The Impact of Antigen Processing on CD8$^+$ T Cell Memory Inflation

Submission for Degree of Doctor of Philosophy

JULIA COLSTON

Nuffield Department of Medicine, The Peter Medawar Building for Pathogen Research

Merton College

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Abstract

T cell "memory inflation" is the sustained induction of effector memory cells that home to peripheral tissues and retain their functionality. Defining the mechanisms that drive these non-classical memory responses may contribute towards the development of novel prophylactic or therapeutic vaccines. A model of memory inflation based on responses to β-galactosidase delivered by a non-replicating adenoviral vector provides a robust tool for investigating the underlying mechanisms. This work has shown that these responses are not dependent upon the human cytomegalovirus (HCMV) promoter within the model, this being the only part of the model that is CMV-derived. This model has been used to test the hypothesis that bypassing antigen processing would result in inflationary memory responses to CD8$^+$ T cell epitopes that are not normally the targets of such responses. When the β-gal$_{497\text{-}504}$ restricted epitope (ICPMYARV) was expressed as a minigene in a recombinant adenovirus vector, inflationary CD8$^+$ T cell responses were induced, instead of the classical responses obtained with full-length β-galactosidase. Similar results were obtained with the M45$_{985\text{-}993}$ (HGIRNASFI) epitope from the mouse cytomegalovirus M45 protein. These data demonstrate that the polypeptide context of a CD8$^+$ T cell epitope may determine whether classical or inflating memory responses are induced. This could be relevant to the design of recombinant antigens in adenoviral vectors, which have emerging therapeutic and prophylactic applications.

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TABLE OF CONTENTS

1. INTRODUCTION **1**
1.1 CD8$^+$ T CELLS 2
1.2 WHAT IS "MEMORY INFLATION"? 4
1.3 CYTOMEGALOVIRUS AND MEMORY INFLATION 7
1.4 CMV AND IMMUNE SENESCENCE 11
1.5 ANTIGEN PROCESSING AND MEMORY INFLATION 12
1.5.1 The immunoproteasome 12
1.5.2 Memory inflation and the immunoproteasome 15
1.6 ADENOVIRAL VECTORS 17
1.7 OVERVIEW OF THE ADENOVIRAL MODEL FOR MEMORY INFLATION 26
1.8 THESIS AIMS 29

2. MATERIALS AND METHODS **30**
2.1 ANIMALS 30
2.1.1 Ethics statement 30
2.1.2 Mice 30
2.1.3 Procedures; immunisations, intravenous blood sampling 31
2.2 VIRUSES 33
2.2.1 Adenoviral vectors 33
2.2.2 MCMV 39
2.2.3 Vaccinia LacZ 39
2.3 REAGENTS AND SOLUTIONS, TISSUE CULTURE MEDIA, PEPTIDES, TETRAMERS AND ANTIBODIES 40
2.3.1 Reagents and solutions 40
2.3.2 Tissue culture media 41
2.3.3 Peptides 42
2.3.4 Tetrameric MHC class I peptide complexes 43
2.3.5 Antibodies 44
2.4 FLOW CYTOMETRY 45
2.4.1 Preparation of samples 45
2.4.2 Gating strategy 46
2.5 ISOLATION OF LIVER, LUNG AND SPLEEN LYMPHOCYTES 47
2.6 INTRACELLULAR CYTOKINE STAINING 48
2.7 β-GAL LUMINESCENCE ASSAY 49
2.8 ADENOVIRAL ANTIBODY ELISA 49
2.9 STATISTICAL ANALYSIS 50

3. RESULTS – THE ADENOVIRAL MODEL **51**
3.1 INTRODUCTION 51
3.2 OVERVIEW / AIMS 55
3.3 AD-LACZ REMAINS A GOOD MODEL OF MEMORY INFLATION REGARDLESS OF THE TRANSGENE PROMOTER 56
3.3.1 Comparison of alternative promoters 56
3.3.2 Comparison of different HCMV promoters 63
3.4 NO OTHER KB-RESTRICTED EPITOPES CAN BE IDENTIFIED FROM THE AD-LACZ MODEL 65
3.5 THE IMPORTANCE OF THE ROUTE OF IMMUNISATION 69
3.5.1 Intravenous versus intraperitoneal 69
3.5.2 Intravenous versus intramuscular 72
3.6 CONCLUSIONS 75

4. RESULTS – MINIGENES **78**
4.1 INTRODUCTION 78
4.2 OVERVIEW / AIMS 80
4.3 AD-D8V AND AD-I8V MINIGENES 81
4.3.1 The Ad-D8V and Ad-I8V minigenes compared to Ad-LacZ in blood 81
4.3.2 Phenotype, distribution and functionality of the tetramer-specific $CD8^+$ T cells induced by the Ad-I8V and Ad-D8V minigenes 84
4.4 AD-M45 MINIGENE 90
4.4.1 The Ad-M45 minigene compared to MCMV 90
4.4.2 Phenotype, distribution and functionality of the tetramer-specific $CD8^+$ T cells induced by the Ad-M45 minigene 92
4.5 CONCLUSIONS 96

5. RESULTS – PROCESSING AND PRESENTATION **98**
5.1 INTRODUCTION 98
5.2 OVERVIEW / AIMS 101
5.3 IMMUNOPROTEASOME ($LMP7^{-/-}$) STUDIES 102
5.4 N-TERMINAL EXTENSIONS 105
5.5 C-TERMINAL EXTENSIONS 110
5.6 TCR USAGE 115
5.7 CONCLUSIONS 118

6. RESULTS – CO-INFLATION AND BOOSTING **121**
6.1 INTRODUCTION 121
6.2 OVERVIEW / AIMS 123
6.3 MINIGENES COMBINED – CO-INFLATION / COMPETITION 124
6.3.1 Co-inflation between Ad-I8V and Ad-D8V 124
6.3.2 Co-inflation between I8V and D8V tetramer-specific responses from the same insert on the vector 127
6.3.3 Co-inflation between Ad-I8V, Ad-D8V and Ad-M45 130
6.4 BOOSTING 132
6.5 CONCLUSIONS 134

7. DISCUSSION AND FUTURE WORK **138**
7.1 AD-LACZ: THE HCMV PROMOTER AND THE ROLE OF THE ADHU5 VECTOR 138
7.2 THE IMPACT OF ANTIGEN PROCESSING ON $CD8^+$ T CELL MEMORY INFLATION 141
7.2.1 Minigenes 141
7.2.2 Co-inflation, boosting and protection 143
7.3 FUTURE WORK 147
7.4 POTENTIAL BENEFITS AND APPLICATION OF THE CURRENT FINDINGS AND FUTURE WORK 152

