated (CD8 Null) tetramers
Representative FACs plot analysis showing PBMCs from one individual stained with either the normal (left) or CD8 null mutated (right) tetramers, which identify a normal or high affinity CD8$^+$ T cell population respectively. Numbers shown represent total percentage of CD8$^+$ T cells identified by relevant tetramer.

6.3.4 CMV-specific $CD8^+$ T cell avidity and ageing

The next step was to determine any influence of donor age on the avidity of the CMVpp65-specific $CD8^+$ T cell population. If indeed 'quantity over quality' is a hallmark of the $CD8^+$ T cell response to CMV in older individuals, those subjects with larger CMVpp65-specific $CD8^+$ TCEs may have a smaller percentage of high avidity T cells compared with younger individuals, with smaller CMVpp65-specific $CD8^+$ TCEs. This was therefore investigated.

6.3.4.1 Low avidity NLV-specific $CD8^+$ T cell populations in the old

All donors who were an HLA-A*02 or HLA-B*07 match were stained with both normal and CD8null tetramers specific for NLV- or TPR-specific CMVpp65 peptide respectively, and the size of these populations recorded (figures 6.5A & 6.5B). The population binding CD8null tetramer was a fraction of that binding normal tetramer (0-100%), which varied between individuals. When dividing this data into young or old age groups and by NLV- or TPR-specific populations (figure 6.5C & 6.5D); the normal NLV population and CD8null NLV population was comparable in the young, whereas in the old, the CD8null NLV population was significantly lower (<0.01) than the normal NLV population. This pattern was not seen for the TPR population. Results suggest the NLV-specific $CD8^+$ T cell population in old HLA-A*02^+ individuals is of lower avidity than in the young.

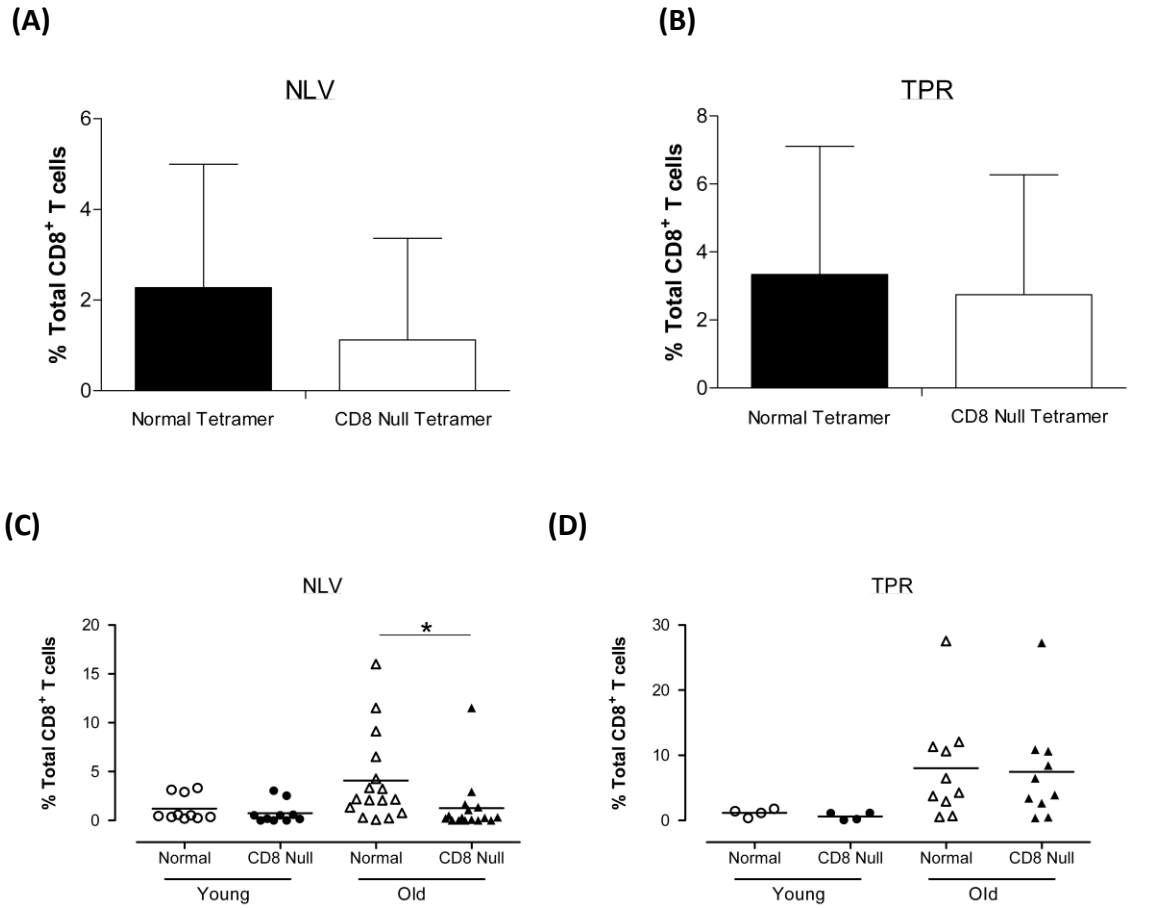


Figure 6-5. Frequency of normal and CD8null binding populations within donors
Bar chart comparing normal and CD8null tetramer binding populations identified within donors. (A) total NLV-specific normal and high CD8null populations from 30 donors. (B) TPR-specific normal and CD8null populations from 20 donors. (C) NLV-specific normal and CD8null populations by age: 10 young and 16 old donors. (D) TPR-specific normal and CD8null populations by age: 4 young and 10 old donors. Significant differences represented by asterisks; *<0.01;**<0.001;***<0.0001.

6.3.5 The relationship between avidity and CD45RA re-expression

The next step was to investigate any association between CD45RA re-expression and avidity within CMVpp65-specific $CD8^+$ T cell populations.

6.3.5.1 CD45RA expression is associated with low avidity

Expression of CD45RA/CD45RO in the normal and high avidity CMVpp65-specific populations was compared within the same individuals. In the representative example of figure 6.6A; the characteristic CD45RA/CD45RO spread in the total normal CMVpp65-specific population can be seen, with a predominately $CD45RA^+$ (i.e. 73%) subpopulation. However, within the same individual, the high avidity CMVpp65-specific population is just 15% $CD45RA^+$; the dominant subpopulation being $CD45RO^+$ (i.e. 66%). When data on 40 individuals were pooled (figure 6.6B), a highly significant negative correlation was found between the percentage of the $CD45RA^+$ memory subpopulation within the total CMVpp65-specific population and the percentage of the CMVpp65-specific population being high avidity i.e. binding the null tetramer ($p=0.0002$, $R^2=0.30$). The reverse was also true, i.e. a positive correlation when plotting the percentage of $CD45RO^+$ memory in the total CMVpp65-specific population, against the percentage of CMVpp65-specific population being high avidity (not shown). Furthermore, when the data was divided into NLV or TPR specific CMVpp65 populations, it was NLV-specific response that seemed to drive this highly significant relationship ($p<0.0001$ $R^2=0.53$, Figure 6.6C) and not the TPR-specific response ($p=0.2727$, $R^2=0.09$, Figure 6.6D).

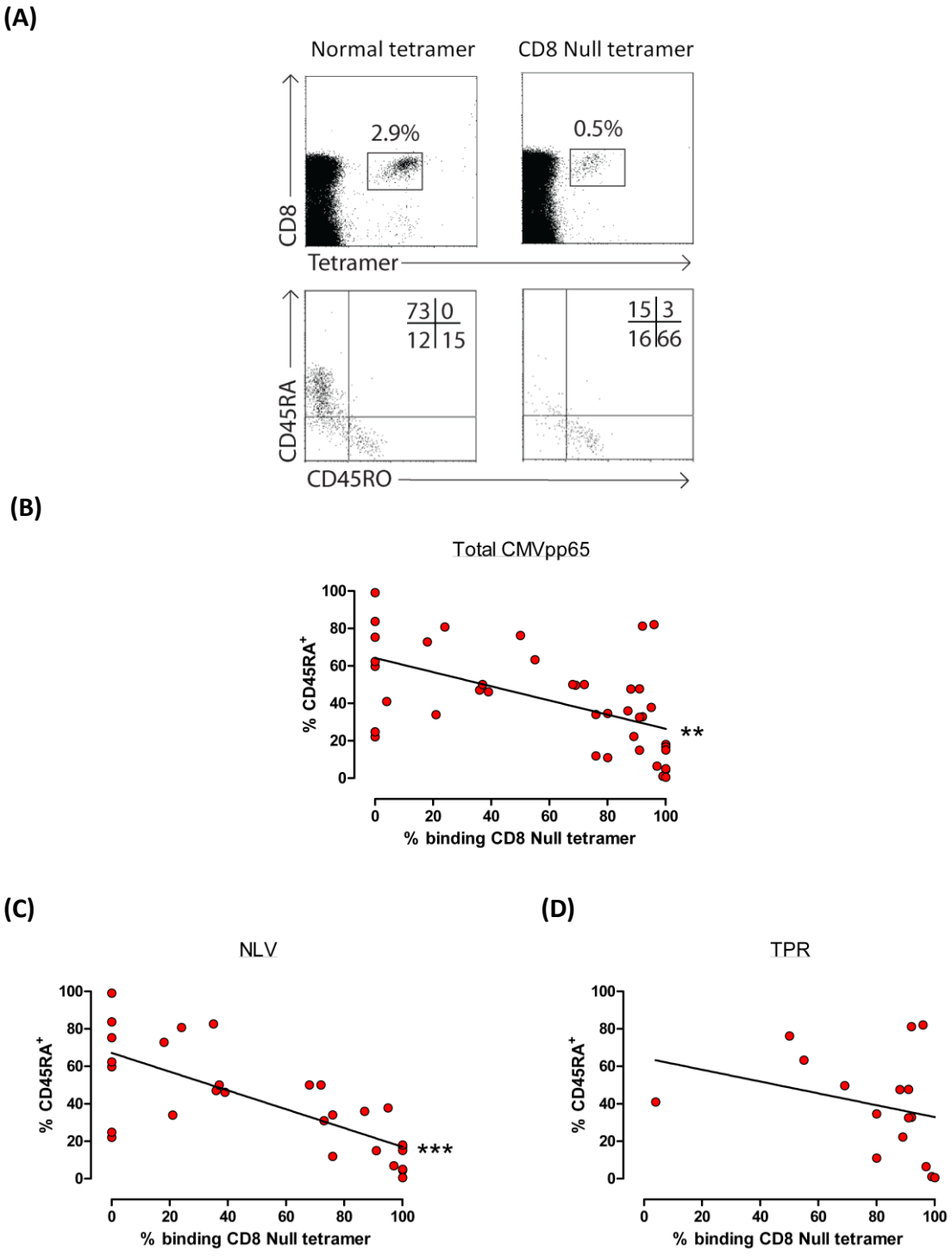


Figure 6-6. CD45RA$^+$ memory CMVpp65-specific CD8$^+$ T cells are associated with low avidity.
(A) Representative example of staining in one individual with either the Normal or High affinity NLV-specific tetramer with CD45RA and CD45RO. (B) Pooled data showing relationship between percentages CD45RA$^+$ within total (NLV- or TPR-specific populations); 45 donors shown. (C) Pooled data showing relationship between percentages CD45RA$^+$ within NLV-specific populations; 29 donors shown. (D) Pooled data showing relationship between percentages CD45RA$^+$ within TPR-specific populations; 16 donors shown. Significant correlations represented by asterisks; *<0.01;**<0.001;***<0.0001.

Although the association between CD45RA expression and T cell avidity was seen in HLA-A*02 individuals, the correlation was not significant in HLA-B*07 individuals. However, some HLA-B*07 individuals had two separate populations upon staining with the normal tetramer (figure 6.7). When comparing the CD45RA/CD45RO phenotype of these two populations, the higher MFI population showed a predominately $CD45RO^+$ phenotype and the lower MFI showed a predominately $CD45RA^+$ phenotype. This suggests that these individuals may also have CD45RA re-expressing CMV-specific T cells that are associated with lower affinity on the basis of less binding of the tetramer equating to lower MFI.

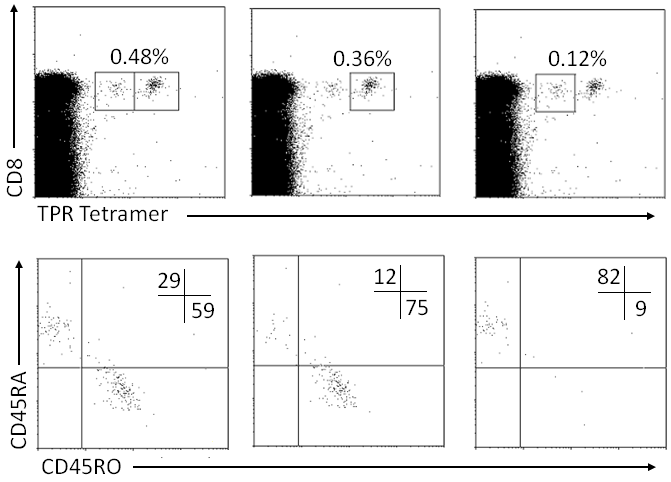


Figure 6-7. Dual populations identified with TPR-specific tetramer show alternate phenotypes
Representative example of staining in one individual with TPR-specific tetramer. From left to right, CD45RA/CD45RO expression is shown on each population of total TPR-specific, high MFI TPR-specific, and low MFI TPR-specific populations. Representative of at least 3 individuals.

6.3.6 Co-receptor availability on CD8$^+$ and CMV-specific CD8$^+$ T cells

In order for CD8$^+$ T cells to become activated, peptide must be presented to its TCR via the MHC class I molecules on the surface of the APC. To stabilise this interaction it is imperative there is adequate CD8 co-receptor availability on the T cell (Daniels and Jameson, 2000, Villacres et al., 2003). To investigate if CD8 co-receptor availability may determine T cell binding to the mutated (CD8null) tetramer observed by CD45RA$^+$ subpopulations, the surface expression levels of CD8 co-receptor was analysed on both CD8$^+$ and CMV-specific CD8$^+$ T cell subpopulations (figures 6.8A & 6.8B respectively). No difference in CD8 levels was found between the memory subpopulations, indicating no subpopulation exhibited greater CD8 availability which may account for enhanced binding to the null tetramer.

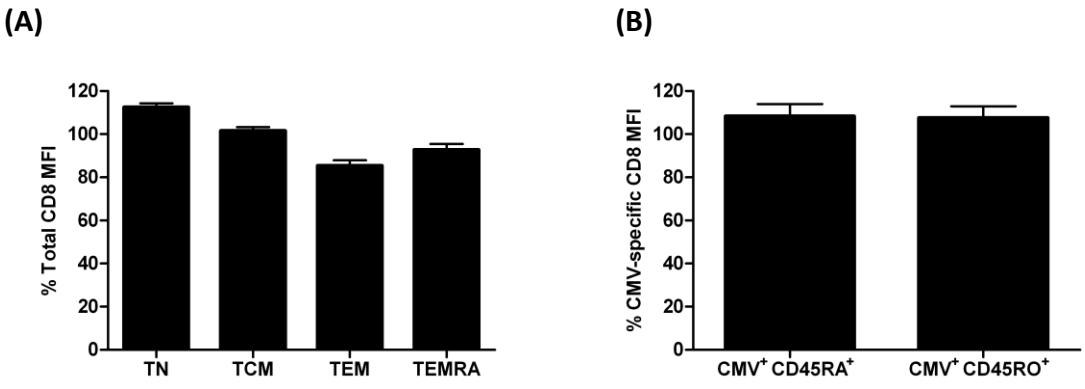


Figure 6-8. CD8 co-receptor expression in global CD8$^+$ and CMV-specific CD8$^+$ T cell subpopulations
(A) CD8 levels within the CD8$^+$ T cell subpopulations relative to total CD8 levels (set to 100%) from 10 individuals. (B) Relative levels of CD8 in CD45RA$^+$ and CD45RO$^+$ CMV-specific populations relative to total CMV-specific CD8 levels (set to 100%) from 6 individuals.

6.3.6.1 CD8null tetramers identify T cells with superior polyfunctionality

Polyfunctional responses by CMV-specific $CD8^+$ T cells have been correlated with control of CMV viremia during organ transplantation (Gratama et al., 2001, Kirchner et al., 2008, Nebbia et al., 2008). Although CMV reactivation in healthy individuals is rare, older individuals have been associated with dysfunctional responses to CMV (Almanzar et al., 2005, Ouyang et al., 2003b, Khan et al., 2004). Null tetramers identify a population of T cells with high affinity for antigen, however it is unclear whether this population also exhibits a superior functional response upon activation. It has been shown previously that $CD8^+$ T cells recognising very low antigen concentrations i.e. high-avidity $CD8^+$ T cells, are the most efficient at eliminating viruses (Alexander-Miller et al. 1996; Almeida et al. 2007; Derby et al. 2001). However, one study has shown that low-avidity $CD8^+$ T cells may be more beneficial in controlling chronic infections (Harari et al. 2007).

Whether high or low avidity CMV-specific $CD8^+$ T cells show the superior functional responses were therefore investigated, as the association of T cell avidity between age and phenotype found in the last section may have functional consequences. The functional responses of normal and high avidity CMVpp65-specific populations to CMVpp65 peptide were compared to investigate this. IFN-γ, CD107a and TNF-α were used as functional indicators of their response to activation. The outline of this experiment is summarised in figure 6.9.

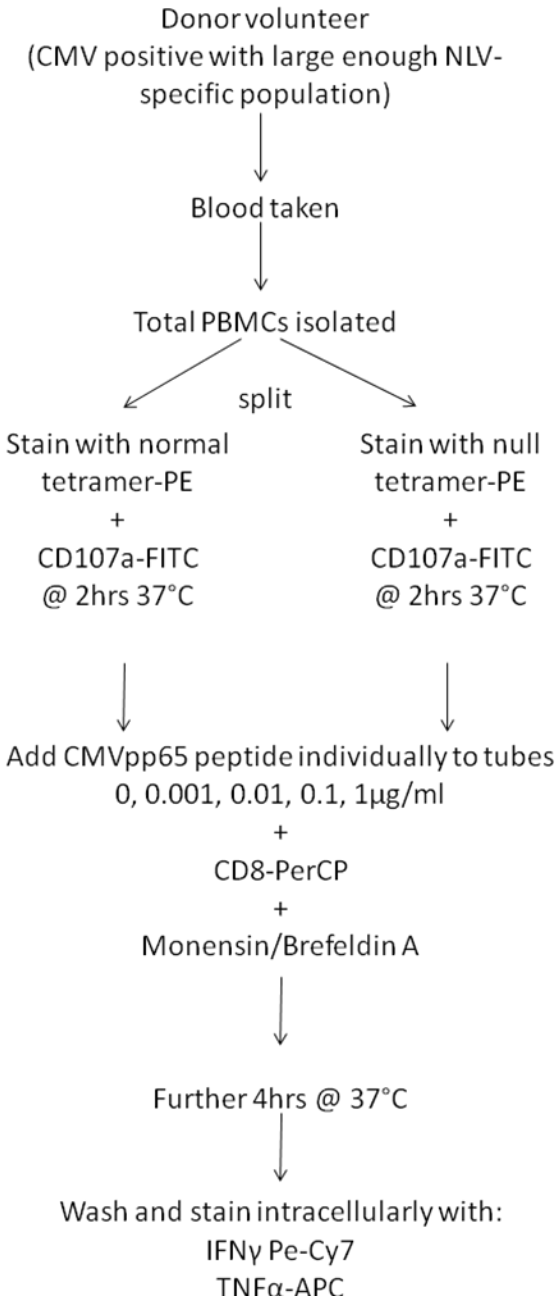


Figure 6-9. Normal vs. high avidity polyfunctionality comparison experiment
Schematic summarising the experiment used to compare the polyfunctional read-out of CMV-specific high avidity versus total normal population.