REFERENCES **154**

APPENDICES **173**

LIST OF FIGURES

FIGURE 1.1: GROUPING OF CD8$^+$ T CELLS ACCORDING TO THEIR CD62L/CD127 PHENOTYPE INTO T_{EM}, T_E AND T_{CM} POOLS. 6
FIGURE 1.2: THE MHC CLASS I ANTIGEN PRESENTATION PATHWAY. 13
FIGURE 1.3: ADENOVIRUS (SPECIES C) ENTRY INTO TARGET CELLS. 20
FIGURE 1.4: MEMORY INFLATION USING THE ADENOVIRAL (AD-LACZ) MODEL. 27
FIGURE 2.1: TYPICAL IN VIVO EXPERIMENTAL SCHEDULE. 32
FIGURE 2.2: ADHU5 TEMPLATE FOR ALL VECTORS USED. 33
FIGURE 3.1: COMPARISON OF THE IMMUNE RESPONSES OF MICE TO AD-LACZ VIRUSES UTILISING HCMV, RSV AND EF1α PROMOTERS TO DRIVE LACZ EXPRESSION. 58
FIGURE 3.2: INFLATIONARY D8V-SPECIFIC CD8$^+$ T CELLS INDUCED BY AD-LACZ VIRUSES WITH THREE DIFFERENT PROMOTERS HAVE SIMILAR T_{EM} PHENOTYPES. 59
FIGURE 3.3: DISTRIBUTION AND FUNCTIONALITY OF THE INFLATIONARY D8V-SPECIFIC CD8$^+$ T CELLS ACROSS ALL THREE AD-LACZ VIRUSES WITH THE DIFFERENT PROMOTERS. 60
FIGURE 3.4: β-GAL PRODUCTION IN VITRO FROM THE THREE AD-LACZ VIRUSES WITH DIFFERENT PROMOTERS. 62
FIGURE 3.5: COMPARISON OF AD-LACZ CONSTRUCTS WITH DIFFERING HCMV PROMOTERS: LONG VERSUS SHORT. 64
FIGURE 3.6: 12KB PEPTIDE RESPONSES EX VIVO. 66
FIGURE 3.7: FOCUSED KB-RESTRICTED PEPTIDE STIMULATION EX VIVO. 67
FIGURE 3.8: E8V TETRAMER-SPECIFIC CD8$^+$ T CELL RESPONSES IN VIVO. 68
FIGURE 3.9: COMPARISON BETWEEN INFLATIONARY RESPONSES FOLLOWING INTRAVENOUS OR INTRAPERITONEAL AD-LACZ ADMINISTRATION. 70
FIGURE 3.10: PHENOTYPE AND DISTRIBUTION OF INFLATIONARY RESPONSES FOLLOWING INTRAVENOUS OR INTRAPERITONEAL AD-LACZ ADMINISTRATION. 71
FIGURE 3.11: COMPARISON OF INFLATIONARY RESPONSES FOLLOWING INTRAVENOUS OR INTRAMUSCULAR AD-LACZ ADMINISTRATION. 73
FIGURE 3.12: PHENOTYPE, DISTRIBUTION AND FUNCTIONALITY OF INFLATIONARY RESPONSES FOLLOWING INTRAVENOUS OR INTRAMUSCULAR AD-LACZ ADMINISTRATION. 74
FIGURE 4.1: THE AD-D8V MINIGENE TETRAMER-SPECIFIC CD8$^+$ T CELL RESPONSES IN VIVO COMPARED TO AD-LACZ. 82
FIGURE 4.2: THE AD-I8V MINIGENE TETRAMER-SPECIFIC CD8$^+$ T CELL RESPONSES IN VIVO COMPARED TO AD-LACZ. 83

FIGURE 4.3: THE PHENOTYPE FOR THE AD-D8V MINIGENE INDUCED CD8+ T CELLS. 85
FIGURE 4.4: THE PHENOTYPE FOR THE AD-I8V MINIGENE INDUCED CD8+ T CELLS......86
FIGURE 4.5: DISTRIBUTION OF AD-D8V INDUCED CD8+ T CELLS IN THE PERIPHERAL TISSUES......87
FIGURE 4.6: DISTRIBUTION OF AD-I8V INDUCED CD8+ T CELLS IN THE PERIPHERAL TISSUES......88
FIGURE 4.7: FUNCTIONALITY OF AD-D8V AND AD-I8V INDUCED CD8+ T CELLS IN ICS AT DAY 100-POST IMMUNISATION. 89
FIGURE 4.8: THE AD-M45 MINIGENE TETRAMER-SPECIFIC CD8+ T CELL RESPONSES IN VIVO COMPARED TO MCMV. 91
FIGURE 4.9: THE PHENOTYPE FOR THE AD-M45 INDUCED CD8+ T CELLS. 93
FIGURE 4.10: DISTRIBUTION OF AD-M45 INDUCED CD8+ T CELLS IN THE PERIPHERY. 94
FIGURE 4.11: FUNCTIONALITY OF AD-M45 INDUCED CD8+ T CELLS IN ICS AT DAY 75-POST IMMUNISATION......95
FIGURE 5.1: IMMUNOPROTEASOME STUDIES AND AD-D8V. 103
FIGURE 5.2: IMMUNOPROTEASOME STUDIES AND AD-I8V. 104
FIGURE 5.3: AD-5AA-I8V......106
FIGURE 5.4: AD-7AA-I8V......107
FIGURE 5.5: AD-10AA-I8V......108
FIGURE 5.6: AD-10AA-I8V PHENOTYPE......109
FIGURE 5.7: AD-10AA-I8V-10AA. 111
FIGURE 5.8: AD-10AA-I8V-10AA ADENOVIRUS ANTIBODY ELISA. 112
FIGURE 5.9: AD-I8V-10AA......113
FIGURE 5.10: AD-I8V-10AA AND AD-10AA-I8V-10AA INDUCED CD8+ T CELL RESPONSES FOLLOWING PEPTIDE STIMULATION IN AN ICS ASSAY. 114
FIGURE 5.11: COMPARISON OF VΒ TCR USAGE FOLLOWING AD-LACZ IMMUNISATION..116
FIGURE 5.12: COMPARISON OF VΒ TCR USAGE FOLLOWING AD-D8V IMMUNISATION. ..117
FIGURE 6.1: AD-I8V AND AD-D8V ADMINISTERED INTRAVENOUSLY IN THE SAME IMMUNISATION. 125
FIGURE 6.2: AD-I8V AND AD-D8V ADMINISTERED INTRAMUSCULARLY IN THE SAME IMMUNISATION. 126
FIGURE 6.3: VECTORS CONTAINING AN INSERT OF BOTH EPITOPES WITH A GLYCINE-PROLINE LINKER WITH EITHER EPITOPE FIRST. 128
FIGURE 6.4: AD-ICD AND AD-DAI ADENOVIRUS ANTIBODY ELISA. 129
FIGURE 6.5: AD-M45, AD-I8V AND AD-D8V ADMINISTERED INTRAVENOUSLY IN THE SAME IMMUNISATION. 131

FIGURE 6.6: D8V AND I8V-SPECIFIC RESPONSES IN PREVIOUSLY AD-LACZ IMMUNISED MICE, FOLLOWING CHALLENGE WITH VV-LACZ. 133
FIGURE 7.1: SUMMATIVE MODEL OF THE MECHANISMS OF MEMORY INFLATION. 146
FIGURE 7.2: REPRESENTATION OF THE FLEX ON/OFF SYSTEM. 148
FIGURE 7.3: SCHEMATIC OF THE LCMV CHALLENGE/PROTECTION STUDIES. 151
FIGURE A2.1: AD-LACZ (RSV) TITRATED IN B6 MICE. 179
FIGURE A2.2: AD-LACZ (EF1α) TITRATED IN B6 MICE. 180
FIGURE A2.3: AD-D8V TITRATED IN B6 MICE. 181
FIGURE A2.4: AD-I8V TITRATED IN B6 MICE. 182
FIGURE A2.5: GATING STRATEGY – TETRAMER SPECIFIC POPULATIONS 183
FIGURE A2.6: GATING STRATEGY – ANTIBODY PANEL 184

LIST OF TABLES

TABLE 1.1: ADENOVIRAL VACCINE DEVELOPMENTS IN HUMAN TRIALS: HUMAN ADENOVIRUS VECTORS. 23

TABLE 1.2: ADENOVIRAL VACCINE DEVELOPMENTS IN HUMAN TRIALS: SIMIAN ADENOVIRUS VECTORS. 24

TABLE 2.1: SUMMARY OF THE CONSTRUCTS USED IN THIS WORK. 37

TABLE 2.2: AMINO ACID SEQUENCES FOR EXTENDED AD-I8V MINIGENE VECTORS AND AD-M45. 38

TABLE 2.3: AMINO ACID SEQUENCES AND β-GAL POSITION FOR 12 KB-RESTRICTED EPITOPES IN β-GALACTOSIDASE. 42

TABLE 7.1: SUMMARY OF AD-LACZ VECTORS TO BE TESTED ACCORDING TO SEROTYPE. 149

LIST OF ABBREVIATIONS

Aa	Amino acid
Ab	Antibody
ACK	Ammonium-Chloride-Potassium
AdHu5	Human adenovirus, serotype 5
Ag	Antigen
APCs	Antigen Presenting Cells
APC (fluorochrome)	Allophycocyanin
B6	C57BL/6 mouse
β2M	β-2 Microglobulin
β-gal	β-galactosidase
CD	Cluster of Differentiation
Class I	MHC class I
Class II	MHC class II
CMV	Cytomegalovirus
CPE	Cytopathic effect
CTL	Cytotoxic T Lymphocyte
D8V	Peptide DAPIYTNV (βgal_{96-103})
ddH_20	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylene diamine tetra-acetic acid
E	Early gene
EF1α (EF1a)	Elongation Factor 1-alpha
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
HCMV	Human Cytomegalovirus
HLA	Human Leucocyte Antigen
HSV-1	Herpes Simplex Virus-1
I8V	Peptide ICPMYARV ($\beta gal_{497-504}$)
ICS	Intracellular cytokine staining
IE	Immediate early gene