Results comparing the polyfunctional responses of the normal vs. high avidity populations are shown in figure 6.10. A representative example of one donor response (figure 6.10A), shows with stimulation at the low CMVpp65 peptide concentration of 0.001µg; the high avidity population (identified by CD8null tetramer) responded with greater production of all functional markers, relative to the normal population (identified by non-mutated tetramer), as depicted in the relevant histograms. When comparing functional responses over a peptide titration, the high avidity population produced elevated levels of each functional marker relative to the normal population at all concentrations tested (figure 6.10B).

Despite inter-individual variations in responses, findings were consistent in three different donors. When comparing responses to stimulation with 0.1µg CMVpp65 peptide, the high avidity population showed significantly higher expression of all functional markers (figure 6.10C). Results suggest that the high avidity population also exhibits superior polyfunctionality over the total normal population. Therefore, the use of CD8null tetramers gave a novel and efficient way of identifying CMVpp65-specific $CD8^+$ T cells exhibiting high polyfunctionality within a given CMVpp65-specific $CD8^+$ T cell population.

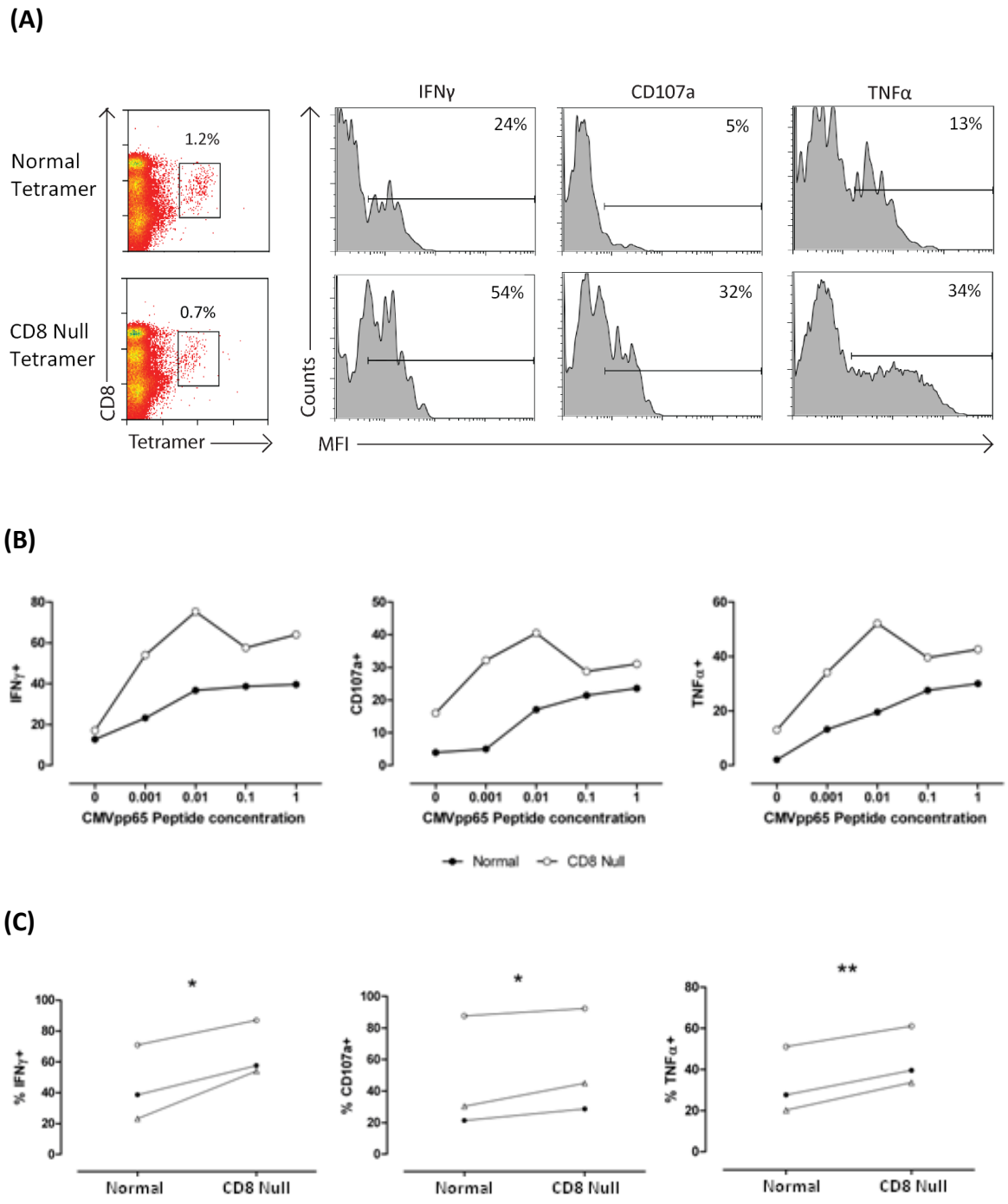


Figure 6-10. CMVpp65-specific CD8$^+$ T cell functional responses identified by avidity tetramers

(A) Representative staining of one donor identifying the normal (top) or high avidity (bottom) CMVpp65specific populations by regular tetramer and CD8 Null tetramer respectively. Relative expression of IFNγ, CD107a and TNFα following stimulation with 0.001µg CMVpp65peptide is shown for each population by relevant histograms, as percentage positivity vs. isotype control. (B) Full titration showing functional readouts following the stimulation of normal or high affinity populations with CMVpp65 peptide concentrations at 0µg, 0.001µg, 0.01µg, 0.1, and 1µg/ml. (C) Example comparison of functional output by either population following stimulation with 0.1µg CMVpp65 peptide for 3 donors. One-tailed paired T test used. Significant correlations represented by asterisks; *<0.01;**<0.001;***<0.0001.

6.4 Discussion

The aim of this chapter was to investigate if $CD8^+$ T_{EMRA} cells represented an exhausted subpopulation within the $CD8^+$ T cell compartment. The purpose was to specifically address whether $CD8^+$ T_{EMRA} cells can respond functionally upon activation. In conclusion, results showed both $CD8^+$ and CMVpp65-specific T_{EMRA} cells can exhibit a broad polyfunctional profile upon activation. However, they do so only when given a greater stimulus. Furthermore, evidence suggests it is a low avidity for antigen that may explain their higher requirement for activation. Therefore CMVpp65-specific T_{EMRA} cells are associated with lower functional avidity.

$CD8^+$ T_{EMRA} cells were found to react less efficiently to a broad antigenic stimulus relative to the other memory $CD8^+$ T cells in terms of percentage of responding cells. This was further illustrated by the fact that CMV-specific $CD45RA^+$ memory $CD8^+$ T cells responded to CMV antigen less efficiently than CMV-specific $CD45RO^+$ memory $CD8^+$ T cells based on IFN-γ production. Work presented in this chapter showed that this is likely due to an association between CD45RA re-expression and low affinity TCR for antigen within this CMVpp65-specific $CD8^+$ T cell subpopulation. It has been shown previously that some memory $CD8^+$ T cells require greater CD8 help for activation than others (Price et al., 2005, Khan et al., 2010). However, CD8 co-receptor levels in the present study were found to be similar across all $CD8^+$ and CMV-specific $CD8^+$ T cell memory subpopulations regardless of CD45RA expression, suggesting this is not the

reason for the difference in activation in this instance. However CD8 antibody blocking experiments will be needed to confirm this.

As discussed in the introduction of this chapter, debate surrounds whether a high or low avidity T cell response is most beneficial during a chronic viral infection (Almeida et al., 2007, Harari et al., 2007). In the present study it was found that high avidity $CD8^+$ T cells (as identified by binding CD8null tetramer) displayed the superior functional responses to CMV peptide. These observations are consistent with findings showing CMV-specific $CD8^+$ T cells with high avidity express all effector functions upon activation *ex-vivo* compared with low avidity $CD8^+$ T cells, which exhibited no *ex-vivo* cytotoxicity, secreted minimal IFN-γ, and did not recognise autologous infected targets (Villacres et al., 2003).

So how do CD45RA re-expressing memory T cells accumulate if they show lower functional avidity? Usually during an immune response $CD8^+$ T-cell clones that initially expand bear TCRs with a broad range of avidities for the same antigen and this is subsequently followed by a strong contraction of those T cells expressing low avidity TCR (Zehn et al). However, chronic CMV infection may lead to the exhaustion of CMV-specific $CD8^+$ T cells, as has been observed in older individuals (Ouyang et al., 2003b). Studies into antigen avidity during persistent CMV infection has found CD8 clonotypes exhibiting high antigen avidity were preferentially driven towards terminal differentiation compared with those of low antigen avidity (Price et al., 2005). Interestingly, the same authors also found low avidity clonotypes could co-exist

competitively with their high avidity counterparts. In the context of animal studies, it has been shown that mice receiving repeated challenges with Listeria monocytogenes (L.m.) can lead to L.m-specific $CD8^+$ T_{EM} cells becoming ablated and impaired while L.m-specific $CD8^+$ T_{CM} cells remain unaffected (Huster et al., 2009). This further highlights how one $CD8^+$ T cell memory subpopulation may be outlived by another during chronic infection. In this respect, CMV-specific cells with high functional avidity may be lost and replaced by CMV-specific cells with low functional avidity. Indeed, there is a finite 'immune space' and this may result in competition between such T cells (Akbar and Fletcher, 2005, Franceschi et al., 2000, Pawelec et al., 2005, Vasto et al., 2007, Ku et al., 2001).

The functional avidity of CMVpp65-specific $CD8^+$ TCEs was also analysed on the basis of donor age. Old HLA-A*02^+ individuals showed a lower frequency of cells with high functional avidity compared with younger individuals. Furthermore, the percentage of CMVpp65-specific $CD45RA^+$ memory cells was associated with a significantly lower functional avidity population within these individuals. Furthermore, on retrospective analysis, the old individual with a large CD8null binding NLV-specific population of 11.5% (figure 6.5C) was subsequently found to be 82% $CD45RO^+$, which was consistent with the association of CD45RO expression and high affinity. However, the highly significant correlation between functional avidity and CD45RA expression was due to individuals who were HLA-A*02^+ (targeting NLV peptide) although there was evidence for a similar association for those who were HLA-B*07^+ (targeting TPR peptide) in certain individuals.

Such individuals showed dual staining with tetramer that led to the identification of two distinct populations exhibiting different CD45RA/CD45RO phenotype. Indeed, the identification of dual populations with tetramer has been shown elsewhere, and may represent populations with different TCR affinities (Moss and Khan, 2004).

These results suggest different HLA-types may equate with different T cell functional avidity for antigen. Indeed, there are similar reports associating HLA type with T cell functional avidity during chronic viral infection (Almeida et al., 2007, Harari et al., 2007). Furthermore, it is known that susceptibility to various diseases can be dependent on HLA type (Blackwell et al., 2009, Burgner et al., 2006). Although CMV disease is rare in immune-competent individuals, HLA type can still influence the T cell response of these individuals (Hollsberg, 2002, Lacey et al., 2003). Indeed, a minimal number of $CD8^+$ CMVpp65-specific T cells have been estimated to be needed for protection against CMV reactivation during organ transplantations (Aubert et al., 2001, Cwynarski et al., 2001). Interestingly, these numbers seem to differ on the basis of one's HLA type (Giest et al., 2010, Koehl et al., 2008). For example, HLA-B*3501/pp65-specific $CD8^+$ T cells confer protection from CMV reactivation with significantly lower cell numbers than HLA-A*0201/pp65-specific $CD8^+$ T cells (Giest et al., 2010).

Furthermore, individuals having both HLA-A*02 and HLA-B*07 alleles show preferential and dominant T cell numbers specific for the TPR epitope of CMVpp65 over the NLV epitope (Lacey et al., 2003 and our observations). In addition, while not tested in the

present study, these HLA-A*02/B*07 positive individuals show TPR-specific T cell responses with higher cytokine production and preferential expansion over their NLV-specific T cell responses (Lacey et al., 2003). Interestingly, when stimulating with NLV peptide only (instead of overlapping pp65 peptide pool which would contain TPR peptide), NLV responses are comparable to those of HLA-A*02 single positive individuals (Lacey et al., 2003). This suggests, in the absence of immunodominant TPR stimulus, the NLV response can compensate. This is consistent with findings in this chapter, as it was shown TPR-specific T cells were uniformly high avidity T cells, which showed the greater production of cytokine compared with the total population. Immunodominance of HLA-B*07^{+} restricted epitopes over HLA-A*02^{+} restricted epitopes has also been reported in EBV immune responses (Hollsberg, 2002). One possible mechanism for this immunodominance is enhanced peptide-MHC association and processing. Evidence supporting this shows different HLA-B44 subtypes processing the same epitope do so with different speeds of epitope assembly and transportation (Khanna et al., 1997).

In conclusion, this chapter shows that CD8^{+} T_{EMRA} cells retain polyfunctionality upon activation, suggesting they are not an exhausted population. However, it seems they require a higher degree of activation to display these functional properties, and whether CMV-specific CD45RA^{+} memory CD8^{+} T cells can reach the levels displayed by CMV-specific CD45RO^{+} memory CD8^{+} T cells is questionable, and requires further investigation. Significantly, it seems that a lower avidity for antigen may be instrumental in this association.

7 General Discussion

This thesis aimed to address the impact of one proposed driver of senescence, CMV infection, on age-related changes seen within the $CD8^+$ T cell compartment. The results of this investigation will now be discussed in the light of current evidence to formulate a hypothesis on how CMV may impact immune senescence.

7.1 CMV and the Immune Risk Phenotype

The OCTO and NONA longitudinal studies (Wikby et al., 2005) identified CMV seropositivity as a significant factor within the immune risk phenotype (IRP), a cluster of immune parameters associated with a 2-4 year decreased survival rate. The expansion of highly differentiated CMV-specific $CD8^+$ T cell memory populations may provide the link between CMV and the IRP (Pawelec et al., 2006a). Therefore, data presented here showing that the age-related accumulation of highly differentiated $CD8^+$ T_{EMRA} cells within the $CD8^+$ T cell compartment is accelerated in CMV positive individuals is important. Indeed, some old individuals had >50% of their global $CD8^+$ T cell population consisting of these $CD8^+$ T_{EMRA} cells, all of which were CMV positive. Furthermore, results have shown that CMV-specific $CD8^+$ T_{EMRA} cells dominate within CMV-specific $CD8^+$ TCEs of older individuals. This is consistent with similar findings that CMVpp65-specific $CD8^+$ T cells in the elderly were predominantly $CD45RA^+$ but in the young they were mostly $CD45RO^+$ (Colonna-Romano et al., 2007). The increase in this highly differentiated $CD8^+$ T cell population may serve to impact the CD4:CD8 ratio and lower

CD27/CD28 expression of the $CD8^+$ T cell compartment, both of which are significant factors defining the IRP.

The association between CMV seropositivity, the IRP and premature mortality is currently being disputed through the large Leiden longevity study (Maier et al. unpublished observations). Furthermore, one study showed a group of long-lived individuals with a 30% decreased mortality risk appeared to be more resistant to CMV infection, and even after CMV infection were less susceptible to the CMV related immune changes (Derhovanessian et al., 2010). Interestingly, this long-lived population did not show the age-related accumulation of CD45RA re-expressing memory T cells (Derhovanessian et al., 2010). Therefore, the impact of CMV may require further definition. Are some individuals more susceptible to CMV related changes? When considering the significance of CMV seropositivity on the IRP, it must be remembered that seroprevalence varies considerably depending on racial group and socioeconomic status (Dowd et al., 2009). Therefore, CMV seropositivity may be a restrictive biomarker, identifying only those who are more likely to have encountered other infections or complications, or even those who have less competent immune responses generally. This could this explain why CMV seropositivity is associated with increased incidences of cardiovascular disease, frailty, cognitive decline, depression, stress and anxiety (Simanek et al., 2011, Schmaltz et al., 2005, Strandberg et al., 2004, Phillips et al., 2008) but is negatively associated with wealth, education, and longevity (Dowd et al., 2009, Cannon et al., 2010, Bate et al., 2010).

One proposed mechanism for the detrimental impact of CMV infection during ageing is that large CMV-specific $CD8^+$ TCEs may compete for survival niches that lead to the loss of T cells of other specificities (Akbar and Fletcher 2005; Moss and Khan 2004). The analysis of CMV-specific TCEs within individuals may therefore yield more appropriate information than just CMV seropositivity alone. Indeed, there has been much work characterising these CMV-specific TCEs (Gillespie et al., 2000, Kern et al., 1999, Khan et al., 2004, Moss and Khan, 2004, Nebbia et al., 2008, Ouyang et al., 2003a, Ouyang et al., 2004, Pita-Lopez et al., 2009, Wills et al., 1996, Wills et al., 1999, Ouyang et al., 2003b). One key question is whether these represent senescent or exhausted T cells and if these fill up the immune space requiring a large share of survival signals. Indeed, CMV-specific T cells in older individuals show lower IFN-γ responses (Hadrup et al., 2006, Ouyang et al., 2002, Ouyang et al., 2004, Khan et al., 2004) indicative of exhaustion and higher KLRG-1 (Ouyang et al., 2003a) indicative of senescence.

7.2 Senescence vs. exhaustion in T_{EMRA} cells

Results in this study showed $CD8^+$ T_{EMRA} cells are relatively prone to apoptosis through low Bcl-2 levels, and exhibit low proliferation both directly *ex-vivo* and in response to stimulation. In addition, low telomerase levels and high DNA damage through γH2AX expression was also found in this subpopulation. This collectively suggests $CD8^+$ T_{EMRA} cells are a senescent population. Similar results have also been observed with $CD4^+$ T_{EMRA} cells, which show low ki67, low Bcl-2 and low telomerase activity (Libri et al., 2011). Relatively long telomere lengths for a memory population however, indicate they may be approaching senescence through a telomere-independent mechanism, having

undergone less rounds of cell division. Indeed, turnover studies using *in-vivo* deuterated glucose labelling, suggests CMV-specific $CD8^+$ $CD45RA^+$ memory T cells may persist through an extended lifespan rather than through an increase in proliferation (Wallace et al., 2004). In doing so, have they accumulated greater DNA damage? The high γH2AX expression found in results presented here suggests they have, although γH2AX expression in CMV-specific $CD8^+$ T cells needs to be investigated also in or order to address this.

p38 MAP kinase is one of the key molecules that regulates both telomere-dependent and telomere-independent senescence (Iwasa et al., 2003, Ono and Han, 2000). Recently in our group, it has been shown that $CD4^+$ T_{EMRA} cells express high levels of total and phosphorylated forms of p38 (Dimitri et al., submitted). Furthermore, the addition of BIRB 796, an inhibitor of p38 signaling (Bagley et al., 2007), resulted in enhanced cell recovery, reduced apoptosis and enhanced telomerase activity by up to 4 fold (Dimitri et al., submitted). This suggests p38 regulates senescence-associated defects in T cells, specifically in $CD4^+$ T_{EMRA} cells. Preliminary results suggest similar findings in $CD8^+$ T_{EMRA} cells also, and work is ongoing.