IFN	Interferon
IL	Interleukin
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
iu	Infectious units
kb	Kilobase
Kb	Mouse H-2K (haplotype b) – equivalent MHC class I
LacZ	LacZ Operon – encoding for β-galactosidase
L	Late gene
LCMV	Lymphocytic Choriomeningitis
LMP7	Immunoproteasome subunit – also know as β5i
$LMP7^{-/-}$ (LMP7ko)	LMP7 knock out mouse
LP	Long Promoter
LPS	Lipopolysaccharide
MCMV	Murine Cytomegalovirus
MECL-1	Multicatalytic endopeptidase-like complex 1
MIE	Major Immediate Early gene
MFI	Mean fluorescent intensity
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
MVA	Modified Vaccinia Ankara
pAPCs	Professional Antigen Presenting Cells
PB	Pacific Blue
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
Pe-Cy	Phycoerythrin Cyanine
PerCP-Cy	Peridinin Chlorophyll Protein Cyanine
PMA/I	Phorbol 12-myristate 13-acetate and ionomycin
pNNP	p-nitrophenyl phosphate
RPMI	Roswell Park Memorial Institute media
RSV	Rous Sarcoma Virus
SEM	Standard error of the mean
SV40	Simian (vacuolating) virus 40

TAP	Transporter associated with antigen processing
T_{CM}	T cell central memory
T_E	T cell effector
T_{EM}	T cell effector memory
TCR	T cell receptor
TNF	Tumour Necrosis Factor
Trm	Tissue-resident memory T cells
VV	Vaccinia Virus

List of Publications associated with my DPhil work

Julia M Colston, Beatrice Bolinger, Matthew G Cottingham, Sarah Gilbert and Paul Klenerman. Conversion of classical to inflationary $CD8^+$ T cell memory. Under submission July 2015.

Stuart Sims, Julia Colston, Vince Emery and Paul Klenerman. CD73 is dispensable for the regulation of inflationary $CD8^+$ T-cells after murine cytomegalovirus infection and adenovirus immunisation. PlosONE 2014 Dec 9;9(12):e114323.

1. Introduction

The focus of this thesis is based upon the adenoviral model of $CD8^+$ T cell memory inflation and the role of antigen processing in the production of inflationary populations. The introduction will cover what is already known about memory inflation from work done with the murine cytomegalovirus (MCMV) and adenoviral models, the importance of the processing environment in the production of $CD8^+$ T cell responses to viral infections, and the use of adenoviral vectors currently in vaccine strategies. Presentation of the results generated from this work will then be made in the subsequent chapters. The adenoviral model has been adapted to address key questions around the role of the processing environment in the production of inflationary populations, as well as some of the features of the model itself, including the relevance of the CMV promoter.

1.1 CD8$^+$ T cells

CD8$^+$ T cells are an important part of the adaptive immune response. Activated effector CD8$^+$ T cells (or cytotoxic T lymphocytes) are essential to the immune response against intracellular pathogens (particularly viruses), cancers and more generally damaged cells. During a viral infection, T lymphocytes undergo cytokine-driven phenotypic changes, but only those that recognise pathogen-encoded (cognate) antigen carry on to multiple rounds of replication, generating large numbers of CTL effector progeny. These are the foot soldiers of the adaptive immune response (Zhang and Bevan, 2011).

CD8$^+$ T cells develop from common lymphoid precursors in the bone marrow, which then migrate to the thymus. Here the T cell receptor genes are rearranged to express a specific T cell antigen receptor (TCR). During an infection, the naïve CD8$^+$ T cells are primed by antigen presenting cells (APCs) in the secondary lymphoid organs. APCs present foreign peptides attached to major histocompatibility complex (MHC) class I molecules. The antigen specific pool of CD8$^+$ T cells then expands out (primary responses) in the acute phase of infection. To achieve maximum expansion, these cells need to integrate multiple signals, including TCR, co-stimulatory signals and inflammatory cytokines (Mescher et al., 2006; Parish and Kaech, 2009). Subsequent to activation within the secondary lymphoid tissues, T cells then migrate to non-lymphoid sites where they may continue to proliferate (Masopust et al., 2001; Reinhardt et al., 2001). CD8$^+$ T cells mediate their

cytolytic activity via the granule exocytosis pathway, involving perforin, granzyme A and B and other cytotoxic granules, and the granule-independent pathway, involving death receptors on the target cell (Kagi et al., 1996). After the acute phase response to the infection, this is then followed by the secondary responses: apoptotic loss of the majority of the effector cells and the development of a stable memory pool (Wherry and Ahmed, 2004).

The phase at which the $CD8^+$ T cell is in can be determined from the unique pattern of cell surface molecules that it expresses. These are numerous, but critically, naïve T cells require L-selectin (CD62L) in order to exit the blood and home to the lymph nodes. After activation CTLs downregulate lymphoid organ homing receptors (CD62L, CCR7) and upregulate surface expression markers (CD44), which home the cell to peripheral sites of inflammation and facilitate cell adhesion.

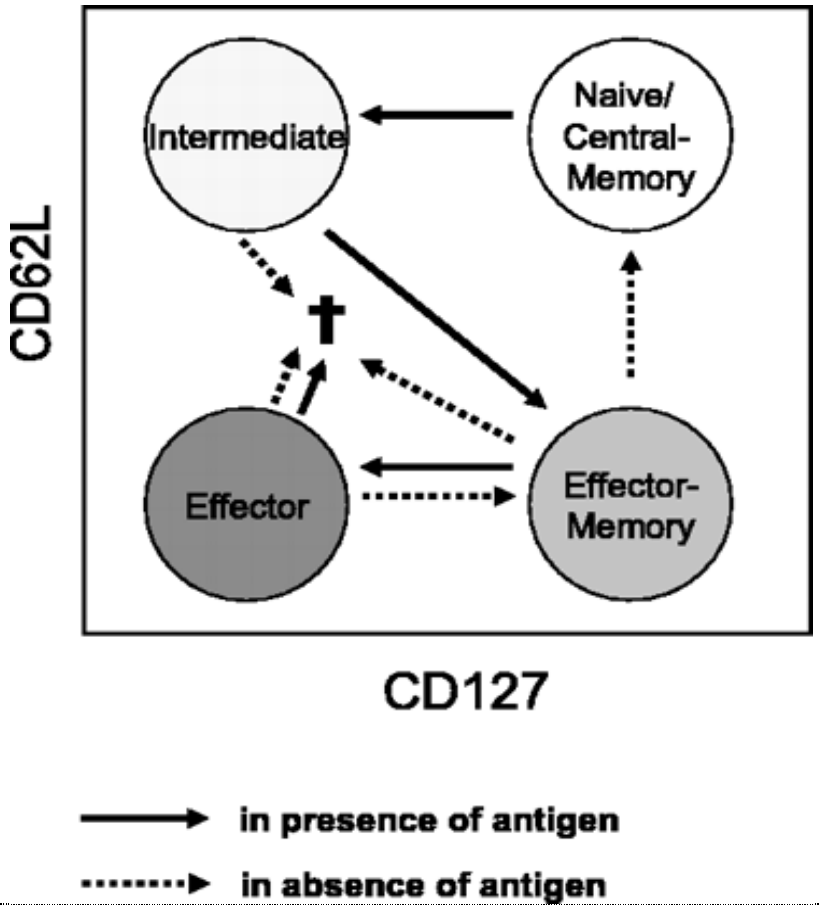
1.2 What is "Memory Inflation"?

T cell "memory inflation" is a striking immunological response, first reported in murine cytomegalovirus (MCMV) infection (Karrer et al., 2003). Certain epitope-specific $CD8^+$ T cell populations (see forward) can be tracked *in vivo*, using MHC class I peptide tetramers (Altman et al., 1996). These responses are noted to continue to expand in the periphery after an initial viral infection and remain functional over the life span of the host (Gillespie et al., 2000; Holtappels et al., 2000; Karrer et al., 2003; Komatsu et al., 2003; Munks et al., 2006a). This is in contrast to what is seen in most other viral infections, with the usual $CD8^+$ T cell contraction to a central memory pool after the acute phase of infection, and the exhaustion of any remaining peripheral $CD8^+$ T cells (Arens et al., 2011; Gallimore et al., 1998; Lang and Nikolich-Zugich, 2011; O'Hara et al., 2012; Sierro et al., 2005; Zajac et al., 1998). These inflationary populations in mice parallel the huge expansion of $CD8^+$ T cells seen in those infected with human CMV (HCMV), especially in the elderly, where such exaggerated responses are thought to be relevant to immune senescence. The phenomenon of memory inflation has now become well established and similar features have been described by a number of groups, from studies of CMV and other low-level persistent viruses (Jones et al., 2010; Komatsu et al., 2003; Lang et al., 2009; Norbeck et al., 2005; Snyder et al., 2008; Torti et al., 2011b; Walton et al., 2011).

The distinct kinetics of memory inflation are accompanied by modifications of T cell phenotype. These include changes in homing, such that the populations can readily home to peripheral tissues, and loss of the requirement for co-stimulation through CD28 and CD27. The inflationary $CD8^+$ T cells typically have an effector memory phenotype, characterised by being $CD44^+$, $CD62L^-$, $CD127^{lo/hi}$, $CD27^{lo}$ and $IL2^{+/-}$ (Bachmann et al., 2005; Bolinger et al., 2013). A number of other markers can be used to additionally define these populations ($CCR7^-$, KLRG-1^{hi} and NKG2A/D^{hi} for example), but through out this work the panel of CD44, CD62L, CD127 and CD27 has been used. Figure 1.1 demonstrates the ability to group $CD8^+$ T cell pools using a co-stain of CD127 and CD62L.

The populations also maintain strong functionality: killing, cytokine release and proliferation (Karrer et al., 2003). This is in contrast with the other prevailing models of chronic virus infection, especially lymphocytic choriomeningitis (LCMV), which lead to T cell exhaustion (Gallimore et al., 1998; Zajac et al., 1998).

Figure 1.1: Grouping of $CD8^+$ T cells according to their CD62L/CD127 phenotype into T_{EM}, T_E and T_{CM} pools.



Staining, in mice, for CD62L and CD127 allows for the differentiation of $CD8^+$ T cells into naïve/central memory, effector memory and effector pools. In addition to this classification, T_{EM} $CD8^+$ T cells are $CD27/CD28^{low}$ and $CD44^{hi}$.

1.3 Cytomegalovirus and memory inflation

Cytomegalovirus belongs to the *Herpesviridae* family. These are a group of large double-stranded DNA viruses, where infection in the host is characterised by latency and reactivation. Herpesviruses broadly divide into α-Herpesviruses and β-Herpesviruses, the latter of which CMV is a member. Different mammalian species are infected by different CMVs. Their genomes share a central mutually homologous section encoding structural proteins and proteins involved in viral replication. These are then flanked by non-homologous sequences that hold the species-specific adaptations. As such, MCMV is an established model for studying HCMV infection (Krmpotic et al., 2003).

Initial work with MCMV and memory inflation was carried out in the BALB/c mouse model, where MCMV infection leads to the production of the pp89-specific inflationary CD8+ T cell responses (Karrer et al., 2003). Subsequent work looking into memory inflation in MCMV infection has been mostly in the C57BL/6 mouse model. C57BL/6 (B6) mice mount a response to at least 20 viral antigens during acute infection with MCMV (Munks et al., 2006b). Most of those responses, including the M45 immunodominant Ag, then decline to small central memory populations, or non-inflationary populations. In contrast, the inflationary populations in B6 mice are represented by only 3 responses; M38, m139 and IE3 (Munks et al., 2006a).

MCMV has complex transcriptional machinery with a range of genes expressed at earlier and later time points. This starts with transcription of immediate early (IE) genes, followed by early (E) and then late (L) gene products.

CMV and immune evasion

The innate and adaptive immune responses to MCMV are well described. Critical to working with CMV and understanding memory inflation are the immune evasion mechanisms of the virus. MCMV encodes for numerous proteins whose function it is to evade the innate immune system. These proteins are able to aid evasion of the virus from typical innate responses through NK cells (m157), macrophages (chemokine expression and the US22 gene family), the complement system and induction of receptors for the Fc domain of murine immunoglobulin G molecules. (Arase et al., 2002; Davis-Poynter et al., 1997; MacDonald et al., 1997; Menard et al., 2003; Nomura et al., 2002; Thale et al., 1994). It also has a number of ways in which it evades the adaptive immune response. These include mechanisms for suppression of the MHC class II antigen presentation pathway (Miller et al., 2001), inhibition of presentation through the MHC class I antigen presentation pathway (Reddehase et al., 2002) and inhibition of IFNγ signaling, which affects both antigen presentation pathways (Khan et al., 2004b). Overall, CMV encodes a wide array of immune evasion genes (Jackson et al., 2011), which limit antigen presentation through interference with antigen processing. It has therefore been very difficult to define a virological basis for the difference between “inflationary” and “non-inflationary” epitopes, given the complexities

of working with the MCMV model (Munks et al., 2007). We know that only certain epitopes drive inflationary responses (Munks et al., 2006a; Sierro et al., 2005) and that these epitopes are derived from different parts of the viral genome.

Maintenance of inflationary responses

The many factors that drive and/or maintain memory inflation have been the subject of intense scrutiny by different groups (Klenerman and Dunbar, 2008). In CMV, it is clear that the underlying feature of long-term persistence of the virus, and therefore viral antigen, remains important (Snyder et al., 2008). If the same antigen is delivered with a non-persistent vector, vaccinia virus, there is no inflation even though acute responses are comparable (Munks et al., 2006a). However, if the replicative capacity of the MCMV is impaired, inflation is still induced (Snyder et al., 2011). It seems likely that other mechanisms are involved. Systemic administration, the specific processing environment and the types of antigen presenting cells involved in inflation are likely critical, and discussed in further detail ahead. Also of interest is the increasing body of evidence around $CD8^+$ T cell “stemness” (Buchholz et al., 2013; Gattinoni et al., 2009; Graef et al., 2014), as well as tissue-resident memory T (Trm) cells (Schenkel and Masopust, 2014; Thome and Farber, 2015). $CD8^+$ T cell “stemness” refers to the recent findings that memory T cells display core molecular signatures and functional attributes characteristic of stem cells. It is possible that memory inflation could be fuelled by either of a stem-like cell pool or Trm pool, but further work in this field is required.

A number of other factors have been investigated and found necessary or redundant for memory inflation. Using a range of knockout mice, the role of $CD4^+$ T cells, co-stimulation, cytokines and cross-presentation have been analysed (Arens et al., 2011; Jones et al., 2010; Munks et al., 2007; Snyder et al., 2011; Torti et al., 2011b). This thesis will not go into detail in these areas, but it is important to remember the role of other factors outside of $CD8^+$ T cells in memory inflation. A caveat of these experiments is that modification of the host response can lead to viral reactivation, which then triggers further antigen release.