Evidence indicates $CD8^+$ T_{EMRA} cells are a senescent population, but are they exhausted? Exhaustion is defined by the progressive loss of T cell function, leading to the deletion of the exhausted cell. This develops when there is a high antigenic load and non-functional antigen-specific $CD8^+$ T cells have been observed during HIV (Day et al., 2006), hepatitis

B virus (HBV) (Das et al., 2008) and hepatitis C virus (HCV) (Urbani et al., 2006, Nakamoto et al., 2009) infections. Exhausted T cells are subject to negative regulation by signaling through multiple inhibitory receptors that inhibit functional and proliferative responses (Blackburn et al., 2009). Exhaustion therefore is different from senescence in that it is initiated by signaling events and not through DNA damage responses as is found in senescent T cells (Akbar and Henson, 2011). Programmed cell death 1 (PD-1) has been shown to be the most highly expressed cell and best studied surface inhibitory receptor that regulates exhaustion in $CD8^+$ T cells (Day et al., 2006, Petrovas et al., 2006, Trautmann et al., 2006, Barber et al., 2006, Blackburn et al., 2009). PD-1 is expressed highly on highly differentiated T cells (Appay et al., 2008, Sauce et al., 2007, Shen et al., 2010, Henson et al., 2008) and the expression of PD-1 on both $CD4^+$ and $CD8^+$ T cells has been associated with CMV disease and reactivation during transplantation (Gallez-Hawkins et al., 2009). Also during transplantation, PD-1 expression on CMV-specific T cells was associated with poor functional responses and viremia in patients (Sester et al., 2008). Interestingly, expression of PD-1 is lower on $CD8^+$ T_{EMRA} cells compared with T_{EM} cells (Appay et al., 2008, Sauce et al., 2007, Shen et al., 2010) suggesting $CD8^+$ T_{EMRA} cells are not the most exhausted of the memory $CD8^+$ T cell populations according to this pathway. Indeed, high avidity $CD45RO^+$ T cells could be induced to undergo apoptosis through more frequent antigenic stimulation, which would be reflected by higher PD-1 levels compared to T_{EMRA} cells (Alexander-Miller et al., 1996).

Indeed, results presented here have shown that $CD8^+$ and CMV-specific $CD8^+$ T_{EMRA} cells are capable of exhibiting broad polyfunctional responses, including IFN-γ, CD107a, and TNF-α, supporting evidence that they are not an exhausted population. $CD4^+$ T_{EMRA} cells, despite showing signs of senescence, have also been shown to retain polyfunctionality (Libri et al., 2011). However, results from this thesis showed the polyfunctional responses to activation were marked by a lower percentage of $CD8^+$ T_{EMRA} cells responding compared to the other memory $CD8^+$ T cell subpopulations. Furthermore, the CMV-specific IFN-γ response to activation was significantly greater in $CD45RO^+$ versus $CD45RA^+$ CMV-specific $CD8^+$ T cells.

Work using mutated avidity tetramers also revealed that despite CMV-specific $CD8^+$ T cells being able to mount polyfunctional responses, the high avidity population exhibited significantly greater numbers producing each functional marker compared with the low avidity population. This is important as CD45RA re-expression on CMV-specific $CD8^+$ T cells was associated with lower functional avidity. Therefore, evidence collectively suggests CMV-specific $CD8^+$ T_{EMRA} cells may be responding less efficiently than the CMV-specific population as a whole. This may explain how the accumulation of T_{EMRA} cells within the CMV-specific compartment of old individuals could lead to an overall reduction (dysfunctional responses and senescence) of the CMV-specific compartment as a whole, compared with that in the young (Hadrup et al., 2006, Ouyang et al., 2003a, Ouyang et al., 2004, Khan et al., 2004).

7.3 Generation of T_{EMRA} cells

There is debate whether T_{EMRA} cells are driven to proliferate by antigen or are homeostatically maintained by cytokine. Initial evidence suggesting they are driven by antigen came from TCR vβ repertoire screening, which showed T_{EMRA} cells differed significantly from un-primed naive $CD8^+$ T cells and often contained large clonal expansions (Hamann et al., 1999a). $CD8^+$ T_{EMRA} cells also have shorter telomere lengths than un-primed naive T cells (Faint et al., 2001, Hamann et al., 1999a), exhibit low expression of CD27/CD28, and display various effector type properties (Wills et al., 2002, van Leeuwen et al., 2002), collectively indicating they have undergone repeated antigenic stimulation. However, the appearance of CMV-specific $CD8^+$ T_{EMRA} cells coincides with the resolution of primary CMV infection (Miles et al., 2007, Wills et al., 1999, van de Berg et al., 2008) and also correlate with clearance of CMV from the blood of pregnant women with primary CMV infection (Lilleri et al., 2008). Interestingly, lower CMV-specific $CD8^+$ T_{EMRA} cell numbers in the few months post infection, also correlated with higher chances of transmission to the new born (Lilleri et al., 2008) suggesting active CMV infection was still occurring at this point. Altogether, this suggests that T_{EMRA} cells may appear in the absence of active CMV infection.

Data presented here shows T_{EMRA} cells dominate the CMV-specific $CD8^+$ T cell compartment later in life, which suggests they appear more frequently later in infection. The argument against cytokine driven, rather than antigen driven, proliferation of T_{EMRA} cells is the prediction that cytokine would cause the expansion of all memory T cells

equally; however TCR screening of EBV-specific and CMV-specific $CD8^+$ TCEs reveals preferentially expansion of certain clones (Iancu et al., 2009, Trautmann et al., 2005). Therefore, it is possible that T_{EMRA} cells are generated from previously proliferating memory cells which are subsequently maintained by cytokine. In support of this hypothesis, IL-15 plus IL-7 was shown to cause the reversion of $CD8^+$ T cells from a $CD45RO^+CCR7^+$ (T_{CM}) to $CD45RA^+CCR7^-$ (T_{EMRA}) phenotype (Geginat et al., 2001) and IL-15 treatment alone caused EBV-specific $CD8^+CD45RO^+$ T cells to revert back to a $CD45RA^+$ phenotype *in-vitro* (Dunne et al., 2002). Further confirmation comes from data in the present study, showing CMV-specific $CD8^+CD45RO^+$ T cells can re-express CD45RA during IL-15 treatment.

Older humans have increased serum levels of pro-inflammatory cytokines such as IL-6 and TNF-α, which correlate with increased frailty and reduced life expectancy (Franceschi et al. 2007). Previous studies have shown that TNF-α can accelerate T cell differentiation *in-vitro* (Bryl et al. 2005) and inhibit telomerase activity in human $CD8^+$ T cells (Parish et al. 2009). It has also been shown that CMV can lead to increased IFN-α levels, causing T cell differentiation and telomerase down-regulation in $CD4^+$ T cells (Fletcher et al., 2005). Acute viral infections also have the potential to drive the bystander activation and proliferation of CMV and EBV specific $CD8^+$ T cells (Sandalova et al., 2010). Evidence therefore suggests the involvement of pro-inflammatory cytokines in the generation and activation of T_{EMRA} cells. Indirect evidence for this is that anti-TNF-α therapy in patients with rheumatoid arthritis depletes $CD8^+$ T_{EMRA} cells (Bruns

et al. 2009). Furthermore, supporting the hypothesis that an inflammatory cytokine environment may lead to the accumulation of T_{EMRA} cells come from results in this thesis, showing that both IFN-α and CMV lysate treatment can increase IL-15 mRNA levels *in-vitro*. Therefore a hypothesis for the generation of CMV-specific $CD8^+$ T_{EMRA} cells is proposed (figure 7.1). Based on results from this thesis and the evidence discussed so far, it is hypothesised that both CMV and ageing act synergistically to create an environment favouring the generation of CMV-specific $CD8^+$ T_{EMRA} cells from low avidity memory $CD8^+$ T cell populations.

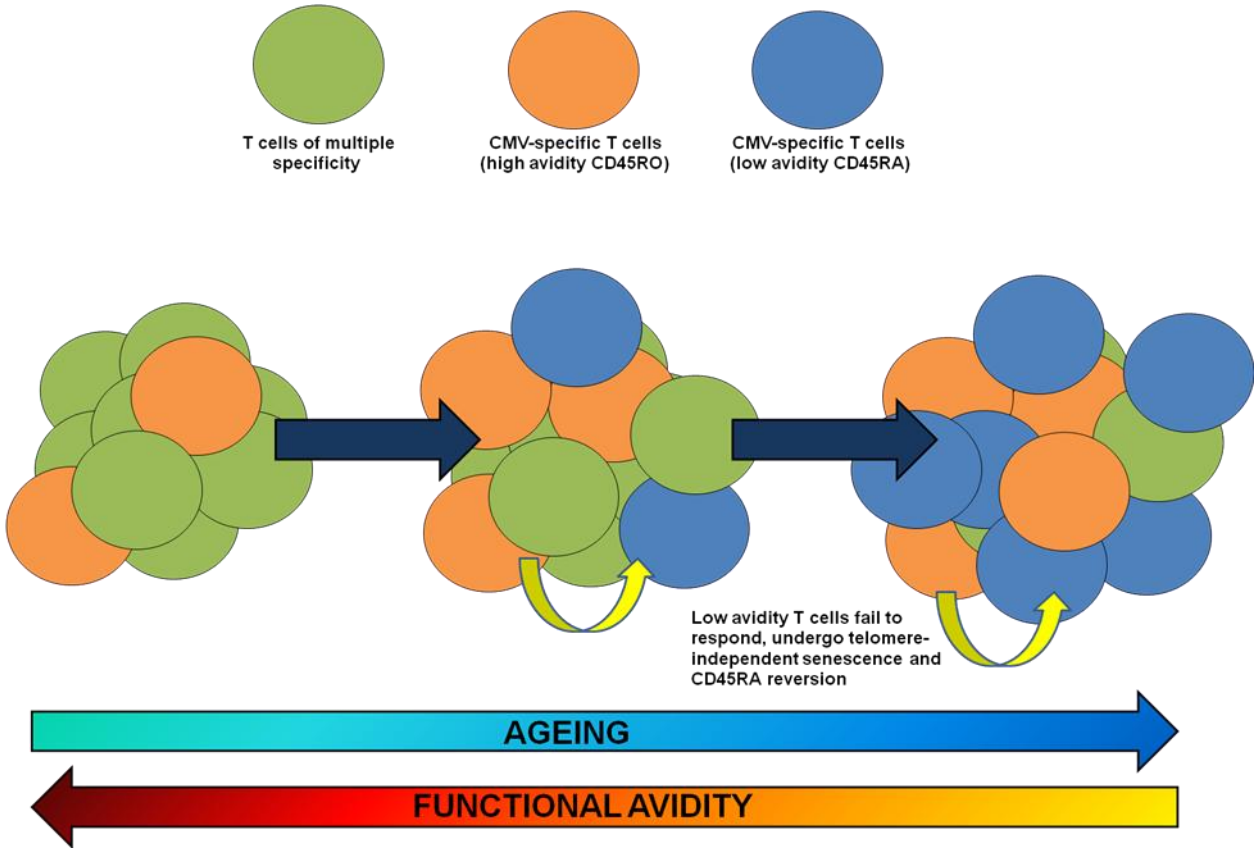


Figure 7-1. Hypothesis for the generation of CMV-specific TEMRA cells during ageing

7.4 Mechanism and purpose for reversion

What purpose does CD45RA re-expression serve? Differential expression of CD45 isoforms have been reported to influence biological function and cell signalling events, however, experiments with CD45-defective cells show the addition of chimeric molecules containing only the intracellular CD45 PTPase domain was sufficient to restore TCR-CD3 mediated cell signalling (Volarevic et al., 1993). This questions the role of the CD45 glycoprotein extracellular domain. The immune synapse formed between a T cell and its APC allows sustained MHC-TCR interaction with subsequent downstream signalling for complete activation, the large size of CD45R relative to other participants of the IS led to proposals that it is excluded from the immune synapse (Penninger et al., 2001). In support of this, CD45R was reportedly absent from membrane lipid rafts involved with signalling (Rodgers and Rose, 1996). However reversion to the larger $CD45RA^+$ glycoprotein-rich isoform could still theoretically influence activation by APCs at the immune synapse, by creating a glycoprotein barrier absent during communication of $CD45RO^+$ isoform expressing T cells with APC. Such a model compares the CD45RA isoform to a break or rheostat on the cell surface, preventing surplus activation. In fact, there is experimental evidence to support this in the form of the kinetic-segregation model (Choudhuri et al., 2005). In this respect T_{EMRA} cells may be a 'pseudo-naive' population, requiring greater and/or longer stimulation for activation than required by the traditional memory cells. The association between CD45RA re-expression and low functional avidity on CMV-specific $CD8^+$ T cells may reflect this. However, whether it is $CD8^+$ T cells expressing low avidity that are preferentially re-expressing CD45RA or

whether the process of CD45RA re-expression mechanically causes lower avidity needs further investigation. However, experiments using mouse models have shown that a single cell can differentiate into either high or low avidity $CD8^+$ T cells (Kroger and Alexander-Miller, 2007).

7.5 The impact of CMV on telomere length

The benefit of having longer telomere lengths in blood leukocytes is associated with better health (Terry et al., 2008) and more years of life (Njajou et al., 2009). The consequence of having short lymphocyte telomeres on the other hand is associated with an 8-fold increase in death from infectious diseases and a greater risk of heart disease (Cawthon et al., 2003, Spyridopoulos et al., 2009). Therefore, the finding that CMV infection in healthy individuals is associated with shorter telomeres is important and we have recently published this (van de Berg et al., 2010). The age-related attrition of telomeres was most prominent in $CD8^+$ T cells and CMV positivity was shown to significantly increase the rate of telomere attrition in $CD8^+$ T cells. Furthermore, the effect of CMV infection upon telomere length is less marked in the young age group, but highly significant in the old age group, suggesting both the decrease in naive cells (having relative long telomeres) and accumulation of memory cells (having relatively shorter telomeres) may be the root cause. Indeed, telomere lengths were longest in $CD8^+$ T_N cells and shorter in the memory populations, which was consistent with previous observations (Akbar and Vukmanovic-Stejic, 2007). However $CD8^+$ T_{EMRA} cell telomere lengths were not the shortest of the memory subpopulations but actually significantly longer than the $CD8^+$ T_{EM} cells, which is consistent with another report

(Plunkett et al., 2005). Furthermore CMV-specific T_{EMRA} cells showed significantly longer telomeres than CMV-specific $CD8^+CD45RO^+$ T cells. Despite this, these populations represent T cells with significantly shorter telomere lengths compared with naive T cells, and these highly differentiated cells correlate with shorter telomere lengths (van de Berg et al., 2010). Therefore, their accumulation contributes to lowering overall telomere length.

7.6 Clinical relevance of the study

The effective control of CMV in a clinical setting gives an insight into how CMV may be optimally controlled in healthy ageing individuals. In one study the absolute number of responding CMV-specific $CD8^+$ T cells was inversely correlated to the amount of pre-emptive ganciclovir treatment required following hematopoietic stem cell transplantation (Gratama et al., 2001), which suggests a minimal number of cells is required to confer protection. Indeed, evidence suggests a minimal number of $CD8^+$ CMVpp65-specific T cells are needed for protection against CMV reactivation during organ transplantations (Aubert et al., 2001, Cwynarski et al., 2001) and these numbers have been found to differ on the basis of an individual's HLA type (Giest et al., 2010, Koehl et al., 2008). However an adequate functional response also seems to be key in the control of CMV replication: In one study a significantly lower frequency of IFN-γ/IL-2 multi-cytokine producing $CD4^+$ and $CD8^+$ T cells were found in liver transplant recipients experiencing CMV DNAemia compared to those not having CMV DNAemia (Nebbia et al., 2008). Furthermore, in a study of renal transplant recipients, poor control of CMV replication was linked to impairments in IFN-γ production by CMV-specific $CD8^+$ T cells

which was associated with a 14-fold increased risk of progression to high level CMV replication (Mattes et al., 2008). Additionally, the authors highlighted a possible age-associated decline in function as patients >40 years of age were more likely to progress to high level CMV infection. CMV reactivation as defined by symptomatic CMV disease has also been associated with quality T cells showing high IFN-γ production versus those with symptomatic CMV reactivation which showed poor IFN-γ responses (Crough et al., 2007).

Therefore the identification of optimal CMV-specific $CD8^+$ T cells with high functional avidity presents an opportunity for the selection of those T cells in a therapeutic scenario. Recently, what started as small scale trials using adoptive transfer of CMV-specific $CD4^+$ and $CD8^+$ T cells for patients undergoing transplantation has spread due to promising preliminary results conferring protection from CMV reactivation (Feuchtinger et al., 2010, Kotton, 2010, Peggs et al., 2003, Schmitt et al., 2011, Emery et al., 2011). The engineering of TCRs specific for viral epitopes also presents a possible avenue for adoptive transfer of functionally optimal T cells (Perro et al., 2010). The benefits of which have been shown in Hepatitis B virus (HBV) infection wherein polyfunctional HBV-specific $CD8^+$ T cells can be generated (Gehring et al., 2010); this offers the possibility of reconstituting the immune response which is found to be dysfunctional in some patients with chronic HBV infection. In a previous report high avidity $CD8^+$ T cells were shown to express all effector functions upon activation *ex-vivo* compared with low avidity $CD8^+$ T cells which exhibited no *ex-vivo* cytotoxicity, secreted minimal IFNγ and did not

recognise autologous infected targets (Villacres et al., 2003). This is confirmed in the present study, as the high avidity population also showed better activation and polyfunctional potential. Therefore, in addition to the polyfunctionality of a T cell, the functional avidity of a T cell may prove a defining attribute when selecting the most optimal T cells.

Such criteria could also be applied to tackling the large CMV-specific TCEs found in older individuals. Indeed, the purging of large CMV-specific $CD8^+$ TCEs from older individuals to 'free up space' has been proposed (Koch et al., 2006, Pawelec et al., 2006b). Many of the $CD8^+$ T cells in the old have been reported as being dysfunctional when compared with those in the young (Almanzar et al., 2005, Ouyang et al., 2003b) and are believed to be disposable due to the lack of unique clonotypes contained within them (Weinberger et al., 2009). Furthermore CMV-specific $CD8^+$ TCEs often express KLRG-1, a marker identifying cells unable to undergo further division (Ouyang et al., 2003a, Voehringer et al., 2002) and senescence marker CD57 (Kern et al., 1996). Therefore purging CMV-specific $CD8^+$ TCEs on the basis of KLRG-1/CD57 co-expression could be an option, while retaining a core of polyfunctional, long-lived CMV-specific memory T cells. The latter could be evaluated therefore on the basis of their functional avidity for antigen

In addition, the senescent or exhausted cells may be reinvigorated through the blocking of markers including p38 (senescence) as well as PD-1 (exhaustion) in highly differentiated T cells (Henson et al., 2008, Akbar and Henson, 2011). An important

observation is that PD-1 signalling blockade can restore antigen-specific T cell responses (both proliferation and cytokine secretion) in LCMV-infected mice (Barber et al., 2006) and in humans with chronic viral infections (Day et al., 2006, Petrovas et al., 2006, Trautmann et al., 2006)'

Telomerase can slow senescence in cells through telomere elongation and therefore represents another possible target for intervention. Gene transduction with the catalytic component of telomerase (hTERT) was shown to result in telomere length stabilization and reduced expression of cyclin-dependent kinase inhibitors in *in-vitro* cell cultures (Effros et al., 2005). Furthermore telomerase reactivation has been successful in reversing the age-associated damage observed in aged mice (Jaskelioff et al., 2011). CMV-specific T cells with low telomerase levels could therefore benefit from such methods, however the risk of malignancy is ever present and like all potential therapies the benefits must outweigh the risks. Several diseases that are manifested by telomerase deficiencies may benefit from such therapy e.g. DKC, but the treatment of healthy individuals may be less justified. Although there are ongoing trials of a natural telomerase activator being used as part of a supplementary diet (Harley et al., 2011).