1.4 CMV and immune senescence

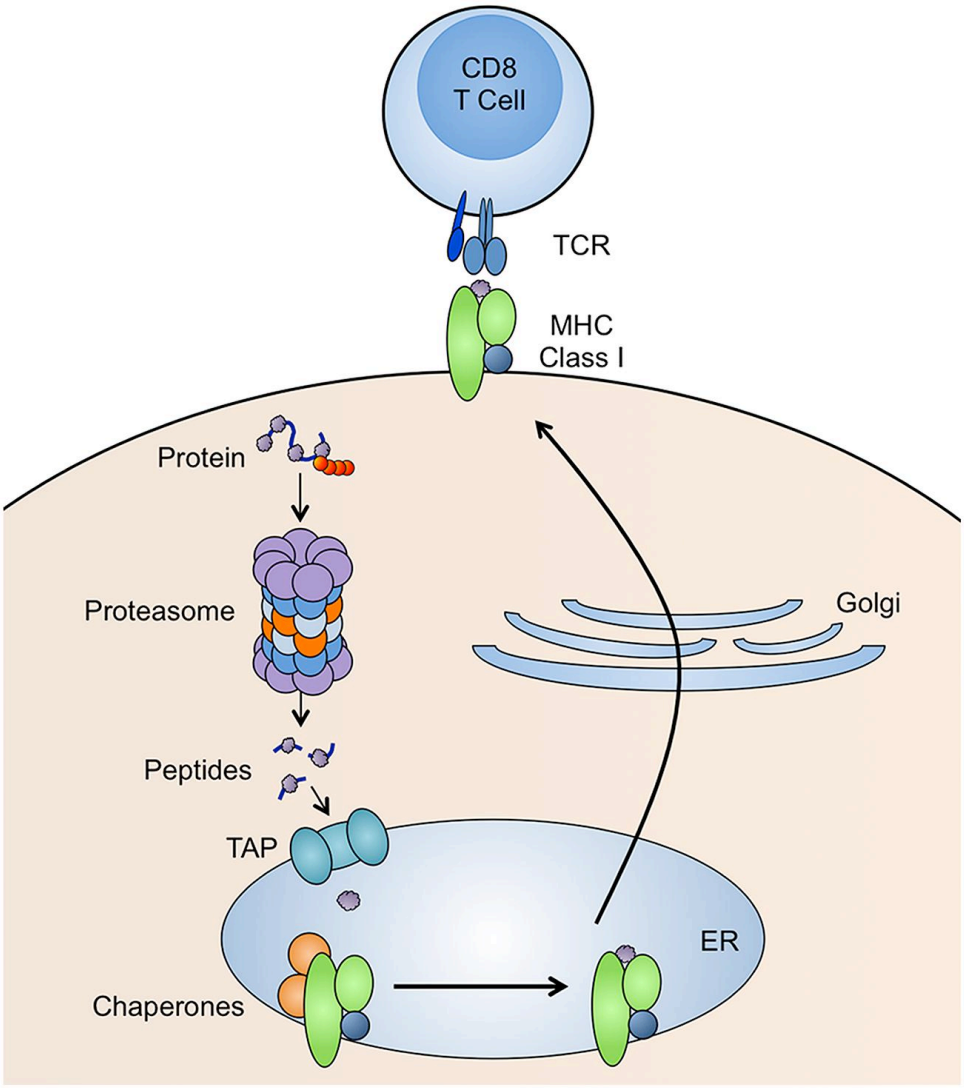
Immune senescence is the impairment of immunity as a result of age-associated changes in a variety of cells. It is a phenomenon of decreased function, involving changes to both innate and adaptive immunity and an imbalance between the two (Le Saux et al., 2012; McElhaney et al., 2012; Nikolich-Zugich, 2014; Pawelec et al., 2010; Wills et al., 2011). The contribution of CMV to immune senescence is becoming increasingly more apparent. CMV-infected individuals have been shown to develop large populations of $CD8^+$ and $CD4^+$ T cells (Sylwester et al., 2005). These virus-specific populations have been shown to increase with age, and to dominate the memory compartments in elderly CMV seropositive individuals (Khan et al., 2002; Wills et al., 2011). This may have an impact on the ability of these individuals to respond to other infections and immunisations (Khan et al., 2004a; Pera et al., 2015). The inflated populations seen with CMV infection appear to be irrefutably linked as a contributory factor in the development of immune senescence (Pawelec et al., 2009). Murine CMV infection leads to a similar expansion of these $CD8^+$ T cells that demonstrate memory inflation and these in turn have been shown to propagate immune senescence with an impact on immunity in ageing mice (Mekker et al., 2012). Indeed, there is some evidence that antiviral treatment of CMV infection can reverse these effects on immune senescence (Beswick et al., 2013).

1.5 Antigen processing and memory inflation

1.5.1 The immunoproteasome

Viruses are obligate intracellular parasites and rely on the host cell machinery for protein synthesis. Viral proteins are then cleaved in the cytoplasm by the proteasome. Protein degradation by proteasomes is the source of most antigenic peptide presented on MHC class I molecules (Goldberg and Rock, 1992; Rock et al., 1994). Within mammalian cells, proteins are typically broken down into 8- to 11-residue peptides (Falk et al., 1991), which can then be presented on class I molecules (Rock and Goldberg, 1999). Most intracellular protein degradation is handled by the ubiquitin-proteasome pathway (Ciechanover et al., 2000; Glickman and Ciechanover, 2002). The ubiquitin tag on the protein serves as a signal for their degradation. Once generated in the cytosol, peptides are transported by the TAP (transporter associated with antigen processing) complex into the endoplasmic reticulum (Neefjes et al., 1993). Here they bind with class I molecules and are transported to the cell surface (Cascio et al., 2001) allowing for interaction with $CD8^+$ T cells. This is summarised in figure 1.2.

Figure 1.2: The MHC class I antigen presentation pathway.



Proteins with ubiquitin tags (red spheres) are degraded by proteasomes and the resulting peptides are transported into the endoplasmic reticulum (ER) by TAP. In the ER, the peptide is loaded onto MHC class I molecules by many molecular chaperones. The peptide-MHC class I complex is then transported to the cell surface for presentation to $CD8^+$ T cells (McCarthy and Weinberg, 2015).

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Proteasomes exist in many forms in cells, with different regulatory or activator cap complexes that associate with the 20S core to control access to the proteolytic inner chamber (Baumeister et al., 1998). The immunoproteasome is an inducible form of the proteasome, thought to be relevant for the production of class I ligands (Goldberg et al., 2002; Groettrup et al., 2010; Rock et al., 1994). Proteolysis is conducted in the core particle, or 20S proteasome. This is composed of two outer α-rings and two inner β-rings. Constituent proteasomes have 14 structural subunits and 6 catalytic (β) subunits (2 each of β1, β2 and β5) (Coux et al., 1996). There are then three alternative beta-type proteasome subunits (β1i, β2i and β5i), which are constitutively expressed by a number of haematopoetic cells and are otherwise induced in other cell types, as described below. The first two, β1i (also known as LMP2) and β5i (also known as LMP7) were identified in the 1990's in association with MHC class I ligands (Brown et al., 1991; Glynne et al., 1991; Kelly et al., 1991; Ortiz-Navarrete et al., 1991). The production of these proteasome subunits is strongly induced by the proinflammatory cytokines IFNγ and TNFα (Coux et al., 1996; Gaczynska et al., 1993; Rock and Goldberg, 1999; Tanaka, 1994). Subsequently, a third proteasome subunit, also induced by inflammatory cytokines, β2i (also called LMP10 or MECL1) was described (Groettrup et al., 1996; Hisamatsu et al., 1996; Nandi et al., 1996). Following exposure to IFNγ, these three subunits are strongly expressed and the constitutive proteasome is switched almost completely to the production of the immunoproteasome (Akiyama et al., 1994; Boes et al., 1994). The reasons and exact mechanisms for this exchange are still not fully

understood. It has been demonstrated that the immunoproteasome cleaves proteins after hydrophobic residues and less so after acidic residues (Aki et al., 1994; Driscoll et al., 1993; Gaczynska et al., 1993; Gaczynska et al., 1994). The immunoproteasome thus facilitates elimination of virus-infected and tumor cells by cytotoxic T cells (CTLs).