7.7 Future studies

7.7.1 Generation of high vs. low avidity T cell populations

Do low avidity CMV-specific $CD8^+$ T cells preferentially revert to a CD45RA re-expressing phenotype in response to IL-15? This may be investigated by combining the use of mutated avidity tetramers and the IL-15 treatment experiments demonstrated in this thesis.

7.7.2 Investigating other CMV antigens

Work presented here has focused on CMVpp65-specific T cell responses, as CMVpp65 has consistently been shown to illicit large T cell clonal expansions. However, T cell immune responses to CMV peptide pools has revealed a broad response, with $CD8^+$ T cells targeting many different CMV peptides (Gandhi and Khanna, 2004). Therefore, extending analysis to include different CMV antigens may reveal more on the relationship between T cell functional avidity and phenotype. IE-1 in particular has been shown to elicit large T cell numbers, which can often exceed T cell numbers specific for pp65 (Khan et al., 2002a, Kern et al., 2000). Furthermore, IE-1-specific T cell responses correlate with protection from CMV infection (Bunde et al., 2005, Nickel et al., 2009). The question is, whether IE-1-specific T cells or T cells specific for other CMV antigens share the characteristics of pp65-specific $CD8^+$ T cells, and if this is influenced by size of the population or donor age.

7.7.3 Alternate senescence pathways

Are T_{EMRA} cells undergoing a different form of senescence? Preliminary data suggests T_{EMRA} cells show high levels of DNA damage and inhibiting these pathways can up-regulate telomerase. Work will focus on investigating the CMV-specific compartment and what mechanisms may be involved and manipulated.

7.7.4 Telomere length analysis using quantum dots.

The application of quantum dots in the analysis of telomere lengths is currently being continued in lab. Future experiments aim to identify if CMV-specific $CD8^+$ T cells that have critically short telomere lengths are also associated with loss of function or senescence. Further characterisation of telomere lengths on the basis of avidity will also be addressed.

Bibliography

ABO, T. & BALCH, C. M. 1981. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). *J Immunol,* 127**,** 1024-9.

ACUTO, O. & MICHEL, F. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol,* 3**,** 939-51.

ADAMS, J. M. & CORY, S. 1998. The Bcl-2 protein family: arbiters of cell survival. *Science,* 281**,** 1322-6.

ADEMA, G. J., HARTGERS, F., VERSTRATEN, R., DE VRIES, E., MARLAND, G., MENON, S., FOSTER, J., XU, Y., NOOYEN, P., MCCLANAHAN, T., BACON, K. B. & FIGDOR, C. G. 1997. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature,* 387**,** 713-7.

ADHIKARI, N. K., FOWLER, R. A., BHAGWANJEE, S. & RUBENFELD, G. D. 2010. Critical care and the global burden of critical illness in adults. *Lancet,* 376**,** 1339-46.

AGGARWAL, S. & GUPTA, S. 1998. Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Fas ligand, Bcl-2, and Bax. *J Immunol,* 160**,** 1627-37.

AGIUS, E., LACY, K. E., VUKMANOVIC-STEJIC, M., JAGGER, A. L., PAPAGEORGIOU, A. P., HALL, S., REED, J. R., CURNOW, S. J., FUENTES-DUCULAN, J., BUCKLEY, C. D., SALMON, M., TAAMS, L. S., KRUEGER, J., GREENWOOD, J., KLEIN, N., RUSTIN, M. H. & AKBAR, A. N. 2009. Decreased TNF-alpha synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging. *J Exp Med,* 206**,** 1929-40.

AHMED, M., LANZER, K. G., YAGER, E. J., ADAMS, P. S., JOHNSON, L. L. & BLACKMAN, M. A. 2009. Clonal expansions and loss of receptor diversity in the naive CD8 T cell repertoire of aged mice. *J Immunol,* 182**,** 784-92.

AKBAR, A. N., BEVERLEY, P. C. & SALMON, M. 2004. Will telomere erosion lead to a loss of T-cell memory? *Nat Rev Immunol,* 4**,** 737-43.

AKBAR, A. N. & FLETCHER, J. M. 2005. Memory T cell homeostasis and senescence during aging. *Curr Opin Immunol,* 17**,** 480-5.

AKBAR, A. N. & HENSON, S. M. 2011. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol,* 11**,** 289-95.

AKBAR, A. N., TERRY, L., TIMMS, A., BEVERLEY, P. C. & JANOSSY, G. 1988. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. *J Immunol,* 140**,** 2171-8.

AKBAR, A. N. & VUKMANOVIC-STEJIC, M. 2007. Telomerase in T lymphocytes: use it and lose it? *J Immunol,* 178**,** 6689-94.

ALEXANDER-MILLER, M. A. 2000. Differential expansion and survival of high and low avidity cytotoxic T cell populations during the immune response to a viral infection. *Cell Immunol,* 201**,** 58-62.

ALEXANDER-MILLER, M. A., LEGGATT, G. R. & BERZOFSKY, J. A. 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc Natl Acad Sci U S A,* 93**,** 4102-7.

ALMANZAR, G., SCHWAIGER, S., JENEWEIN, B., KELLER, M., HERNDLER-BRANDSTETTER, D., WURZNER, R., SCHONITZER, D. & GRUBECK-LOEBENSTEIN, B. 2005. Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. *J Virol,* 79**,** 3675-83.

ALMEIDA, J. R., PRICE, D. A., PAPAGNO, L., ARKOUB, Z. A., SAUCE, D., BORNSTEIN, E., ASHER, T. E., SAMRI, A., SCHNURIGER, A., THEODOROU, I., COSTAGLIOLA, D., ROUZIOUX, C., AGUT, H., MARCELIN, A. G., DOUEK, D., AUTRAN, B. & APPAY, V. 2007. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med,* 204**,** 2473-85.

ALTMAN, J. D. & DAVIS, M. M. 2003. MHC-peptide tetramers to visualize antigen-specific T cells. *Curr Protoc Immunol,* Chapter 17**,** Unit 17 3.

APPAY, V., DUNBAR, P. R., CALLAN, M., KLENERMAN, P., GILLESPIE, G. M., PAPAGNO, L., OGG, G. S., KING, A., LECHNER, F., SPINA, C. A., LITTLE, S., HAVLIR, D. V., RICHMAN, D. D., GRUENER, N., PAPE, G., WATERS, A., EASTERBROOK, P., SALIO, M., CERUNDOLO, V., MCMICHAEL, A. J. & ROWLAND-JONES, S. L. 2002. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med,* 8**,** 379-85.

APPAY, V. & ROWLAND-JONES, S. L. 2004. Lessons from the study of T-cell differentiation in persistent human virus infection. *Semin Immunol,* 16**,** 205-12.

APPAY, V., VAN LIER, R. A., SALLUSTO, F. & ROEDERER, M. 2008. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A,* 73**,** 975-83.

ASHKENAZI, A. & DIXIT, V. M. 1998. Death receptors: signaling and modulation. *Science,* 281**,** 1305-8.

AUBERT, G., HASSAN-WALKER, A. F., MADRIGAL, J. A., EMERY, V. C., MORTE, C., GRACE, S., KOH, M. B., POTTER, M., PRENTICE, H. G., DODI, I. A. & TRAVERS, P. J. 2001. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. *J Infect Dis,* 184**,** 955-63.

AYDAR, Y., BALOGH, P., TEW, J. G. & SZAKAL, A. K. 2002. Age-related depression of FDC accessory functions and CD21 ligand-mediated repair of co-stimulation. *Eur J Immunol,* 32**,** 2817-26.

AZUMA, M., PHILLIPS, J. H. & LANIER, L. L. 1992. CD28 co-stimulation of T-cell-mediated cytotoxicity. *Int J Cancer Suppl,* 7**,** 33-5.

AZUMA, M., PHILLIPS, J. H. & LANIER, L. L. 1993. CD28- T lymphocytes. Antigenic and functional properties. *J Immunol,* 150**,** 1147-59.

BAGLEY, M. C., DAVIS, T., DIX, M. C., ROKICKI, M. J. & KIPLING, D. 2007. Rapid synthesis of VX-745: p38 MAP kinase inhibition in Werner syndrome cells. *Bioorg Med Chem Lett,* 17**,** 5107-10.

BAIRD, D. M. 2005. New developments in telomere length analysis. *Exp Gerontol,* 40**,** 363-8.

BANCHEREAU, J. & STEINMAN, R. M. 1998. Dendritic cells and the control of immunity. *Nature,* 392**,** 245-52.

BARBER, D. L., WHERRY, E. J., MASOPUST, D., ZHU, B., ALLISON, J. P., SHARPE, A. H., FREEMAN, G. J. & AHMED, R. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature,* 439**,** 682-7.

BATE, S. L., DOLLARD, S. C. & CANNON, M. J. 2010. Cytomegalovirus seroprevalence in the United States: the national health and nutrition examination surveys, 1988-2004. *Clin Infect Dis,* 50**,** 1439-47.

BEERMAN, I., BHATTACHARYA, D., ZANDI, S., SIGVARDSSON, M., WEISSMAN, I. L., BRYDER, D. & ROSSI, D. J. 2010. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proc Natl Acad Sci U S A,* 107**,** 5465-70.

BESTILNY, L. J., GILL, M. J., MODY, C. H. & RIABOWOL, K. T. 2000. Accelerated replicative senescence of the peripheral immune system induced by HIV infection. *AIDS,* 14**,** 771-80.

BETTS, M. R., NASON, M. C., WEST, S. M., DE ROSA, S. C., MIGUELES, S. A., ABRAHAM, J., LEDERMAN, M. M., BENITO, J. M., GOEPFERT, P. A., CONNORS, M., ROEDERER, M. & KOUP, R. A. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood,* 107**,** 4781-9.

BEVERLEY, P. C. 2010. Immune memory: the basics and how to trigger an efficient long-term immune memory. *J Comp Pathol,* 142 Suppl 1**,** S91-5.

BLACK, F. L., HIERHOLZER, W. J., PINHEIRO, F., EVANS, A. S., WOODALL, J. P., OPTON, E. M., EMMONS, J. E., WEST, B. S., EDSALL, G., DOWNS, W. G. & WALLACE, G. D. 1974. Evidence for persistence of infectious agents in isolated human populations. *Am J Epidemiol,* 100**,** 230-50.

BLACKBURN, S. D., SHIN, H., HAINING, W. N., ZOU, T., WORKMAN, C. J., POLLEY, A., BETTS, M. R., FREEMAN, G. J., VIGNALI, D. A. & WHERRY, E. J. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol,* 10**,** 29-37.

BLACKWELL, J. M., JAMIESON, S. E. & BURGNER, D. 2009. HLA and infectious diseases. *Clin Microbiol Rev,* 22**,** 370-85, Table of Contents.

BLANKENBERG, S., RUPPRECHT, H. J., BICKEL, C., ESPINOLA-KLEIN, C., RIPPIN, G., HAFNER, G., OSSENDORF, M., STEINHAGEN, K. & MEYER, J. 2001. Cytomegalovirus infection with interleukin-6 response predicts cardiac mortality in patients with coronary artery disease. *Circulation,* 103**,** 2915-21.

BOISE, L. H., GONZALEZ-GARCIA, M., POSTEMA, C. E., DING, L., LINDSTEN, T., TURKA, L. A., MAO, X., NUNEZ, G. & THOMPSON, C. B. 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell,* 74**,** 597-608.

BOSCH, J. A., FISCHER, J. E. & FISCHER, J. C. 2009. Psychologically adverse work conditions are associated with CD8+ T cell differentiation indicative of immunesenescence. *Brain Behav Immun,* 23**,** 527-34.

BOUCHER, N., DUFEU-DUCHESNE, T., VICAUT, E., FARGE, D., EFFROS, R. B. & SCHACHTER, F. 1998. CD28 expression in T cell aging and human longevity. *Exp Gerontol,* 33**,** 267-82.

BOYD, J. M., GALLO, G. J., ELANGOVAN, B., HOUGHTON, A. B., MALSTROM, S., AVERY, B. J., EBB, R. G., SUBRAMANIAN, T., CHITTENDEN, T., LUTZ, R. J. & ET AL. 1995. Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene,* 11**,** 1921-8.

BOYMAN, O., LETOURNEAU, S., KRIEG, C. & SPRENT, J. 2009. Homeostatic proliferation and survival of naive and memory T cells. *Eur J Immunol,* 39**,** 2088-94.

BROUILETTE, S., SINGH, R. K., THOMPSON, J. R., GOODALL, A. H. & SAMANI, N. J. 2003. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol,* 23**,** 842-6.

BROUILETTE, S. W., MOORE, J. S., MCMAHON, A. D., THOMPSON, J. R., FORD, I., SHEPHERD, J., PACKARD, C. J. & SAMANI, N. J. 2007. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. *Lancet,* 369**,** 107-14.

BROWN, M. G., DOKUN, A. O., HEUSEL, J. W., SMITH, H. R., BECKMAN, D. L., BLATTENBERGER, E. A., DUBBELDE, C. E., STONE, L. R., SCALZO, A. A. & YOKOYAMA, W. M. 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science,* 292**,** 934-7.

BRZEZINSKA, A. 2005. Does in vitro replicative senescence of human CD8+ cells reflect the phenotypic changes observed during in vivo ageing? *Acta Biochim Pol,* 52**,** 931-5.

BUNDE, T., KIRCHNER, A., HOFFMEISTER, B., HABEDANK, D., HETZER, R., CHEREPNEV, G., PROESCH, S., REINKE, P., VOLK, H. D., LEHMKUHL, H. & KERN, F. 2005. Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. *J Exp Med,* 201**,** 1031-6.

BURGNER, D., JAMIESON, S. E. & BLACKWELL, J. M. 2006. Genetic susceptibility to infectious diseases: big is beautiful, but will bigger be even better? *Lancet Infect Dis,* 6**,** 653-63.

BUTCHER, E. C. & PICKER, L. J. 1996. Lymphocyte homing and homeostasis. *Science,* 272**,** 60-6.

CALADO, R. T. & YOUNG, N. S. 2009. Telomere diseases. *N Engl J Med,* 361**,** 2353-65.

CALLAHAN, J. E., KAPPLER, J. W. & MARRACK, P. 1993. Unexpected expansions of CD8-bearing cells in old mice. *J Immunol,* 151**,** 6657-69.

CAMPISI, J. & D'ADDA DI FAGAGNA, F. 2007. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol,* 8**,** 729-40.

CANNON, M. J., SCHMID, D. S. & HYDE, T. B. 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Rev Med Virol,* 20**,** 202-13.

CASAZZA, J. P., BETTS, M. R., PRICE, D. A., PRECOPIO, M. L., RUFF, L. E., BRENCHLEY, J. M., HILL, B. J., ROEDERER, M., DOUEK, D. C. & KOUP, R. A. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med,* 203**,** 2865-77.

CAWTHON, R. M., SMITH, K. R., O'BRIEN, E., SIVATCHENKO, A. & KERBER, R. A. 2003. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet,* 361**,** 393-5.

CELLERAI, C., HARARI, A., STAUSS, H., YERLY, S., GERETTI, A. M., CARROLL, A., YEE, T., AINSWORTH, J., WILLIAMS, I., SWEENEY, J., FREEDMAN, A., JOHNSON, M., PANTALEO, G. & LOES, S. K. 2011. Early and Prolonged Antiretroviral Therapy Is Associated with an HIV-1-Specific T-Cell Profile Comparable to That of Long-Term Non-Progressors. *PLoS One,* 6**,** e18164.

CESARE, A. J. & REDDEL, R. R. 2010. Alternative lengthening of telomeres: models, mechanisms and implications. *Nat Rev Genet,* 11**,** 319-30.

CHAMPAGNE, P., OGG, G. S., KING, A. S., KNABENHANS, C., ELLEFSEN, K., NOBILE, M., APPAY, V., RIZZARDI, G. P., FLEURY, S., LIPP, M., FORSTER, R., ROWLAND-JONES, S., SEKALY, R. P., MCMICHAEL, A. J. & PANTALEO, G. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature,* 410**,** 106-11.

CHANG, J. T., PALANIVEL, V. R., KINJYO, I., SCHAMBACH, F., INTLEKOFER, A. M., BANERJEE, A., LONGWORTH, S. A., VINUP, K. E., MRASS, P., OLIARO, J., KILLEEN, N., ORANGE, J. S., RUSSELL, S. M., WENINGER, W. & REINER, S. L. 2007. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science,* 315**,** 1687-91.

CHAO, D. T. & KORSMEYER, S. J. 1998. BCL-2 family: regulators of cell death. *Annu Rev Immunol,* 16**,** 395-419.

CHEE, M. S., BANKIER, A. T., BECK, S., BOHNI, R., BROWN, C. M., CERNY, R., HORSNELL, T., HUTCHISON, C. A., 3RD, KOUZARIDES, T., MARTIGNETTI, J. A. & ET AL. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol,* 154**,** 125-69.

CHEN, W. F., LIU, S. L., GAO, X. M. & PANG, X. W. 1986. The capacity of lymphokine production by peripheral blood lymphocytes from aged humans. *Immunol Invest,* 15**,** 575-83.

CHIDRAWAR, S., KHAN, N., WEI, W., MCLARNON, A., SMITH, N., NAYAK, L. & MOSS, P. 2009. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. *Clin Exp Immunol,* 155**,** 423-32.

CHITTENDEN, T., HARRINGTON, E. A., O'CONNOR, R., FLEMINGTON, C., LUTZ, R. J., EVAN, G. I. & GUILD, B. C. 1995. Induction of apoptosis by the Bcl-2 homologue Bak. *Nature,* 374**,** 733-6.

CHIU, W. K., FANN, M. & WENG, N. P. 2006. Generation and growth of CD28nullCD8+ memory T cells mediated by IL-15 and its induced cytokines. *J Immunol,* 177**,** 7802-10.

CHOUDHURI, K., WISEMAN, D., BROWN, M. H., GOULD, K. & VAN DER MERWE, P. A. 2005. T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature,* 436**,** 578-82.

CHOWERS, M. Y., LANG, R., NASSAR, F., BEN-DAVID, D., GILADI, M., RUBINSHTEIN, E., ITZHAKI, A., MISHAL, J., SIEGMAN-IGRA, Y., KITZES, R., PICK, N., LANDAU, Z., WOLF, D., BIN, H., MENDELSON, E., PITLIK, S. D. & WEINBERGER, M. 2001.

Clinical characteristics of the West Nile fever outbreak, Israel, 2000. *Emerg Infect Dis,* 7**,** 675-8.

CHRISTENSEN, K., DOBLHAMMER, G., RAU, R. & VAUPEL, J. W. 2009. Ageing populations: the challenges ahead. *Lancet,* 374**,** 1196-208.

CICIN-SAIN, L., SMYK-PEARSON, S., CURRIER, N., BYRD, L., KOUDELKA, C., ROBINSON, T., SWARBRICK, G., TACKITT, S., LEGASSE, A., FISCHER, M., NIKOLICH-ZUGICH, D., PARK, B., HOBBS, T., DOANE, C. J., MORI, M., AXTHELM, M. K., LEWINSOHN, D. A. & NIKOLICH-ZUGICH, J. 2010. Loss of naive T cells and repertoire constriction predict poor response to vaccination in old primates. *J Immunol,* 184**,** 6739-45.