The thymus is a specialised lymphoid organ, within which T cell maturation occurs. There is an additional type of specialised proteasome, termed the thymoproteasome. This is made up of β1i and β2i as well as the cTEC (cortical thymic epithelial cells) specific proteasome subunit β5t. β5t has a role in generating $CD8^+$ T cells in the thymus. In β5t-deficient mice it has been shown that class I peptides generated through the thymoproteasome are of higher affinity. Given positive selection is assumed to result from low-affinity interactions, this suggests that β5t is essential for positive selection of T cells (Murata et al., 2007; Nitta et al., 2010; Xing et al., 2013).

1.5.2 Memory inflation and the immunoproteasome

Both murine and human cytomegaloviruses encode immunoevasins that affect the MHC class I processing pathway. These inhibit upregulation of the immunoproteasome in response to IFNγ (Khan et al., 2004b). Inhibition of immunoproteasome formation occurs at a pretranscriptional level. The effect is mediated by the MCMV protein M27 (Zimmermann et al., 2005). When cells are infected with a virus lacking M27 (which acts as a STAT2 inhibitor,

interfering with IFNγ receptor signaling) immunoproteasome expression is no longer inhibited.

One recent finding of interest is that “inflationary” epitopes appear to be relatively independent of the immunoproteasome (Hutchinson et al., 2011). However, once again the CMV model is complex, since in most cases the epitopes are derived from different proteins, expressed by different promoters. Whilst we know that specific epitopes can show immunoproteasome dependence or independence, the overall effect on T cell responses is not fully understood.

The fact that the inflationary epitopes show independence from the immunoproteasome is perhaps just a pointer at the possibility that these epitopes are being presented on non-classical APCs. Evidence that supports the likelihood of a non-haematopoetic APC in the generation of inflationary $CD8^+$ T cells seems increasingly more convincing (Smith et al., 2014; Torti et al., 2011a). Alternatively, it may be that inflationary epitopes favour a proteasome over the immunoproteasome. This remains a field that requires further investigation.

1.6 Adenoviral vectors

Adenoviruses have a broad range of vertebrate hosts. The *Adenoviridae* family is divided into five genera, of which human adenoviruses fall into the *Mastadenovirus* genus (Davison et al., 2003). They are 90-100nm non-enveloped icosahedral viruses with linear double-stranded DNA genomes of around 34-43kb, encoding between ~30-40 genes (Hall et al., 2010; Tatsis and Ertl, 2004). There are around 57 human adenovirus serotypes, which are divided into seven species (A-G), mostly based upon their haemagglutination properties and phylogenetic analysis. These different serotypes are associated with different clinical manifestations of disease, causing respiratory illness, conjunctivitis and gastroenteritis most commonly. Species C adenoviruses, and mainly serotypes 2 and 5 (AdHu2 and AdHu5), are the ones for which the majority of our understanding of host interactions are based, these typically causing respiratory illness.

Human adenoviruses have been extensively used in the development of vectors for gene transfer (Liu, 2010b). Their genome is well characterised and easy to manipulate for this purpose. Importantly, there are also very efficient methods for production of high titres of virus. Adenoviral vectors give robust transgene expression, and this can be enhanced through strong heterologous promoters (Schaack et al., 2011). Most importantly, they are able to infect many cell types and do not integrate their genome into host-cell

chromosomes, eliminating the risk of insertional mutagenesis (Bangari and Mittal, 2006).

Viral genome

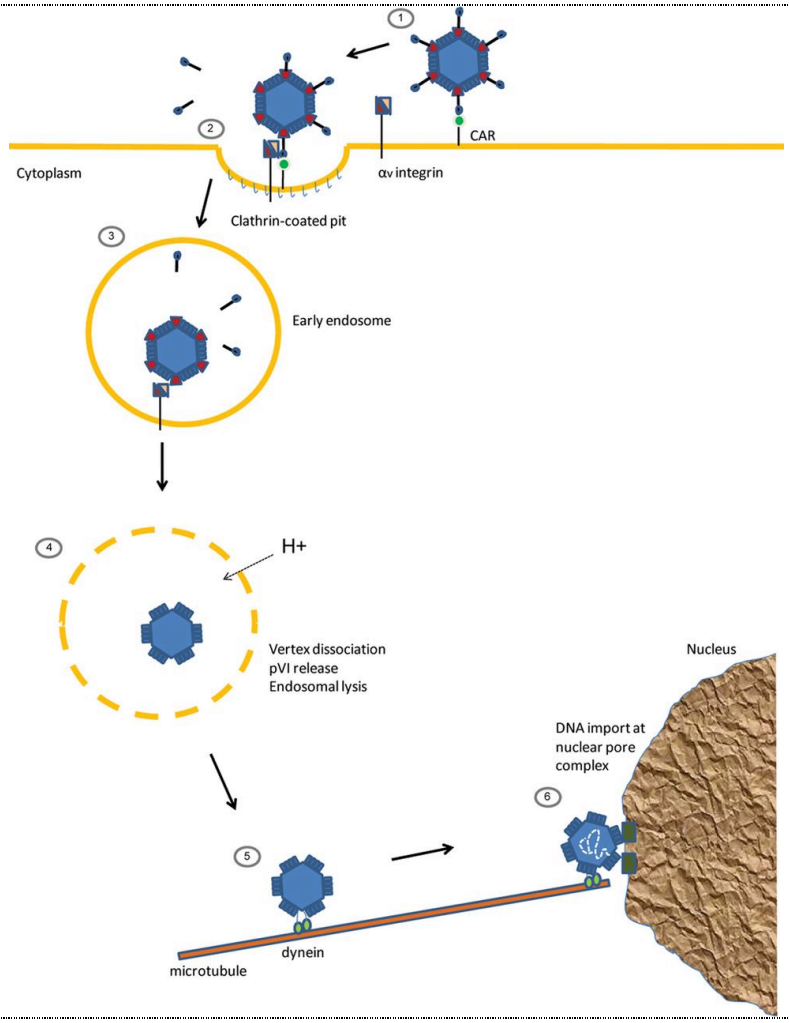
The linear genome is flanked by inverted terminal repeats (ITRs). The 5' terminus is linked to a terminal protein (TP), and this plays a role in initiation of virus replication (Russell, 2009). The adenovirus genome shows marked diversity across the species, but is organised in a general manner which is conserved amongst species (Tibbetts, 1977). The genome carries the early units (E1A, E1B, E2A, E2B, E3 and E4), two units that are expressed with a delay after initiation of viral replication (IX and Iva2) and the late genes (L1-L5) (Hall et al., 2010; Tatsis and Ertl, 2004). There are other minor transcription units present, as well as some virus-associated RNAs, with functions in the inhibition of host protein synthesis in infected cells. E1A deletion typically renders the virus replication-defective, unless the E1A gene is supplied *in trans* by the cell line used for production (Tatsis and Ertl, 2004).

Viral structure and tropism

The adenovirus particle is made up of 13 structural proteins. Hexon, penton base and fibre constitute the major capsid proteins. The minor capsid proteins include IIIa, VI, VIII and IX. The dsDNA genome is found in association with the core proteins V, VII, Mu, TP, Iva2 and the viral protease. The fibre and penton base proteins mediate uptake into host cells (Nemerow et al., 2009). All but the species B and certain serotypes of species D adenoviruses act upon the coxsackie and adenovirus receptor (CAR) (Bergelson et al., 1997;

Roelvink et al., 1998). These other serotypes associate with CD46 (Gaggar et al., 2003), CD80 and CD86 (Short et al., 2004). The CAR receptor is found on many cell types, including hepatocytes, epithelial cells, endothelial cells, myoblasts and heart muscle. Figure 1.3 demonstrates the likely entry mechanism of a species C adenovirus into the target cell, and its transport to the cell nucleus.

Figure 1.3: Adenovirus (species C) entry into target cells.