COLONNA-ROMANO, G., AKBAR, A. N., AQUINO, A., BULATI, M., CANDORE, G., LIO, D., AMMATUNA, P., FLETCHER, J. M., CARUSO, C. & PAWELEC, G. 2007. Impact of CMV and EBV seropositivity on CD8 T lymphocytes in an old population from West-Sicily. *Exp Gerontol,* 42**,** 995-1002.

COLONNA-ROMANO, G., BULATI, M., AQUINO, A., SCIALABBA, G., CANDORE, G., LIO, D., MOTTA, M., MALAGUARNERA, M. & CARUSO, C. 2003. B cells in the aged: CD27, CD5, and CD40 expression. *Mech Ageing Dev,* 124**,** 389-93.

COLOVAI, A. I., MIRZA, M., VLAD, G., WANG, S., HO, E., CORTESINI, R. & SUCIU-FOCA, N. 2003. Regulatory CD8+CD28- T cells in heart transplant recipients. *Hum Immunol,* 64**,** 31-7.

COTTER, T. G. 2009. Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer,* 9**,** 501-7.

CRAIG, J. M., MACAULEY, J. C., WELLER, T. H. & WIRTH, P. 1957. Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease. *Proc Soc Exp Biol Med,* 94**,** 4-12.

CRETEL, E., VEEN, I., PIERRES, A., BINAN, Y., ROBERT, P., LOUNDOU, A. D., BAUMSTARCK-BARRAU, K., HUBERT, A. M., BONGRAND, P. & HEIM, M. 2010. [Immune profile of elderly patients admitted in a geriatric short care unit.]. *Rev Med Interne*.

CROUGH, T., FAZOU, C., WEISS, J., CAMPBELL, S., DAVENPORT, M. P., BELL, S. C., GALBRAITH, A., MCNEIL, K. & KHANNA, R. 2007. Symptomatic and asymptomatic viral recrudescence in solid-organ transplant recipients and its relationship with the antigen-specific CD8(+) T-cell response. *J Virol,* 81**,** 11538-42.

CROWLEY, B., DEMPSEY, J., OLUJOHUNGBE, A., KHAN, A., MUTTON, K. & HART, C. A. 2002. Unusual manifestations of primary cytomegalovirus infection in patients without HIV infection and without organ transplants. *J Med Virol,* 68**,** 237-40.

CWYNARSKI, K., AINSWORTH, J., COBBOLD, M., WAGNER, S., MAHENDRA, P., APPERLEY, J., GOLDMAN, J., CRADDOCK, C. & MOSS, P. A. 2001. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. *Blood,* 97**,** 1232-40.

D'ADDA DI FAGAGNA, F. 2008. Living on a break: cellular senescence as a DNA-damage response. *Nat Rev Cancer,* 8**,** 512-22.

DANIELS, M. A. & JAMESON, S. C. 2000. Critical role for CD8 in T cell receptor binding and activation by peptide/major histocompatibility complex multimers. *J Exp Med,* 191**,** 335-46.

DAS, A., HOARE, M., DAVIES, N., LOPES, A. R., DUNN, C., KENNEDY, P. T., ALEXANDER, G., FINNEY, H., LAWSON, A., PLUNKETT, F. J., BERTOLETTI, A., AKBAR, A. N. & MAINI, M. K. 2008. Functional skewing of the global CD8 T cell population in chronic hepatitis B virus infection. *J Exp Med,* 205**,** 2111-24.

DAY, C. L., KAUFMANN, D. E., KIEPIELA, P., BROWN, J. A., MOODLEY, E. S., REDDY, S., MACKEY, E. W., MILLER, J. D., LESLIE, A. J., DEPIERRES, C., MNCUBE, Z., DURAISWAMY, J., ZHU, B., EICHBAUM, Q., ALTFELD, M., WHERRY, E. J., COOVADIA, H. M., GOULDER, P. J., KLENERMAN, P., AHMED, R., FREEMAN, G. J. & WALKER, B. D. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature,* 443**,** 350-4.

DE MARTINIS, M., FRANCESCHI, C., MONTI, D. & GINALDI, L. 2005. Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. *FEBS Lett,* 579**,** 2035-9.

DERBY, M., ALEXANDER-MILLER, M., TSE, R. & BERZOFSKY, J. 2001. High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL. *J Immunol,* 166**,** 1690-7.

DERHOVANESSIAN, E., MAIER, A. B., BECK, R., JAHN, G., HAHNEL, K., SLAGBOOM, P. E., DE CRAEN, A. J., WESTENDORP, R. G. & PAWELEC, G. 2010. Hallmark features of immunosenescence are absent in familial longevity. *J Immunol,* 185**,** 4618-24.

DOISNE, J. M., URRUTIA, A., LACABARATZ-PORRET, C., GOUJARD, C., MEYER, L., CHAIX, M. L., SINET, M. & VENET, A. 2004. CD8+ T cells specific for EBV, cytomegalovirus, and influenza virus are activated during primary HIV infection. *J Immunol,* 173**,** 2410-8.

DONNELLY, C. A., GHANI, A. C., LEUNG, G. M., HEDLEY, A. J., FRASER, C., RILEY, S., ABU-RADDAD, L. J., HO, L. M., THACH, T. Q., CHAU, P., CHAN, K. P., LAM, T. H., TSE, L. Y., TSANG, T., LIU, S. H., KONG, J. H., LAU, E. M., FERGUSON, N. M. & ANDERSON, R. M. 2003. Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in Hong Kong. *Lancet,* 361**,** 1761-6.

DOWD, J. B., AIELLO, A. E. & ALLEY, D. E. 2009. Socioeconomic disparities in the seroprevalence of cytomegalovirus infection in the US population: NHANES III. *Epidemiol Infect,* 137**,** 58-65.

DUNNE, P. J., BELARAMANI, L., FLETCHER, J. M., FERNANDEZ DE MATTOS, S., LAWRENZ, M., SOARES, M. V., RUSTIN, M. H., LAM, E. W., SALMON, M. & AKBAR, A. N. 2005. Quiescence and functional reprogramming of Epstein-Barr virus (EBV)-specific CD8+ T cells during persistent infection. *Blood,* 106**,** 558-65.

DUNNE, P. J., FAINT, J. M., GUDGEON, N. H., FLETCHER, J. M., PLUNKETT, F. J., SOARES, M. V., HISLOP, A. D., ANNELS, N. E., RICKINSON, A. B., SALMON, M. & AKBAR, A. N. 2002. Epstein-Barr virus-specific CD8(+) T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. *Blood,* 100**,** 933-40.

DUTTAGUPTA, P. A., BOESTEANU, A. C. & KATSIKIS, P. D. 2009. Costimulation signals for memory CD8+ T cells during viral infections. *Crit Rev Immunol,* 29**,** 469-86.

EDDLESTON, M., PEACOCK, S., JUNIPER, M. & WARRELL, D. A. 1997. Severe cytomegalovirus infection in immunocompetent patients. *Clin Infect Dis,* 24**,** 52-6.

EFFROS, R. B. 2004. Impact of the Hayflick Limit on T cell responses to infection: lessons from aging and HIV disease. *Mech Ageing Dev,* 125**,** 103-6.

EFFROS, R. B., BOUCHER, N., PORTER, V., ZHU, X., SPAULDING, C., WALFORD, R. L., KRONENBERG, M., COHEN, D. & SCHACHTER, F. 1994. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: a possible cause for both in vivo and in vitro immunosenescence. *Exp Gerontol,* 29**,** 601-9.

EFFROS, R. B., DAGARAG, M., SPAULDING, C. & MAN, J. 2005. The role of CD8+ T-cell replicative senescence in human aging. *Immunol Rev,* 205**,** 147-57.

EMERY, V. C. 2000. Tuning to the right frequency: cytotoxic T lymphocytes and cytomegalovirus. *Transplantation,* 69**,** 2241-2.

EMERY, V. C. 2001. Investigation of CMV disease in immunocompromised patients. *J Clin Pathol,* 54**,** 84-8.

EMERY, V. C., EINSELE, H., ATABANI, S. & HAQUE, T. 2011. Immunotherapy and vaccination after transplant: the present, the future. *Hematol Oncol Clin North Am,* 25**,** 215-29.

ENDL, E., STEINBACH, P., KNUCHEL, R. & HOFSTADTER, F. 1997. Analysis of cell cycle-related Ki-67 and p120 expression by flow cytometric BrdUrd-Hoechst/7AAD and immunolabeling technique. *Cytometry,* 29**,** 233-41.

EPEL, E. S., BLACKBURN, E. H., LIN, J., DHABHAR, F. S., ADLER, N. E., MORROW, J. D. & CAWTHON, R. M. 2004. Accelerated telomere shortening in response to life stress. *Proc Natl Acad Sci U S A,* 101**,** 17312-5.

FAGNONI, F. F., VESCOVINI, R., MAZZOLA, M., BOLOGNA, G., NIGRO, E., LAVAGETTO, G., FRANCESCHI, C., PASSERI, M. & SANSONI, P. 1996. Expansion of cytotoxic CD8+ CD28- T cells in healthy ageing people, including centenarians. *Immunology,* 88**,** 501-7.

FAINT, J. M., ANNELS, N. E., CURNOW, S. J., SHIELDS, P., PILLING, D., HISLOP, A. D., WU, L., AKBAR, A. N., BUCKLEY, C. D., MOSS, P. A., ADAMS, D. H., RICKINSON, A. B. & SALMON, M. 2001. Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotypic and migratory characteristics. *J Immunol,* 167**,** 212-20.

FEUCHTINGER, T., OPHERK, K., BETHGE, W. A., TOPP, M. S., SCHUSTER, F. R., WEISSINGER, E. M., MOHTY, M., OR, R., MASCHAN, M., SCHUMM, M., HAMPRECHT, K., HANDGRETINGER, R., LANG, P. & EINSELE, H. 2010. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood,* 116**,** 4360-7.

FIERRO, M. T., NOVELLI, M., QUAGLINO, P., COMESSATTI, A., FAVA, P., ORTONCELLI, M., PONTI, R. & BERNENGO, M. G. 2008. Heterogeneity of circulating CD4+ memory T-cell subsets in erythrodermic patients: CD27 analysis can help to distinguish cutaneous T-cell lymphomas from inflammatory erythroderma. *Dermatology,* 216**,** 213-21.

FLETCHER, J. M., VUKMANOVIC-STEJIC, M., DUNNE, P. J., BIRCH, K. E., COOK, J. E., JACKSON, S. E., SALMON, M., RUSTIN, M. H. & AKBAR, A. N. 2005. Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion. *J Immunol,* 175**,** 8218-25.

FOCOSI, D., BESTAGNO, M., BURRONE, O. & PETRINI, M. 2010. CD57+ T lymphocytes and functional immune deficiency. *J Leukoc Biol,* 87**,** 107-16.

FRANCESCHI, C., BONAFE, M., VALENSIN, S., OLIVIERI, F., DE LUCA, M., OTTAVIANI, E. & DE BENEDICTIS, G. 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci,* 908**,** 244-54.

FRANCESCHI, C., CAPRI, M., MONTI, D., GIUNTA, S., OLIVIERI, F., SEVINI, F., PANOURGIA, M. P., INVIDIA, L., CELANI, L., SCURTI, M., CEVENINI, E., CASTELLANI, G. C. & SALVIOLI, S. 2007. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mech Ageing Dev,* 128**,** 92-105.

FREEMAN, R. B., JR. 2009. The 'indirect' effects of cytomegalovirus infection. *Am J Transplant,* 9**,** 2453-8.

FRY, A. M., SHAY, D. K., HOLMAN, R. C., CURNS, A. T. & ANDERSON, L. J. 2005. Trends in hospitalizations for pneumonia among persons aged 65 years or older in the United States, 1988-2002. *JAMA,* 294**,** 2712-9.

GALLEZ-HAWKINS, G. M., THAO, L., PALMER, J., DAGIS, A., LI, X., FRANCK, A. E., TEGTMEIER, B., LACEY, S. F., DIAMOND, D. J., FORMAN, S. J. & ZAIA, J. A. 2009. Increased programmed death-1 molecule expression in cytomegalovirus disease and acute graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant,* 15**,** 872-80.

GANDHI, M. K. & KHANNA, R. 2004. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect Dis,* 4**,** 725-38.

GANGEMI, S., BASILE, G., MONTI, D., MERENDINO, R. A., DI PASQUALE, G., BISIGNANO, U., NICITA-MAURO, V. & FRANCESCHI, C. 2005. Age-related modifications in circulating IL-15 levels in humans. *Mediators Inflamm,* 2005**,** 245-7.

GARCIA, S., DISANTO, J. & STOCKINGER, B. 1999. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity,* 11**,** 163-71.

GEGINAT, J., LANZAVECCHIA, A. & SALLUSTO, F. 2003. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood,* 101**,** 4260-6.

GEGINAT, J., SALLUSTO, F. & LANZAVECCHIA, A. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med,* 194**,** 1711-9.

GEHRING, A. J., XUE, S. A., HO, Z. Z., TEOH, D., RUEDL, C., CHIA, A., KOH, S., LIM, S. G., MAINI, M. K., STAUSS, H. & BERTOLETTI, A. 2010. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. *J Hepatol*.

GIBSON, L., HOLMGREEN, S. P., HUANG, D. C., BERNARD, O., COPELAND, N. G., JENKINS, N. A., SUTHERLAND, G. R., BAKER, E., ADAMS, J. M. & CORY, S. 1996. bcl-w, a novel member of the bcl-2 family, promotes cell survival. *Oncogene,* 13**,** 665-75.

GIEST, S., GRACE, S., SENEGAGLIA, A. C., PASQUINI, R., GONZALO-DAGANZO, R. M., FERNANDEZ, M. N., MACKINNON, S., MADRIGAL, J. A. & TRAVERS, P. J. 2010. Cytomegalovirus-specific CD8(+) T cells targeting different HLA/peptide combinations correlate with protection but at different threshold frequencies. *Br J Haematol,* 148**,** 311-22.

GILLESPIE, G. M., WILLS, M. R., APPAY, V., O'CALLAGHAN, C., MURPHY, M., SMITH, N., SISSONS, P., ROWLAND-JONES, S., BELL, J. I. & MOSS, P. A. 2000. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. *J Virol,* 74**,** 8140-50.

GILLIS, S., KOZAK, R., DURANTE, M. & WEKSLER, M. E. 1981. Immunological studies of aging. Decreased production of and response to T cell growth factor by lymphocytes from aged humans. *J Clin Invest,* 67**,** 937-42.

GOLDEN-MASON, L., KLARQUIST, J., WAHED, A. S. & ROSEN, H. R. 2008. Cutting edge: programmed death-1 expression is increased on immunocytes in chronic hepatitis C virus and predicts failure of response to antiviral therapy: race-dependent differences. *J Immunol,* 180**,** 3637-41.

GOMEZ, C. R., NOMELLINI, V., FAUNCE, D. E. & KOVACS, E. J. 2008. Innate immunity and aging. *Exp Gerontol,* 43**,** 718-28.

GORINA, Y., KELLY, T., LUBITZ, J. & HINES, Z. 2008. Trends in influenza and pneumonia among older persons in the United States. *Aging Trends***,** 1-11.

GRATAMA, J. W., VAN ESSER, J. W., LAMERS, C. H., TOURNAY, C., LOWENBERG, B., BOLHUIS, R. L. & CORNELISSEN, J. J. 2001. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood,* 98**,** 1358-64.

HADRUP, S. R., STRINDHALL, J., KOLLGAARD, T., SEREMET, T., JOHANSSON, B., PAWELEC, G., THOR STRATEN, P. & WIKBY, A. 2006. Longitudinal studies of clonally expanded CD8 T cells reveal a repertoire shrinkage predicting mortality and an increased number of dysfunctional cytomegalovirus-specific T cells in the very elderly. *J Immunol,* 176**,** 2645-53.

HAMANN, D., BAARS, P. A., REP, M. H., HOOIBRINK, B., KERKHOF-GARDE, S. R., KLEIN, M. R. & VAN LIER, R. A. 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med,* 186**,** 1407-18.

HAMANN, D., KOSTENSE, S., WOLTHERS, K. C., OTTO, S. A., BAARS, P. A., MIEDEMA, F. & VAN LIER, R. A. 1999a. Evidence that human CD8+CD45RA+CD27- cells are induced by antigen and evolve through extensive rounds of division. *Int Immunol,* 11**,** 1027-33.

HAMANN, D., ROOS, M. T. & VAN LIER, R. A. 1999b. Faces and phases of human CD8 T-cell development. *Immunol Today,* 20**,** 177-80.

HAMMARLUND, E., LEWIS, M. W., HANSEN, S. G., STRELOW, L. I., NELSON, J. A., SEXTON, G. J., HANIFIN, J. M. & SLIFKA, M. K. 2003. Duration of antiviral immunity after smallpox vaccination. *Nat Med,* 9**,** 1131-7.

HANSHAW, J. B., STEINFELD, H. J. & WHITE, C. J. 1968. Fluorescent-antibody test for cytomegalovirus macroglobulin. *N Engl J Med,* 279**,** 566-70.

HARARI, A., CELLERAI, C., ENDERS, F. B., KOSTLER, J., CODARRI, L., TAPIA, G., BOYMAN, O., CASTRO, E., GAUDIERI, S., JAMES, I., JOHN, M., WAGNER, R., MALLAL, S. & PANTALEO, G. 2007. Skewed association of polyfunctional antigen-specific CD8 T cell populations with HLA-B genotype. *Proc Natl Acad Sci U S A,* 104**,** 16233-8.

HARARI, A., ZIMMERLI, S. C. & PANTALEO, G. 2004. Cytomegalovirus (CMV)-specific cellular immune responses. *Hum Immunol,* 65**,** 500-6.

HARLEY, C. B., LIU, W., BLASCO, M., VERA, E., ANDREWS, W. H., BRIGGS, L. A. & RAFFAELE, J. M. 2011. A natural product telomerase activator as part of a health maintenance program. *Rejuvenation Res,* 14**,** 45-56.

HAYFLICK, L. 1965. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res,* 37**,** 614-36.

HENSON, S. M., FRANZESE, O., MACAULAY, R., LIBRI, V., AZEVEDO, R. I., KIANI-ALIKHAN, S., PLUNKETT, F. J., MASTERS, J. E., JACKSON, S., GRIFFITHS, S. J., PIRCHER, H. P., SOARES, M. V. & AKBAR, A. N. 2009. KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. *Blood,* 113**,** 6619-28.

HENSON, S. M., MACAULAY, R., KIANI-ALIKHAN, S. & AKBAR, A. N. 2008. The use of the inhibitory receptors for modulating the immune responses. *Curr Pharm Des,* 14**,** 2643-50.

HERMISTON, M. L., XU, Z. & WEISS, A. 2003. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol,* 21**,** 107-37.

HINTZEN, R. Q., DE JONG, R., LENS, S. M., BROUWER, M., BAARS, P. & VAN LIER, R. A. 1993. Regulation of CD27 expression on subsets of mature T-lymphocytes. *J Immunol,* 151**,** 2426-35.

HIYAMA, K., HIRAI, Y., KYOIZUMI, S., AKIYAMA, M., HIYAMA, E., PIATYSZEK, M. A., SHAY, J. W., ISHIOKA, S. & YAMAKIDO, M. 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol,* 155**,** 3711-5.

HO, M. 2008. The history of cytomegalovirus and its diseases. *Med Microbiol Immunol,* 197**,** 65-73.

HODES, R. J., HATHCOCK, K. S. & WENG, N. P. 2002. Telomeres in T and B cells. *Nat Rev Immunol,* 2**,** 699-706.