The entry of species C Ads has been proposed to involve the following stages. **(1)** Attachment of the fibre knob to the primary receptor CAR. **(2/3)** Subsequent interaction of the penton base with αv integrins. This leads to clathrin-mediated endocytosis resulting in virus internalisation within endosomes. **(4)** The virus begins to dissociate in the low pH environment of the endosome and releases the vertex proteins including pVI. Protein pVI can disrupt the endosomal membrane, allowing the partially dismantled virus particle to escape from the endosome. **(5)** The partially disassembled virus is then transported along microtubules by dynein to the nuclear pore complex. **(6)** At the nuclear pore, viral DNA is imported into the nucleus.

Adenoviral vectored vaccines

Viral vectors allow for several advantages over established protein-based vaccination methods. They induce antibody responses, but additionally elicit CTLs, which is critical to the control of intracellular pathogens and cancers (Liu, 2010a; Rollier et al., 2011). Molecular virology has allowed for recombinant viruses that have been rendered highly attenuated or replication-defective, giving them an excellent safety profile. That said, the host has also evolved with the viruses, adenoviruses being highly immunogenic, impacting on vector efficacy (Liu, 2010a). A number of adenoviral-vectored vaccines against infectious diseases have progressed through from animal to human trials, and it seems relevant to highlight these and discuss some of the successes and failures around the use of these vaccine platforms. Tables 1.1 and 1.2 detail some of the adenoviral vaccines trialed in humans (for human adenovirus vectors and simian adenovirus vectors respectively). Of note, adenoviral vaccines for use in cancer therapies have also shown some promise (Lubaroff et al., 2009), but will not be discussed in any further detail here.

Critically, the initial studies utilising AdHu5 vectors highlighted issues with pre-existing adenoviral antibodies in humans (Buchbinder et al., 2008; Gray et al., 2011). Within the adenoviral vaccine field, there is now a strong body of evidence to support the use of simian adenoviruses or the less common human adenovirus serotypes, in order to avoid the issues around pre-existing immunity. This is especially important given the evidence of decreased immunogenicity associated with pre-existing neutralising antibodies (Geisbert

et al., 2011; Kobinger et al., 2006; Sumida et al., 2005). There are some vaccine candidates that have utilised AdHu26 and AdHu35 vectors, which segregate genetically from AdHu5 and exhibit lower seroprevalence in humans, making them attractive vaccine vector alternatives (Geisbert et al., 2011; Hokey et al., 2014). There is an increasing use of different simian adenoviral vectors (Colloca et al., 2012). There are now a number of vaccines in development using this technology (Antrobus et al., 2014; Capone et al., 2013; Ledgerwood et al., 2014; Pearson et al., 2015; Pierantoni et al., 2015; Rampling et al., 2015; Sheehy et al., 2011; Swadling et al., 2014).

Table 1.1: Adenoviral vaccine developments in human trials: human adenovirus vectors.

Vector	Details	References
AdHu5 - Step Study	HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. MRKAd5 HIV-1 gag/pol/nef vaccine. This did not reduce plasma viraemia after infection, and HIV-1 incidence was higher (although non-significantly) in one subgroup of vaccine-treated compared to placebo-treated men with pre-existing adenovirus serotype 5 immunity.	(Buchbinder et al., 2008; McElrath et al., 2008)
AdHu5 - HVTN 503/Phambili Study	Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. The MRKAd5 HIV-1 vaccine did not prevent HIV-1 infection or lower viral-load setpoint. However, stopping the trial early probably compromised ability to draw conclusions.	(Gray et al., 2011)
AdHu26	A prototype Ad26 vector-based vaccine expressing clade A HIV-1 Env (Ad26.ENVA.01). Ad26.ENVA.01 elicited a broad diversity of humoral and cellular immune responses in humans and was well tolerated.	(Baden et al., 2013; Barouch et al., 2013)
AdHu35	Several trial candidates, including malaria, tuberculosis and HIV vaccines. These are all in different phases of development, but show good safety data.	(Churchyard et al., 2015; Ockenhouse et al., 2015; Omosa-Manyonyi et al., 2015)

Table 1.2: Adenoviral vaccine developments in human trials: simian adenovirus vectors.

Vector	Details	References
ChAd63	Phase Ia clinical evaluation of the Plasmodium falciparum blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. A phase Ia clinical trial was conducted in healthy adults of a ChAd63-MVA MSP1 heterologous prime-boost immunisation regime. The vaccine was safe and generally well tolerated. (ChAd63 also used with other malaria antigens, AMA1 and METRAP, both of which have reached phase II trials with some promise.)	(Biswas et al., 2014; Biswas et al., 2011; Hodgson et al., 2015; Ogwang et al., 2015; Sheehy et al., 2011)
ChAdY25 (or ChAdOx1)	A novel chimpanzee adenovirus vector with low human seroprevalence: improved systems for vector derivation and comparative immunogenicity. The prevalence of virus neutralising antibodies (titre >1:200) against ChAdY25 in serum samples collected from two human populations in the UK and Gambia was particularly low compared to published data for other chimpanzee adenoviruses. (ChAdOx1 is also used in other vaccines, such as influenza.)	(Antrobus et al., 2014; Dicks et al., 2012)
ChAd3	ChAd3 has been used in trials for HCV vaccines as well as the most recent Ebola vaccines. These have all shown good safety data. Of note, better immunogenicity is noted from heterologous prime-boost strategies involving ChAd3 primes and MVA boosts in HCV trials (compared to homologous prime-boost).	(Barnes et al., 2012; Ledgerwood et al., 2014; Rampling et al., 2015; Swadling et al., 2014)
PanAd3	Development in RSV vaccines. Safe and immunogenic, warranting further clinical evaluation.	(Green et al., 2015)

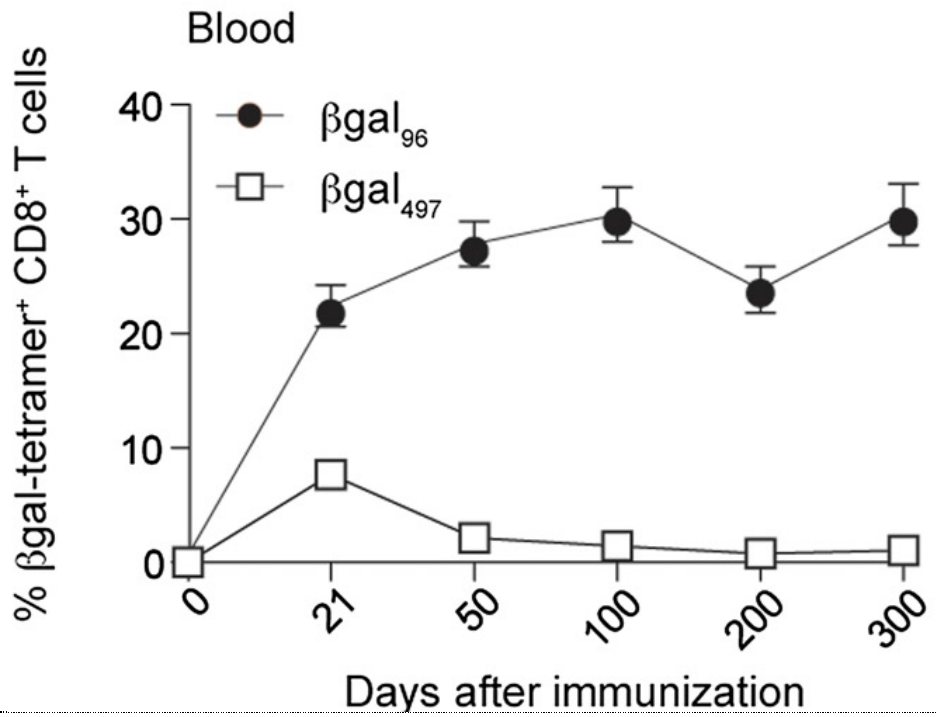
Adenoviral vaccines and protection

Whilst these numerous studies in humans have shown good safety data and immunogenicity, it is important to consider the evidence for protection from adenoviral vaccine candidates. Within the Ad-LacZ (and MCMV) model of memory inflation, it is possible to appreciate a very high magnitude of epitope-specific $CD8^+$ T cell response (20-30% of total $CD8^+$ T cells). This does not however say anything about the possible levels of protection incurred from such responses and more work is needed to address the quality or functionality of these responses, with relation to protection. There is a body of evidence to demonstrate that the magnitude of the response to a vaccine does not determine the outcome of the immunisation, but that the quality of that response is equally or more important (Beverley et al., 2014; Jeyanathan et al., 2013). Many of the human vaccine candidates previously described have not reached the relevant phase in investigation to address their level of protection. For those that have, there is some evidence for partial protection (Biswas et al., 2014; Hodgson et al., 2015; Ogwang et al., 2015) but much work still remains to be done. In animal studies though, there is a good body of evidence for protection from adenoviral-vectored vaccines in a number of infectious disease models (Barouch et al., 2015; Gowen et al., 2014; Grimaldi et al., 2014; O'Brien et al., 2014; Stanley et al., 2014; Wang et al., 2011; Wong et al., 2015; Zhang et al., 2013).