HOLLSBERG, P. 2002. Contribution of HLA class I allele expression to CD8+ T-cell responses against Epstein-Barr virus. *Scand J Immunol,* 55**,** 189-95.

HOU, S., HYLAND, L., RYAN, K. W., PORTNER, A. & DOHERTY, P. C. 1994. Virus-specific CD8+ T-cell memory determined by clonal burst size. *Nature,* 369**,** 652-4.

HUPPERT, F. A., PINTO, E. M., MORGAN, K. & BRAYNE, C. 2003. Survival in a population sample is predicted by proportions of lymphocyte subsets. *Mech Ageing Dev,* 124**,** 449-51.

HUSTER, K. M., STEMBERGER, C., GASTEIGER, G., KASTENMULLER, W., DREXLER, I. & BUSCH, D. H. 2009. Cutting edge: memory CD8 T cell compartment grows in size with immunological experience but nevertheless can lose function. *J Immunol,* 183**,** 6898-902.

HYDE, T. B., SCHMID, D. S. & CANNON, M. J. 2010. Cytomegalovirus seroconversion rates and risk factors: implications for congenital CMV. *Rev Med Virol,* 20**,** 311-26.

IANCU, E. M., CORTHESY, P., BAUMGAERTNER, P., DEVEVRE, E., VOELTER, V., ROMERO, P., SPEISER, D. E. & RUFER, N. 2009. Clonotype selection and composition of human CD8 T cells specific for persistent herpes viruses varies with differentiation but is stable over time. *J Immunol,* 183**,** 319-31.

IWASA, H., HAN, J. & ISHIKAWA, F. 2003. Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. *Genes Cells,* 8**,** 131-44.

JASKELIOFF, M., MULLER, F. L., PAIK, J. H., THOMAS, E., JIANG, S., ADAMS, A. C., SAHIN, E., KOST-ALIMOVA, M., PROTOPOPOV, A., CADINANOS, J., HORNER, J. W., MARATOS-FLIER, E. & DEPINHO, R. A. 2011. Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. *Nature,* 469**,** 102-6.

JONES, R. B., NDHLOVU, L. C., BARBOUR, J. D., SHETH, P. M., JHA, A. R., LONG, B. R., WONG, J. C., SATKUNARAJAH, M., SCHWENEKER, M., CHAPMAN, J. M., GYENES, G., VALI, B., HYRCZA, M. D., YUE, F. Y., KOVACS, C., SASSI, A., LOUTFY, M., HALPENNY, R., PERSAD, D., SPOTTS, G., HECHT, F. M., CHUN, T. W., MCCUNE, J. M., KAUL, R., RINI, J. M., NIXON, D. F. & OSTROWSKI, M. A. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med,* 205**,** 2763-79.

KAECH, S. M., HEMBY, S., KERSH, E. & AHMED, R. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell,* 111**,** 837-51.

KALIA, V., SARKAR, S. & AHMED, R. 2010. CD8 T-cell memory differentiation during acute and chronic viral infections. *Adv Exp Med Biol,* 684**,** 79-95.

KARRER, U., SIERRO, S., WAGNER, M., OXENIUS, A., HENGEL, H., KOSZINOWSKI, U. H., PHILLIPS, R. E. & KLENERMAN, P. 2003. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. *J Immunol,* 170**,** 2022-9.

KAUFMANN, D. E. & WALKER, B. D. 2008. Programmed death-1 as a factor in immune exhaustion and activation in HIV infection. *Curr Opin HIV AIDS,* 3**,** 362-7.

KERN, F., BUNDE, T., FAULHABER, N., KIECKER, F., KHATAMZAS, E., RUDAWSKI, I. M., PRUSS, A., GRATAMA, J. W., VOLKMER-ENGERT, R., EWERT, R., REINKE, P., VOLK, H. D. & PICKER, L. J. 2002. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. *J Infect Dis,* 185**,** 1709-16.

KERN, F., FAULHABER, N., FROMMEL, C., KHATAMZAS, E., PROSCH, S., SCHONEMANN, C., KRETZSCHMAR, I., VOLKMER-ENGERT, R., VOLK, H. D. & REINKE, P. 2000. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. *Eur J Immunol,* 30**,** 1676-82.

KERN, F., KHATAMZAS, E., SUREL, I., FROMMEL, C., REINKE, P., WALDROP, S. L., PICKER, L. J. & VOLK, H. D. 1999. Distribution of human CMV-specific memory T cells

among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms. *Eur J Immunol,* 29**,** 2908-15.

KERN, F., ODE-HAKIM, S., VOGT, K., HOFLICH, C., REINKE, P. & VOLK, H. D. 1996. The enigma of CD57+CD28- T cell expansion--anergy or activation? *Clin Exp Immunol,* 104**,** 180-4.

KHALED, A. R. & DURUM, S. K. 2002. Lymphocide: cytokines and the control of lymphoid homeostasis. *Nat Rev Immunol,* 2**,** 817-30.

KHAN, N., COBBOLD, M., CUMMERSON, J. & MOSS, P. A. 2010. Persistent viral infection in humans can drive high frequency low-affinity T-cell expansions. *Immunology,* 131**,** 537-48.

KHAN, N., COBBOLD, M., KEENAN, R. & MOSS, P. A. 2002a. Comparative analysis of CD8+ T cell responses against human cytomegalovirus proteins pp65 and immediate early 1 shows similarities in precursor frequency, oligoclonality, and phenotype. *J Infect Dis,* 185**,** 1025-34.

KHAN, N., HISLOP, A., GUDGEON, N., COBBOLD, M., KHANNA, R., NAYAK, L., RICKINSON, A. B. & MOSS, P. A. 2004. Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. *J Immunol,* 173**,** 7481-9.

KHAN, N., SHARIFF, N., COBBOLD, M., BRUTON, R., AINSWORTH, J. A., SINCLAIR, A. J., NAYAK, L. & MOSS, P. A. 2002b. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J Immunol,* 169**,** 1984-92.

KHANNA, R., BURROWS, S. R., NEISIG, A., NEEFJES, J., MOSS, D. J. & SILINS, S. L. 1997. Hierarchy of Epstein-Barr virus-specific cytotoxic T-cell responses in individuals carrying different subtypes of an HLA allele: implications for epitope-based antiviral vaccines. *J Virol,* 71**,** 7429-35.

KINDT, T. J., GOLDSBY, R. A., OSBORNE, B. A. & KUBY, J. 2007. *Kuby immunology,* New York, W.H. Freeman.

KIRCHNER, A., HOFFMEISTER, B., CHEREPNEV, G. G., FUHRMANN, S., STREITZ, M., LACHMANN, R., BUNDE, T., MEIJ, P., SCHONEMANN, C., HETZER, R., LEHMKUHL, H. B., VOLKMER-ENGERT, R., VOLK, H. D., GRATAMA, J. W. & KERN, F. 2008. Dissection of the CMV specific T-cell response is required for optimized cardiac transplant monitoring. *J Med Virol,* 80**,** 1604-14.

KIRIK, O. V., BEZNIN, G. V. & KORZHEVSKII, D. E. 2009. [Proliferation markers used in histological studies]. *Morfologiia,* 136**,** 95-100.

KIRKWOOD, T. B. 2008. A systematic look at an old problem. *Nature,* 451**,** 644-7.

KLENERMAN, P., CERUNDOLO, V. & DUNBAR, P. R. 2002. Tracking T cells with tetramers: new tales from new tools. *Nat Rev Immunol,* 2**,** 263-72.

KLENERMAN, P. & HILL, A. 2005. T cells and viral persistence: lessons from diverse infections. *Nat Immunol,* 6**,** 873-9.

KOCH, S., LARBI, A., OZCELIK, D., SOLANA, R., GOUTTEFANGEAS, C., ATTIG, S., WIKBY, A., STRINDHALL, J., FRANCESCHI, C. & PAWELEC, G. 2007. Cytomegalovirus infection: a driving force in human T cell immunosenescence. *Ann N Y Acad Sci,* 1114**,** 23-35.

KOCH, S., SOLANA, R., DELA ROSA, O. & PAWELEC, G. 2006. Human cytomegalovirus infection and T cell immunosenescence: a mini review. *Mech Ageing Dev,* 127**,** 538-43.

KOEHL, U., DIRKWINKEL, E., KOENIG, M., ERBEN, S., SOERENSEN, J., BADER, P., DOERR, H. W., PREISER, W., WEISSINGER, E., KLINGEBIEL, T., MARTIN, H. & LEHRNBECHER, T. 2008. Reconstitution of cytomegalovirus specific T cells after pediatric allogeneic stem cell transplantation: results from a pilot study using a multi-allele CMV tetramer group. *Klin Padiatr,* 220**,** 348-52.

KOTTON, C. N. 2010. Management of cytomegalovirus infection in solid organ transplantation. *Nat Rev Nephrol,* 6**,** 711-21.

KOVACS, E. J., PALMER, J. L., FORTIN, C. F., FULOP, T., JR., GOLDSTEIN, D. R. & LINTON, P. J. 2009. Aging and innate immunity in the mouse: impact of intrinsic and extrinsic factors. *Trends Immunol,* 30**,** 319-24.

KOVAIOU, R. D., HERNDLER-BRANDSTETTER, D. & GRUBECK-LOEBENSTEIN, B. 2007. Age-related changes in immunity: implications for vaccination in the elderly. *Expert Rev Mol Med,* 9**,** 1-17.

KOVAIOU, R. D., WEISKIRCHNER, I., KELLER, M., PFISTER, G., CIOCA, D. P. & GRUBECK-LOEBENSTEIN, B. 2005. Age-related differences in phenotype and function of CD4+ T cells are due to a phenotypic shift from naive to memory effector CD4+ T cells. *Int Immunol,* 17**,** 1359-66.

KRAMMER, P. H., ARNOLD, R. & LAVRIK, I. N. 2007. Life and death in peripheral T cells. *Nat Rev Immunol,* 7**,** 532-42.

KROGER, C. J. & ALEXANDER-MILLER, M. A. 2007. Cutting edge: CD8+ T cell clones possess the potential to differentiate into both high- and low-avidity effector cells. *J Immunol,* 179**,** 748-51.

KRUISBEEK, A. M. 1993. Development of alpha beta T cells. *Curr Opin Immunol,* 5**,** 227-34.

KU, C. C., KAPPLER, J. & MARRACK, P. 2001. The growth of the very large CD8+ T cell clones in older mice is controlled by cytokines. *J Immunol,* 166**,** 2186-93.

KU, C. C., KOTZIN, B., KAPPLER, J. & MARRACK, P. 1997. CD8+ T-cell clones in old mice. *Immunol Rev,* 160**,** 139-44.

KUIJPERS, T. W., VOSSEN, M. T., GENT, M. R., DAVIN, J. C., ROOS, M. T., WERTHEIM-VAN DILLEN, P. M., WEEL, J. F., BAARS, P. A. & VAN LIER, R. A. 2003. Frequencies of circulating cytolytic, CD45RA+CD27-, CD8+ T lymphocytes depend on infection with CMV. *J Immunol,* 170**,** 4342-8.

KURZ, S. K., RAPP, M., STEFFENS, H. P., GRZIMEK, N. K., SCHMALZ, S. & REDDEHASE, M. J. 1999. Focal transcriptional activity of murine cytomegalovirus during latency in the lungs. *J Virol,* 73**,** 482-94.

LACEY, S. F., VILLACRES, M. C., LA ROSA, C., WANG, Z., LONGMATE, J., MARTINEZ, J., BREWER, J. C., MEKHOUBAD, S., MAAS, R., LEEDOM, J. M., FORMAN, S. J., ZAIA, J. A. & DIAMOND, D. J. 2003. Relative dominance of HLA-B*07 restricted CD8+ T-lymphocyte immune responses to human cytomegalovirus pp65 in persons sharing HLA-A*02 and HLA-B*07 alleles. *Hum Immunol,* 64**,** 440-52.

LAGES, C. S., LEWKOWICH, I., SPROLES, A., WILLS-KARP, M. & CHOUGNET, C. 2010. Partial restoration of T-cell function in aged mice by in vitro blockade of the PD-1/ PD-L1 pathway. *Aging Cell,* 9**,** 785-98.

LAGUARDIA, J. J. & GILDEN, D. H. 2001. Varicella-zoster virus: a re-emerging infection. *J Investig Dermatol Symp Proc,* 6**,** 183-7.

LAICHALK, L. L. & THORLEY-LAWSON, D. A. 2005. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J Virol,* 79**,** 1296-307.

LAMBOTIN, M., RAGHURAMAN, S., STOLL-KELLER, F., BAUMERT, T. F. & BARTH, H. 2010. A look behind closed doors: interaction of persistent viruses with dendritic cells. *Nat Rev Microbiol,* 8**,** 350-60.

LANZAVECCHIA, A. & SALLUSTO, F. 2005. Understanding the generation and function of memory T cell subsets. *Curr Opin Immunol,* 17**,** 326-32.

LARBI, A., PAWELEC, G., WONG, S. C., GOLDECK, D., TAI, J. J. & FULOP, T. 2010. Impact of age on T cell signaling: A general defect or specific alterations? *Ageing Res Rev*.

LAU, L. L., JAMIESON, B. D., SOMASUNDARAM, T. & AHMED, R. 1994. Cytotoxic T-cell memory without antigen. *Nature,* 369**,** 648-52.

LENG, S. X., QU, T., SEMBA, R. D., LI, H., YAO, X., NILLES, T., YANG, X., MANWANI, B., WALSTON, J. D., FERRUCCI, L., FRIED, L. P., MARGOLICK, J. B. & BREAM, J. H. 2011. Relationship between cytomegalovirus (CMV) IgG serology, detectable CMV DNA in peripheral monocytes, and CMV pp65(495-503)-specific CD8 (+) T cells in older adults. *Age (Dordr)*.

LIBRI, V., AZEVEDO, R. I., JACKSON, S. E., DI MITRI, D., LACHMANN, R., FUHRMANN, S., VUKMANOVIC-STEJIC, M., YONG, K., BATTISTINI, L., KERN, F., SOARES, M. V. & AKBAR, A. N. 2011. Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4+ CD45RA+ CD27 T cells: the potential involvement of interleukin-7 in this process. *Immunology,* 132**,** 326-39.

LIDEHALL, A. K., SUND, F., LUNDBERG, T., ERIKSSON, B. M., TOTTERMAN, T. H. & KORSGREN, O. 2005. T cell control of primary and latent cytomegalovirus infections in healthy subjects. *J Clin Immunol,* 25**,** 473-81.

LILLERI, D., FORNARA, C., REVELLO, M. G. & GERNA, G. 2008. Human cytomegalovirus-specific memory CD8+ and CD4+ T cell differentiation after primary infection. *J Infect Dis,* 198**,** 536-43.

LIN, E. Y., ORLOFSKY, A., WANG, H. G., REED, J. C. & PRYSTOWSKY, M. B. 1996. A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. *Blood,* 87**,** 983-92.

LINTON, P. J. & DORSHKIND, K. 2004. Age-related changes in lymphocyte development and function. *Nat Immunol,* 5**,** 133-9.

LIU, Z., TUGULEA, S., CORTESINI, R. & SUCIU-FOCA, N. 1998. Specific suppression of T helper alloreactivity by allo-MHC class I-restricted CD8+CD28- T cells. *Int Immunol,* 10**,** 775-83.

LJUBUNCIC, P. & REZNICK, A. Z. 2009. The evolutionary theories of aging revisited--a mini-review. *Gerontology,* 55**,** 205-16.

LOBETTI-BODONI, C., BERNOCCO, E., GENUARDI, E., BOCCADORO, M. & LADETTO, M. 2010. Telomeres and telomerase in normal and malignant B-cells. *Hematol Oncol,* 28**,** 157-67.

LOONEY, R. J., FALSEY, A., CAMPBELL, D., TORRES, A., KOLASSA, J., BROWER, C., MCCANN, R., MENEGUS, M., MCCORMICK, K., FRAMPTON, M., HALL, W. & ABRAHAM, G. N. 1999. Role of cytomegalovirus in the T cell changes seen in elderly individuals. *Clin Immunol,* 90**,** 213-9.

LUTZ, W., SANDERSON, W. & SCHERBOV, S. 1997. Doubling of world population unlikely. *Nature,* 387**,** 803-5.

LYNCH, H. E., GOLDBERG, G. L., CHIDGEY, A., VAN DEN BRINK, M. R., BOYD, R. & SEMPOWSKI, G. D. 2009. Thymic involution and immune reconstitution. *Trends Immunol,* 30**,** 366-73.

MA, A., KOKA, R. & BURKETT, P. 2006. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu Rev Immunol,* 24**,** 657-79.

MACALLAN, D. C., WALLACE, D., ZHANG, Y., DE LARA, C., WORTH, A. T., GHATTAS, H., GRIFFIN, G. E., BEVERLEY, P. C. & TOUGH, D. F. 2004. Rapid turnover of effector-memory CD4(+) T cells in healthy humans. *J Exp Med,* 200**,** 255-60.

MAFFEI, M., GHIOTTO, F., OCCHINO, M., BONO, M., DE SANTANNA, A., BATTINI, L., GUSELLA, G. L., FAIS, F., BRUNO, S. & CICCONE, E. 2008. Human cytomegalovirus regulates surface expression of the viral protein UL18 by means of two motifs present in the cytoplasmic tail. *J Immunol,* 180**,** 969-79.

MAKEDONAS, G. & BETTS, M. R. 2006. Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. *Springer Semin Immunopathol,* 28**,** 209-19.

MANJUNATH, N., SHANKAR, P., WAN, J., WENINGER, W., CROWLEY, M. A., HIESHIMA, K., SPRINGER, T. A., FAN, X., SHEN, H., LIEBERMAN, J. & VON ANDRIAN, U. H. 2001. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J Clin Invest,* 108**,** 871-8.

MATTES, F. M., VARGAS, A., KOPYCINSKI, J., HAINSWORTH, E. G., SWENY, P., NEBBIA, G., BAZEOS, A., LOWDELL, M., KLENERMAN, P., PHILLIPS, R. E., GRIFFITHS, P. D. & EMERY, V. C. 2008. Functional impairment of cytomegalovirus specific CD8 T cells predicts high-level replication after renal transplantation. *Am J Transplant,* 8**,** 990-9.

MCELHANEY, J. E. & DUTZ, J. P. 2008. Better influenza vaccines for older people: what will it take? *J Infect Dis,* 198**,** 632-4.

MCNEILL, L., SALMOND, R. J., COOPER, J. C., CARRET, C. K., CASSADY-CAIN, R. L., ROCHE-MOLINA, M., TANDON, P., HOLMES, N. & ALEXANDER, D. R. 2007. The differential regulation of Lck kinase phosphorylation sites by CD45 is critical for T cell receptor signaling responses. *Immunity,* 27**,** 425-37.

MCVOY, M. A. & ADLER, S. P. 1989. Immunologic evidence for frequent age-related cytomegalovirus reactivation in seropositive immunocompetent individuals. *J Infect Dis,* 160**,** 1-10.

MELENHORST, J. J., SCHEINBERG, P., CHATTOPADHYAY, P. K., LISSINA, A., GOSTICK, E., COLE, D. K., WOOLDRIDGE, L., VAN DEN BERG, H. A., BORNSTEIN, E., HENSEL, N.

F., DOUEK, D. C., ROEDERER, M., SEWELL, A. K., BARRETT, A. J. & PRICE, D. A. 2008. Detection of low avidity CD8(+) T cell populations with coreceptor-enhanced peptide-major histocompatibility complex class I tetramers. *J Immunol Methods,* 338**,** 31-9.

MESSAOUDI, I., LEMAOULT, J., GUEVARA-PATINO, J. A., METZNER, B. M. & NIKOLICH-ZUGICH, J. 2004. Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. *J Exp Med,* 200**,** 1347-58.

MESSAOUDI, I., WARNER, J. & NIKOLICH-ZUGICH, J. 2006. Age-related CD8+ T cell clonal expansions express elevated levels of CD122 and CD127 and display defects in perceiving homeostatic signals. *J Immunol,* 177**,** 2784-92.

MILES, D. J., VAN DER SANDE, M., JEFFRIES, D., KAYE, S., ISMAILI, J., OJUOLA, O., SANNEH, M., TOURAY, E. S., WAIGHT, P., ROWLAND-JONES, S., WHITTLE, H. & MARCHANT, A. 2007. Cytomegalovirus infection in Gambian infants leads to profound CD8 T-cell differentiation. *J Virol,* 81**,** 5766-76.

MILLER, R. A., GARCIA, G., KIRK, C. J. & WITKOWSKI, J. M. 1997. Early activation defects in T lymphocytes from aged mice. *Immunol Rev,* 160**,** 79-90.

MONTEIRO, J., BATLIWALLA, F., OSTRER, H. & GREGERSEN, P. K. 1996. Shortened telomeres in clonally expanded CD28-CD8+ T cells imply a replicative history that is distinct from their CD28+CD8+ counterparts. *J Immunol,* 156**,** 3587-90.

MOSS, P. & KHAN, N. 2004. CD8(+) T-cell immunity to cytomegalovirus. *Hum Immunol,* 65**,** 456-64.

MOSTASHARI, F., BUNNING, M. L., KITSUTANI, P. T., SINGER, D. A., NASH, D., COOPER, M. J., KATZ, N., LILJEBJELKE, K. A., BIGGERSTAFF, B. J., FINE, A. D., LAYTON, M. C., MULLIN, S. M., JOHNSON, A. J., MARTIN, D. A., HAYES, E. B. & CAMPBELL, G. L. 2001. Epidemic West Nile encephalitis, New York, 1999: results of a household-based seroepidemiological survey. *Lancet,* 358**,** 261-4.

MURALI-KRISHNA, K., ALTMAN, J. D., SURESH, M., SOURDIVE, D. J., ZAJAC, A. J., MILLER, J. D., SLANSKY, J. & AHMED, R. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity,* 8**,** 177-87.

MURASKO, D. M. & GOONEWARDENE, I. M. 1990. T-cell function in aging: mechanisms of decline. *Annu Rev Gerontol Geriatr,* 10**,** 71-96.

NAKAMOTO, N., CHO, H., SHAKED, A., OLTHOFF, K., VALIGA, M. E., KAMINSKI, M., GOSTICK, E., PRICE, D. A., FREEMAN, G. J., WHERRY, E. J. & CHANG, K. M. 2009. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog,* 5**,** e1000313.

NAYLOR, K., LI, G., VALLEJO, A. N., LEE, W. W., KOETZ, K., BRYL, E., WITKOWSKI, J., FULBRIGHT, J., WEYAND, C. M. & GORONZY, J. J. 2005. The influence of age on T cell generation and TCR diversity. *J Immunol,* 174**,** 7446-52.

NEBBIA, G., MATTES, F. M., SMITH, C., HAINSWORTH, E., KOPYCINSKI, J., BURROUGHS, A., GRIFFITHS, P. D., KLENERMAN, P. & EMERY, V. C. 2008. Polyfunctional cytomegalovirus-specific CD4+ and pp65 CD8+ T cells protect against high-level replication after liver transplantation. *Am J Transplant,* 8**,** 2590-9.

NICKEL, P., BOLD, G., PRESBER, F., BITI, D., BABEL, N., KREUTZER, S., PRATSCHKE, J., SCHONEMANN, C., KERN, F., VOLK, H. D. & REINKE, P. 2009. High levels of CMV-IE-1-specific memory T cells are associated with less alloimmunity and improved renal allograft function. *Transpl Immunol,* 20**,** 238-42.

NICKOLOFF, B. J. & TURKA, L. A. 1994. Immunological functions of non-professional antigen-presenting cells: new insights from studies of T-cell interactions with keratinocytes. *Immunol Today,* 15**,** 464-9.

NIJHUIS, E. W., REMARQUE, E. J., HINLOOPEN, B., VAN DER POUW-KRAAN, T., VAN LIER, R. A., LIGTHART, G. J. & NAGELKERKEN, L. 1994. Age-related increase in the fraction of CD27-CD4+ T cells and IL-4 production as a feature of CD4+ T cell differentiation in vivo. *Clin Exp Immunol,* 96**,** 528-34.

NIKOLICH-ZUGICH, J. 2008. Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nat Rev Immunol,* 8**,** 512-22.

NIKOLICH-ZUGICH, J., SLIFKA, M. K. & MESSAOUDI, I. 2004. The many important facets of T-cell repertoire diversity. *Nat Rev Immunol,* 4**,** 123-32.

NJAJOU, O. T., HSUEH, W. C., BLACKBURN, E. H., NEWMAN, A. B., WU, S. H., LI, R., SIMONSICK, E. M., HARRIS, T. M., CUMMINGS, S. R. & CAWTHON, R. M. 2009. Association between telomere length, specific causes of death, and years of healthy life in health, aging, and body composition, a population-based cohort study. *J Gerontol A Biol Sci Med Sci,* 64**,** 860-4.

O'LEARY, D. R., MARFIN, A. A., MONTGOMERY, S. P., KIPP, A. M., LEHMAN, J. A., BIGGERSTAFF, B. J., ELKO, V. L., COLLINS, P. D., JONES, J. E. & CAMPBELL, G. L. 2004. The epidemic of West Nile virus in the United States, 2002. *Vector Borne Zoonotic Dis,* 4**,** 61-70.

OGG, G. S. & MCMICHAEL, A. J. 1998. HLA-peptide tetrameric complexes. *Curr Opin Immunol,* 10**,** 393-6.

OLTVAI, Z. N., MILLIMAN, C. L. & KORSMEYER, S. J. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell,* 74**,** 609-19.

ONO, K. & HAN, J. 2000. The p38 signal transduction pathway: activation and function. *Cell Signal,* 12**,** 1-13.

OPFERMAN, J. T., OBER, B. T. & ASHTON-RICKARDT, P. G. 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science,* 283**,** 1745-8.

OPPENHEIMER-MARKS, N., BREZINSCHEK, R. I., MOHAMADZADEH, M., VITA, R. & LIPSKY, P. E. 1998. Interleukin 15 is produced by endothelial cells and increases the transendothelial migration of T cells In vitro and in the SCID mouse-human rheumatoid arthritis model In vivo. *J Clin Invest,* 101**,** 1261-72.

OUYANG, Q., WAGNER, W. M., VOEHRINGER, D., WIKBY, A., KLATT, T., WALTER, S., MULLER, C. A., PIRCHER, H. & PAWELEC, G. 2003a. Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). *Exp Gerontol,* 38**,** 911-20.

OUYANG, Q., WAGNER, W. M., WIKBY, A., REMARQUE, E. & PAWELEC, G. 2002. Compromised interferon gamma (IFN-gamma) production in the elderly to both

acute and latent viral antigen stimulation: contribution to the immune risk phenotype? *Eur Cytokine Netw,* 13**,** 392-4.

OUYANG, Q., WAGNER, W. M., WIKBY, A., WALTER, S., AUBERT, G., DODI, A. I., TRAVERS, P. & PAWELEC, G. 2003b. Large numbers of dysfunctional CD8+ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old. *J Clin Immunol,* 23**,** 247-57.

OUYANG, Q., WAGNER, W. M., ZHENG, W., WIKBY, A., REMARQUE, E. J. & PAWELEC, G. 2004. Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly. *Exp Gerontol,* 39**,** 607-13.

PARISH, C. R. 1999. Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunol Cell Biol,* 77**,** 499-508.

PASSOS, J. F., NELSON, G., WANG, C., RICHTER, T., SIMILLION, C., PROCTOR, C. J., MIWA, S., OLIJSLAGERS, S., HALLINAN, J., WIPAT, A., SARETZKI, G., RUDOLPH, K. L., KIRKWOOD, T. B. & VON ZGLINICKI, T. 2010. Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol Syst Biol,* 6**,** 347.

PASSOS, J. F. & VON ZGLINICKI, T. 2006. Oxygen free radicals in cell senescence: are they signal transducers? *Free Radic Res,* 40**,** 1277-83.

PAWELEC, G., AKBAR, A., CARUSO, C., EFFROS, R., GRUBECK-LOEBENSTEIN, B. & WIKBY, A. 2004. Is immunosenescence infectious? *Trends Immunol,* 25**,** 406-10.

PAWELEC, G., AKBAR, A., CARUSO, C., SOLANA, R., GRUBECK-LOEBENSTEIN, B. & WIKBY, A. 2005. Human immunosenescence: is it infectious? *Immunol Rev,* 205**,** 257-68.

PAWELEC, G., KOCH, S., FRANCESCHI, C. & WIKBY, A. 2006a. Human immunosenescence: does it have an infectious component? *Ann N Y Acad Sci,* 1067**,** 56-65.

PAWELEC, G., KOCH, S., GOUTTEFANGEAS, C. & WIKBY, A. 2006b. Immunorejuvenation in the elderly. *Rejuvenation Res,* 9**,** 111-6.

PEGGS, K. S., VERFUERTH, S., PIZZEY, A., KHAN, N., GUIVER, M., MOSS, P. A. & MACKINNON, S. 2003. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet,* 362**,** 1375-7.

PENNINGER, J. M., IRIE-SASAKI, J., SASAKI, T. & OLIVEIRA-DOS-SANTOS, A. J. 2001. CD45: new jobs for an old acquaintance. *Nat Immunol,* 2**,** 389-96.

PERES, A., BAUER, M., DA CRUZ, I. B., NARDI, N. B. & CHIES, J. A. 2003. Immunophenotyping and T-cell proliferative capacity in a healthy aged population. *Biogerontology,* 4**,** 289-96.

PERRO, M., TSANG, J., XUE, S. A., ESCORS, D., CESCO-GASPERE, M., POSPORI, C., GAO, L., HART, D., COLLINS, M., STAUSS, H. & MORRIS, E. C. 2010. Generation of multi-functional antigen-specific human T-cells by lentiviral TCR gene transfer. *Gene Ther,* 17**,** 721-32.

PETROVAS, C., CASAZZA, J. P., BRENCHLEY, J. M., PRICE, D. A., GOSTICK, E., ADAMS, W. C., PRECOPIO, M. L., SCHACKER, T., ROEDERER, M., DOUEK, D. C. & KOUP, R. A. 2006. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med,* 203**,** 2281-92.

PHILLIPS, A. C., CARROLL, D., KHAN, N. & MOSS, P. 2008. Cytomegalovirus is associated with depression and anxiety in older adults. *Brain Behav Immun,* 22**,** 52-5.

PITA-LOPEZ, M. L., GAYOSO, I., DELAROSA, O., CASADO, J. G., ALONSO, C., MUNOZ-GOMARIZ, E., TARAZONA, R. & SOLANA, R. 2009. Effect of ageing on CMV-specific CD8 T cells from CMV seropositive healthy donors. *Immun Ageing,* 6**,** 11.

PLUNKETT, F. J., FRANZESE, O., BELARAMANI, L. L., FLETCHER, J. M., GILMOUR, K. C., SHARIFI, R., KHAN, N., HISLOP, A. D., CARA, A., SALMON, M., GASPAR, H. B., RUSTIN, M. H., WEBSTER, D. & AKBAR, A. N. 2005. The impact of telomere erosion on memory CD8+ T cells in patients with X-linked lymphoproliferative syndrome. *Mech Ageing Dev,* 126**,** 855-65.

PLUNKETT, F. J., FRANZESE, O., FINNEY, H. M., FLETCHER, J. M., BELARAMANI, L. L., SALMON, M., DOKAL, I., WEBSTER, D., LAWSON, A. D. & AKBAR, A. N. 2007. The loss of telomerase activity in highly differentiated CD8+CD28-CD27- T cells is associated with decreased Akt (Ser473) phosphorylation. *J Immunol,* 178**,** 7710-9.

PLUNKETT, F. J., SOARES, M. V., ANNELS, N., HISLOP, A., IVORY, K., LOWDELL, M., SALMON, M., RICKINSON, A. & AKBAR, A. N. 2001. The flow cytometric analysis of telomere length in antigen-specific CD8+ T cells during acute Epstein-Barr virus infection. *Blood,* 97**,** 700-7.

POLIC, B., HENGEL, H., KRMPOTIC, A., TRGOVCICH, J., PAVIC, I., LUCCARONIN, P., JONJIC, S. & KOSZINOWSKI, U. H. 1998. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. *J Exp Med,* 188**,** 1047-54.

POSNETT, D. N., SINHA, R., KABAK, S. & RUSSO, C. 1994. Clonal populations of T cells in normal elderly humans: the T cell equivalent to "benign monoclonal gammapathy". *J Exp Med,* 179**,** 609-18.

PRICE, D. A., BRENCHLEY, J. M., RUFF, L. E., BETTS, M. R., HILL, B. J., ROEDERER, M., KOUP, R. A., MIGUELES, S. A., GOSTICK, E., WOOLDRIDGE, L., SEWELL, A. K., CONNORS, M. & DOUEK, D. C. 2005. Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. *J Exp Med,* 202**,** 1349-61.

QUINNAN, G. V., JR., KIRMANI, N., ROOK, A. H., MANISCHEWITZ, J. F., JACKSON, L., MORESCHI, G., SANTOS, G. W., SARAL, R. & BURNS, W. H. 1982. Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N Engl J Med,* 307**,** 7-13.

RATTAN, S. I. 2006. Theories of biological aging: genes, proteins, and free radicals. *Free Radic Res,* 40**,** 1230-8.

REED, J. R., VUKMANOVIC-STEJIC, M., FLETCHER, J. M., SOARES, M. V., COOK, J. E., ORTEU, C. H., JACKSON, S. E., BIRCH, K. E., FOSTER, G. R., SALMON, M., BEVERLEY, P. C., RUSTIN, M. H. & AKBAR, A. N. 2004. Telomere erosion in memory T cells induced by telomerase inhibition at the site of antigenic challenge in vivo. *J Exp Med,* 199**,** 1433-43.

REVELLO, M. G. & GERNA, G. 2010. Human cytomegalovirus tropism for endothelial/epithelial cells: scientific background and clinical implications. *Rev Med Virol,* 20**,** 136-55.

ROBERTS, E. T., HAAN, M. N., DOWD, J. B. & AIELLO, A. E. 2010. Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up. *Am J Epidemiol,* 172**,** 363-71.

RODGERS, W. & ROSE, J. K. 1996. Exclusion of CD45 inhibits activity of p56lck associated with glycolipid-enriched membrane domains. *J Cell Biol,* 135**,** 1515-23.

ROOS, M. T., VAN LIER, R. A., HAMANN, D., KNOL, G. J., VERHOOFSTAD, I., VAN BAARLE, D., MIEDEMA, F. & SCHELLEKENS, P. T. 2000. Changes in the composition of circulating CD8+ T cell subsets during acute epstein-barr and human immunodeficiency virus infections in humans. *J Infect Dis,* 182**,** 451-8.

RUDOLPH, M. G., STANFIELD, R. L. & WILSON, I. A. 2006. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol,* 24**,** 419-66.

RUFER, N., BRUMMENDORF, T. H., KOLVRAA, S., BISCHOFF, C., CHRISTENSEN, K., WADSWORTH, L., SCHULZER, M. & LANSDORP, P. M. 1999. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J Exp Med,* 190**,** 157-67.

RUFER, N., DRAGOWSKA, W., THORNBURY, G., ROOSNEK, E. & LANSDORP, P. M. 1998. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat Biotechnol,* 16**,** 743-7.

SAKUISHI, K., APETOH, L., SULLIVAN, J. M., BLAZAR, B. R., KUCHROO, V. K. & ANDERSON, A. C. 2010. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med,* 207**,** 2187-94.

SALLUSTO, F., GEGINAT, J. & LANZAVECCHIA, A. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol,* 22**,** 745-63.

SALLUSTO, F., LENIG, D., FORSTER, R., LIPP, M. & LANZAVECCHIA, A. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature,* 401**,** 708-12.

SALMON, M., PILLING, D., BORTHWICK, N. J., VINER, N., JANOSSY, G., BACON, P. A. & AKBAR, A. N. 1994. The progressive differentiation of primed T cells is associated with an increasing susceptibility to apoptosis. *Eur J Immunol,* 24**,** 892-9.

SAMANI, N. J., BOULTBY, R., BUTLER, R., THOMPSON, J. R. & GOODALL, A. H. 2001. Telomere shortening in atherosclerosis. *Lancet,* 358**,** 472-3.

SANDALOVA, E., LACCABUE, D., BONI, C., TAN, A. T., FINK, K., OOI, E. E., CHUA, R., SHAFAEDDIN SCHREVE, B., FERRARI, C. & BERTOLETTI, A. 2010. Contribution of herpesvirus specific CD8 T cells to anti-viral T cell response in humans. *PLoS Pathog,* 6.

SATRA, M., DALEKOS, G. N., KOLLIA, P., VAMVAKOPOULOS, N. & TSEZOU, A. 2005. Telomerase reverse transcriptase mRNA expression in peripheral lymphocytes of patients with chronic HBV and HCV infections. *J Viral Hepat,* 12**,** 488-93.

SAUCE, D., ALMEIDA, J. R., LARSEN, M., HARO, L., AUTRAN, B., FREEMAN, G. J. & APPAY, V. 2007. PD-1 expression on human CD8 T cells depends on both state of differentiation and activation status. *AIDS,* 21**,** 2005-13.

SAUCE, D., LARSEN, M., CURNOW, S. J., LEESE, A. M., MOSS, P. A., HISLOP, A. D., SALMON, M. & RICKINSON, A. B. 2006. EBV-associated mononucleosis leads to long-term global deficit in T-cell responsiveness to IL-15. *Blood,* 108**,** 11-8.

SCHEEL-TOELLNER, D., RAZA, K., ASSI, L., PILLING, D., ROSS, E. J., LEE, W. Y., CURNOW, S. J., BUCKLEY, C. D., AKBAR, A. N., LORD, J. M. & SALMON, M. 2008. Differential regulation of nuclear and mitochondrial Bcl-2 in T cell apoptosis. *Apoptosis,* 13**,** 109-17.

SCHINDOWSKI, K., FROHLICH, L., MAURER, K., MULLER, W. E. & ECKERT, A. 2002. Age-related impairment of human T lymphocytes' activation: specific differences between CD4(+) and CD8(+) subsets. *Mech Ageing Dev,* 123**,** 375-90.

SCHLEISS, M. R. 2008. Comparison of vaccine strategies against congenital CMV infection in the guinea pig model. *J Clin Virol,* 41**,** 224-30.

SCHLUNS, K. S., KIEPER, W. C., JAMESON, S. C. & LEFRANCOIS, L. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol,* 1**,** 426-32.

SCHMALTZ, H. N., FRIED, L. P., XUE, Q. L., WALSTON, J., LENG, S. X. & SEMBA, R. D. 2005. Chronic cytomegalovirus infection and inflammation are associated with prevalent frailty in community-dwelling older women. *J Am Geriatr Soc,* 53**,** 747-54.

SCHMITT, A., TONN, T., BUSCH, D. H., GRIGOLEIT, G. U., EINSELE, H., ODENDAHL, M., GERMEROTH, L., RINGHOFFER, M., RINGHOFFER, S., WIESNETH, M., GREINER, J., MICHEL, D., MERTENS, T., ROJEWSKI, M., MARX, M., VON HARSDORF, S., DOHNER, H., SEIFRIED, E., BUNJES, D. & SCHMITT, M. 2011. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion,* 51**,** 591-599.

SCHOLZEN, T. & GERDES, J. 2000. The Ki-67 protein: from the known and the unknown. *J Cell Physiol,* 182**,** 311-22.

SCHWAB, R., SZABO, P., MANAVALAN, J. S., WEKSLER, M. E., POSNETT, D. N., PANNETIER, C., KOURILSKY, P. & EVEN, J. 1997. Expanded CD4+ and CD8+ T cell clones in elderly humans. *J Immunol,* 158**,** 4493-9.

SCHWARZ, B. A. & BHANDOOLA, A. 2006. Trafficking from the bone marrow to the thymus: a prerequisite for thymopoiesis. *Immunol Rev,* 209**,** 47-57.

SCHWENDEMANN, J., CHOI, C., SCHIRRMACHER, V. & BECKHOVE, P. 2005. Dynamic differentiation of activated human peripheral blood CD8+ and CD4+ effector memory T cells. *J Immunol,* 175**,** 1433-9.

SEDER, R. A., DARRAH, P. A. & ROEDERER, M. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol,* 8**,** 247-58.

SESTER, U., PRESSER, D., DIRKS, J., GARTNER, B. C., KOHLER, H. & SESTER, M. 2008. PD-1 expression and IL-2 loss of cytomegalovirus- specific T cells correlates with viremia and reversible functional anergy. *Am J Transplant,* 8**,** 1486-97.

SHAW, A. C., JOSHI, S., GREENWOOD, H., PANDA, A. & LORD, J. M. 2010. Aging of the innate immune system. *Curr Opin Immunol,* 22**,** 507-13.

SHEN, T., ZHENG, J., XU, C., LIU, J., ZHANG, W., LU, F. & ZHUANG, H. 2010. PD-1 expression on peripheral CD8+ TEM/TEMRA subsets closely correlated with HCV viral load in chronic hepatitis C patients. *Virol J,* 7**,** 310.

SHORTMAN, K., EGERTON, M., SPANGRUDE, G. J. & SCOLLAY, R. 1990. The generation and fate of thymocytes. *Semin Immunol,* 2**,** 3-12.

SIMANEK, A. M., DOWD, J. B., PAWELEC, G., MELZER, D., DUTTA, A. & AIELLO, A. E. 2011. Seropositivity to cytomegalovirus, inflammation, all-cause and cardiovascular disease-related mortality in the United States. *PLoS One,* 6**,** e16103.

SINCLAIR, J. 2008. Human cytomegalovirus: Latency and reactivation in the myeloid lineage. *J Clin Virol,* 41**,** 180-5.

SINCLAIR, J. & SISSONS, P. 2006. Latency and reactivation of human cytomegalovirus. *J Gen Virol,* 87**,** 1763-79.

SMITH-GARVIN, J. E., KORETZKY, G. A. & JORDAN, M. S. 2009. T cell activation. *Annu Rev Immunol,* 27**,** 591-619.

SOARES, M. V., PLUNKETT, F. J., VERBEKE, C. S., COOK, J. E., FAINT, J. M., BELARAMANI, L. L., FLETCHER, J. M., HAMMERSCHMITT, N., RUSTIN, M., BERGLER, W., BEVERLEY, P. C., SALMON, M. & AKBAR, A. N. 2004. Integration of apoptosis and telomere erosion in virus-specific CD8+ T cells from blood and tonsils during primary infection. *Blood,* 103**,** 162-7.

SODERBERG-NAUCLER, C. 2006. Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J Intern Med,* 259**,** 219-46.

SODERBERG-NAUCLER, C., FISH, K. N. & NELSON, J. A. 1997. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell,* 91**,** 119-26.

SODERBERG-NAUCLER, C., FISH, K. N. & NELSON, J. A. 1998. Growth of human cytomegalovirus in primary macrophages. *Methods,* 16**,** 126-38.

SONG, J. Y., CHEONG, H. J., HWANG, I. S., CHOI, W. S., JO, Y. M., PARK, D. W., CHO, G. J., HWANG, T. G. & KIM, W. J. 2010. Long-term immunogenicity of influenza vaccine among the elderly: Risk factors for poor immune response and persistence. *Vaccine,* 28**,** 3929-35.

SPYRIDOPOULOS, I., HOFFMANN, J., AICHER, A., BRUMMENDORF, T. H., DOERR, H. W., ZEIHER, A. M. & DIMMELER, S. 2009. Accelerated telomere shortening in leukocyte subpopulations of patients with coronary heart disease: role of cytomegalovirus seropositivity. *Circulation,* 120**,** 1364-72.

STARR, T. K., JAMESON, S. C. & HOGQUIST, K. A. 2003. Positive and negative selection of T cells. *Annu Rev Immunol,* 21**,** 139-76.

STONE, J. D., CHERVIN, A. S. & KRANZ, D. M. 2009. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology,* 126**,** 165-76.

STOWE, R. P., KOZLOVA, E. V., YETMAN, D. L., WALLING, D. M., GOODWIN, J. S. & GLASER, R. 2007. Chronic herpesvirus reactivation occurs in aging. *Exp Gerontol,* 42**,** 563-70.

STRANDBERG, T. E., PITKALA, K. H., LINNAVUORI, K. & TILVIS, R. S. 2004. Cognitive impairment and infectious burden in the elderly. *Arch Gerontol Geriatr Suppl*, 419-23.

STRINDHALL, J., NILSSON, B. O., LOFGREN, S., ERNERUDH, J., PAWELEC, G., JOHANSSON, B. & WIKBY, A. 2007. No Immune Risk Profile among individuals who reach 100 years of age: findings from the Swedish NONA immune longitudinal study. *Exp Gerontol,* 42, 753-61.

STRIPPOLI, G. F., HODSON, E. M., JONES, C. & CRAIG, J. C. 2006. Preemptive treatment for cytomegalovirus viremia to prevent cytomegalovirus disease in solid organ transplant recipients. *Transplantation,* 81, 139-45.

SYLWESTER, A. W., MITCHELL, B. L., EDGAR, J. B., TAORMINA, C., PELTE, C., RUCHTI, F., SLEATH, P. R., GRABSTEIN, K. H., HOSKEN, N. A., KERN, F., NELSON, J. A. & PICKER, L. J. 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med,* 202, 673-85.

TAYLOR, G. H. 2003. Cytomegalovirus. *Am Fam Physician,* 67, 519-24.

TERRY, D. F., NOLAN, V. G., ANDERSEN, S. L., PERLS, T. T. & CAWTHON, R. 2008. Association of longer telomeres with better health in centenarians. *J Gerontol A Biol Sci Med Sci,* 63, 809-12.

THOMAS, M. L. 1989. The leukocyte common antigen family. *Annu Rev Immunol,* 7, 339-69.

TOUGH, D. F., BORROW, P. & SPRENT, J. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science,* 272, 1947-50.

TOUGH, D. F., SUN, S., ZHANG, X. & SPRENT, J. 2000. Stimulation of memory T cells by cytokines. *Vaccine,* 18, 1642-8.

TRAUTMANN, L., JANBAZIAN, L., CHOMONT, N., SAID, E. A., GIMMIG, S., BESSETTE, B., BOULASSEL, M. R., DELWART, E., SEPULVEDA, H., BALDERAS, R. S., ROUTY, J. P., HADDAD, E. K. & SEKALY, R. P. 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med,* 12, 1198-202.

TRAUTMANN, L., RIMBERT, M., ECHASSERIEAU, K., SAULQUIN, X., NEVEU, B., DECHANET, J., CERUNDOLO, V. & BONNEVILLE, M. 2005. Selection of T cell clones expressing high-affinity public TCRs within Human cytomegalovirus-specific CD8 T cell responses. *J Immunol,* 175, 6123-32.

TRIMBLE, L. A., KAM, L. W., FRIEDMAN, R. S., XU, Z. & LIEBERMAN, J. 2000. CD3zeta and CD28 down-modulation on CD8 T cells during viral infection. *Blood,* 96, 1021-9.

URBANI, S., AMADEI, B., TOLA, D., MASSARI, M., SCHIVAZAPPA, S., MISSALE, G. & FERRARI, C. 2006. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol,* 80, 11398-403.

URRUTICOECHEA, A., SMITH, I. E. & DOWSETT, M. 2005. Proliferation marker Ki-67 in early breast cancer. *J Clin Oncol,* 23, 7212-20.

VALDES, A. M., ANDREW, T., GARDNER, J. P., KIMURA, M., OELSNER, E., CHERKAS, L. F., AVIV, A. & SPECTOR, T. D. 2005. Obesity, cigarette smoking, and telomere length in women. *Lancet,* 366, 662-4.

VAN DE BERG, P. J., GRIFFITHS, S. J., YONG, S. L., MACAULAY, R., BEMELMAN, F. J., JACKSON, S., HENSON, S. M., TEN BERGE, I. J., AKBAR, A. N. & VAN LIER, R. A. 2010. Cytomegalovirus infection reduces telomere length of the circulating T cell pool. *J Immunol,* 184**,** 3417-23.

VAN DE BERG, P. J., VAN STIJN, A., TEN BERGE, I. J. & VAN LIER, R. A. 2008. A fingerprint left by cytomegalovirus infection in the human T cell compartment. *J Clin Virol,* 41**,** 213-7.

VAN DER MERWE, P. A. & DUSHEK, O. 2011. Mechanisms for T cell receptor triggering. *Nat Rev Immunol,* 11**,** 47-55.

VAN LEEUWEN, E. M., GAMADIA, L. E., BAARS, P. A., REMMERSWAAL, E. B., TEN BERGE, I. J. & VAN LIER, R. A. 2002. Proliferation requirements of cytomegalovirus-specific, effector-type human CD8+ T cells. *J Immunol,* 169**,** 5838-43.

VAN LEEUWEN, E. M., VAN BUUL, J. D., REMMERSWAAL, E. B., HORDIJK, P. L., TEN BERGE, I. J. & VAN LIER, R. A. 2005. Functional re-expression of CCR7 on CMV-specific CD8+ T cells upon antigenic stimulation. *Int Immunol,* 17**,** 713-9.

VAN LIER, R. A., TEN BERGE, I. J. & GAMADIA, L. E. 2003. Human CD8(+) T-cell differentiation in response to viruses. *Nat Rev Immunol,* 3**,** 931-9.

VARGAS, A. L., LECHNER, F., KANTZANOU, M., PHILLIPS, R. E. & KLENERMAN, P. 2001. Ex vivo analysis of phenotype and TCR usage in relation to CD45 isoform expression on cytomegalovirus-specific CD8+ T lymphocytes. *Clin Exp Immunol,* 125**,** 432-9.

VASTO, S., COLONNA-ROMANO, G., LARBI, A., WIKBY, A., CARUSO, C. & PAWELEC, G. 2007. Role of persistent CMV infection in configuring T cell immunity in the elderly. *Immun Ageing,* 4**,** 2.

VELU, V., TITANJI, K., ZHU, B., HUSAIN, S., PLADEVEGA, A., LAI, L., VANDERFORD, T. H., CHENNAREDDI, L., SILVESTRI, G., FREEMAN, G. J., AHMED, R. & AMARA, R. R. 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature,* 458**,** 206-10.

VILLACRES, M. C., LACEY, S. F., AUGE, C., LONGMATE, J., LEEDOM, J. M. & DIAMOND, D. J. 2003. Relevance of peptide avidity to the T cell receptor for cytomegalovirus-specific ex vivo CD8 T cell cytotoxicity. *J Infect Dis,* 188**,** 908-18.

VIRGIN, H. W., WHERRY, E. J. & AHMED, R. 2009. Redefining chronic viral infection. *Cell,* 138**,** 30-50.

VOEHRINGER, D., KOSCHELLA, M. & PIRCHER, H. 2002. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). *Blood,* 100**,** 3698-702.

VOLAREVIC, S., NIKLINSKA, B. B., BURNS, C. M., JUNE, C. H., WEISSMAN, A. M. & ASHWELL, J. D. 1993. Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science,* 260**,** 541-4.

WALDMANN, T. A. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol,* 6**,** 595-601.

WALKER, L. J., SEWELL, A. K. & KLENERMAN, P. 2010. T cell sensitivity and the outcome of viral infection. *Clin Exp Immunol,* 159**,** 245-55.

WALLACE, D. L., MASTERS, J. E., DE LARA, C. M., HENSON, S. M., WORTH, A., ZHANG, Y., KUMAR, S. R., BEVERLEY, P. C., AKBAR, A. N. & MACALLAN, D. C. 2010. Human

cytomegalovirus-specific CD8(+) T-cell expansions contain long-lived cells that retain functional capacity in both young and elderly subjects. *Immunology*.

WALLACE, D. L., MASTERS, J. E., DE LARA, C. M., HENSON, S. M., WORTH, A., ZHANG, Y., KUMAR, S. R., BEVERLEY, P. C., AKBAR, A. N. & MACALLAN, D. C. 2011. Human cytomegalovirus-specific CD8(+) T-cell expansions contain long-lived cells that retain functional capacity in both young and elderly subjects. *Immunology,* 132**,** 27-38.

WALLACE, D. L., ZHANG, Y., GHATTAS, H., WORTH, A., IRVINE, A., BENNETT, A. R., GRIFFIN, G. E., BEVERLEY, P. C., TOUGH, D. F. & MACALLAN, D. C. 2004. Direct measurement of T cell subset kinetics in vivo in elderly men and women. *J Immunol,* 173**,** 1787-94.

WALLER, E. C., MCKINNEY, N., HICKS, R., CARMICHAEL, A. J., SISSONS, J. G. & WILLS, M. R. 2007. Differential costimulation through CD137 (4-1BB) restores proliferation of human virus-specific "effector memory" (CD28(-) CD45RA(HI)) CD8(+) T cells. *Blood,* 110**,** 4360-6.

WANG, K., YIN, X. M., CHAO, D. T., MILLIMAN, C. L. & KORSMEYER, S. J. 1996. BID: a novel BH3 domain-only death agonist. *Genes Dev,* 10**,** 2859-69.

WEEKES, M. P., WILLS, M. R., MYNARD, K., HICKS, R., SISSONS, J. G. & CARMICHAEL, A. J. 1999. Large clonal expansions of human virus-specific memory cytotoxic T lymphocytes within the CD57+ CD28- CD8+ T-cell population. *Immunology,* 98**,** 443-9.

WEINBERGER, B., HERNDLER-BRANDSTETTER, D., SCHWANNINGER, A., WEISKOPF, D. & GRUBECK-LOEBENSTEIN, B. 2008. Biology of immune responses to vaccines in elderly persons. *Clin Infect Dis,* 46**,** 1078-84.

WEINBERGER, B., WELZL, K., HERNDLER-BRANDSTETTER, D., PARSON, W. & GRUBECK-LOEBENSTEIN, B. 2009. CD28(-)CD8(+) T cells do not contain unique clonotypes and are therefore dispensable. *Immunol Lett,* 127**,** 27-32.

WEISKOPF, D., WEINBERGER, B. & GRUBECK-LOEBENSTEIN, B. 2009. The aging of the immune system. *Transpl Int,* 22**,** 1041-50.

WEKSLER, M. E. & SZABO, P. 2000. The effect of age on the B-cell repertoire. *J Clin Immunol,* 20**,** 240-9.

WENG, N. P., LEVINE, B. L., JUNE, C. H. & HODES, R. J. 1996. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J Exp Med,* 183**,** 2471-9.

WHERRY, E. J., HA, S. J., KAECH, S. M., HAINING, W. N., SARKAR, S., KALIA, V., SUBRAMANIAM, S., BLATTMAN, J. N., BARBER, D. L. & AHMED, R. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity,* 27**,** 670-84.

WHERRY, E. J., TEICHGRABER, V., BECKER, T. C., MASOPUST, D., KAECH, S. M., ANTIA, R., VON ANDRIAN, U. H. & AHMED, R. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol,* 4**,** 225-34.

WIERTZ, E., HILL, A., TORTORELLA, D. & PLOEGH, H. 1997. Cytomegaloviruses use multiple mechanisms to elude the host immune response. *Immunol Lett,* 57**,** 213-6.

WIKBY, A., FERGUSON, F., FORSEY, R., THOMPSON, J., STRINDHALL, J., LOFGREN, S., NILSSON, B. O., ERNERUDH, J., PAWELEC, G. & JOHANSSON, B. 2005. An immune risk phenotype, cognitive impairment, and survival in very late life: impact of allostatic load in Swedish octogenarian and nonagenarian humans. *J Gerontol A Biol Sci Med Sci,* 60**,** 556-65.

WIKBY, A., JOHANSSON, B., OLSSON, J., LOFGREN, S., NILSSON, B. O. & FERGUSON, F. 2002. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Exp Gerontol,* 37**,** 445-53.

WIKBY, A., NILSSON, B. O., FORSEY, R., THOMPSON, J., STRINDHALL, J., LOFGREN, S., ERNERUDH, J., PAWELEC, G., FERGUSON, F. & JOHANSSON, B. 2006. The immune risk phenotype is associated with IL-6 in the terminal decline stage: findings from the Swedish NONA immune longitudinal study of very late life functioning. *Mech Ageing Dev,* 127**,** 695-704.

WILLS, M. R., CARMICHAEL, A. J., MYNARD, K., JIN, X., WEEKES, M. P., PLACHTER, B. & SISSONS, J. G. 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J Virol,* 70**,** 7569-79.

WILLS, M. R., CARMICHAEL, A. J., WEEKES, M. P., MYNARD, K., OKECHA, G., HICKS, R. & SISSONS, J. G. 1999. Human virus-specific CD8+ CTL clones revert from CD45ROhigh to CD45RAhigh in vivo: CD45RAhighCD8+ T cells comprise both naive and memory cells. *J Immunol,* 162**,** 7080-7.

WILLS, M. R., OKECHA, G., WEEKES, M. P., GANDHI, M. K., SISSONS, P. J. & CARMICHAEL, A. J. 2002. Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response. *J Immunol,* 168**,** 5455-64.

WOODLAND, D. L. & KOHLMEIER, J. E. 2009. Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat Rev Immunol,* 9**,** 153-61.

WOOLDRIDGE, L., LISSINA, A., COLE, D. K., VAN DEN BERG, H. A., PRICE, D. A. & SEWELL, A. K. 2009. Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology,* 126**,** 147-64.

WREGHITT, T. G., TEARE, E. L., SULE, O., DEVI, R. & RICE, P. 2003. Cytomegalovirus infection in immunocompetent patients. *Clin Infect Dis,* 37**,** 1603-6.

YAGER, E. J., AHMED, M., LANZER, K., RANDALL, T. D., WOODLAND, D. L. & BLACKMAN, M. A. 2008. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J Exp Med,* 205**,** 711-23.

YANG, E., ZHA, J., JOCKEL, J., BOISE, L. H., THOMPSON, C. B. & KORSMEYER, S. J. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell,* 80**,** 285-91.

ZHANG, X., FUJII, H., KISHIMOTO, H., LEROY, E., SURH, C. D. & SPRENT, J. 2002. Aging leads to disturbed homeostasis of memory phenotype CD8(+) cells. *J Exp Med,* 195**,** 283-93.

ZHOU, P., QIAN, L., KOZOPAS, K. M. & CRAIG, R. W. 1997. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. *Blood,* 89**,** 630-43.