1.7 Overview of the adenoviral model for memory inflation

As discussed, the MCMV model is a complex system to work with to better define the mechanisms of memory inflation. Recently the Klenerman laboratory has developed a new inflation model, set up by Beatrice Bolinger (Bolinger et al., 2013; Krebs et al., 2005), which offers a more tractable approach. This model is based upon a recombinant non-replicating human adenovirus serotype 5 (AdHu5). The transgene comprises a human CMV (HCMV) immediate early promoter and a LacZ open reading frame, encoding β-galactosidase (making the construct Ad-LacZ). Within β-galactosidase (β-gal), two Kb-restricted epitopes have been identified. The first is at position $\beta\text{-gal}_{96\text{-}103}$, otherwise known as D8V (DAPIYTNV) (Overwijk et al., 1997), and the second is at $\beta\text{-gal}_{497\text{-}504}$, or I8V (ICPMYARV) (Oukka et al., 1996). These two epitopes elicit $CD8^+$ T cell populations with a typical effector memory and central memory response respectively, when Ad-LacZ is delivered intravenously into a C57BL/6 mouse (figure 1.4). These epitopes are expressed from the same transgene, controlling for expression. The model has been shown to recapitulate all of the features of memory inflation identified in MCMV, despite of the replication deficiency of the virus. These features include frequency, function, phenotype, distribution, immunoproteasome-independency (Bolinger et al., 2013). The model has also been shown to work through the intradermal route of immunisation, albeit at a markedly reduced magnitude of response.

Figure 1.4: Memory Inflation using the adenoviral (Ad-LacZ) model.



Graph taken from data produced from within the Klenerman laboratory, showing the inflation populations in blood from the Ad-LacZ model (Bolinger et al., 2013). The $CD8^+$ T cell responses were tracked over time using MHC class I peptide tetramers to identify responses in blood to the two dominant epitopes derived from the insert. The inflationary epitope (β-gal_{96-103}), which we call "D8V" and the non-inflationary epitope (β-$gal_{497-504}$), which we call "I8V".

The questions around how inflating populations are primed and then maintained remain. The adenoviral model throws up more key questions around the maintenance of these cells, given the replication deficiency of the virus. It is possible that persistent viral antigen may still play a role in maintaining these pools of $CD8^+$ T cells (Bolinger et al., 2013; Tatsis et al., 2007), some of which may additionally maintain "stem-like" qualities (Gattinoni et al., 2009; Graef et al., 2014; Klenerman and Dunbar, 2008; Puleston et al., 2014; Quinn et al., 2015; Smith et al., 2014).

In summary, the adenoviral model is a good model of memory inflation, replicating that seen in MCMV, with the additional advantages of:

1. There is no replication *in vivo*, thus the issues to do with local stochastic reactivation of CMV are avoided.
2. The LacZ vector can be readily tracked *in vivo* by immunohistochemistry if required.
3. The two T cell responses can be readily tracked and are derived from the same transgene, thus controlling for the level and timing of expression.
4. The complex immune evasion genes of CMV are avoided.
5. Most relevant to this project – the vector can be readily manipulated to modify the insert and the promoter.

1.8 Thesis aims

The mechanisms underlying memory inflation are not well understood, particularly why only some epitope-specific responses inflate and not others. The development of an *in vivo* model using a recombinant non-replicating adenovirus (AdHu5) expressing β-galactosidase under an HCMV promoter has allowed for a more tractable approach. This model has been used to explore some of the features of adenoviral vectors and memory inflation. This work is relevant both in terms of understanding the basic molecular biology, but also from a translational perspective. It may contribute towards understanding around the negative implications of memory inflation, in terms of CMV disease and immune senescence. It may also contribute towards harnessing these responses in a positive sense, in the use of novel adenoviral vaccine strategies.

The focused aims of this thesis are:

1) To test the hypothesis that the viral promoter dictates memory inflation in the Ad-LacZ model, and

2) To test the hypothesis that cellular antigen processing dictates memory inflation in the Ad-LacZ model.

2. Materials and Methods

2.1 Animals

2.1.1 Ethics statement

All mouse experiments were performed in Oxford according to UK Home Office regulations (project licence number 30/2744 (until 25/05/2015) and 30/3293 (after 25th May 2015), personal licence number 30/10139) and after review and approval by the local ethical review board at the University of Oxford.

2.1.2 Mice

All mice were bred and maintained in specific pathogen free (SPF) conditions in individually ventilated cages and fed on a normal chow diet at the Biomedical Services Building, Oxford. All experiments were carried out with age and sex matched animals (females, aged 6±2 weeks). Groups were typically made up of 5 animals, unless stated otherwise. Naïve controls were included in all experiments. All experiments have been repeated a minimum of two times unless otherwise stated.

C57BL/6 mice were obtained from Harlan (UK). LMP7$^{-/-}$ mice, (Fehling et al., 1994), were kindly re-derived with the help of Denise Jelfs and Richard Corderoy from embryo stocks previously held by the Klenerman laboratory.

(This mouse line is also available through The Jackson Laboratory: Stock No. 021201 *Psmb8tm1Hjf*/J.)

2.1.3 Procedures; immunisations, intravenous blood sampling

Intravenous immunisations

Mice were restrained in a tail vein restrainer (Braintree Scientific, USA), after having been warmed at 37°C until vasodilation of the lateral tail vein was visible. They were immunised with 200μL of the applicable virus diluted to the correct viral titre in sterile PBS (see table 2.1), using a U-100 29G BD Ultra-Fine™ short insulin syringe (0.3mL) (BD, UK).

Intraperitoneal immunisations

Mice were manually restrained and immunised with 200μL maximum volume into the peritoneal cavity using a U-100 29G 0.3mL insulin syringe.

Intramuscular immunisations

Mice were manually restrained and immunised with 5μL per site (up to a maximum of 20μL) into the lower *musculus tibialis* of each rear leg using a U-100 29G 0.3mL insulin syringe.

Intravenous blood sampling

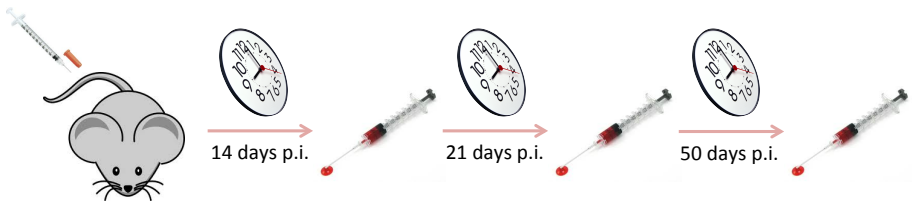
Mice were restrained in a tail vein restrainer (Braintree Scientific, USA), after having been warmed at 37°C until vasodilation of the lateral tail vein was visible. 5-8 drops (~30-60μL) of blood were collected directly into 3mL 1x

FACs buffer following puncture of the vein using a 21G needle (Greiner Bio-one, Germany).

Organ collection

Mice were sacrificed and organs retrieved. Before organ collection, first the animal was perfused with 10-15mL PBS. Typically the spleen, liver and lungs were collected for analysis.

Figure 2.1: Typical in vivo experimental schedule.



Day 0:	Day 14:	Day 21:	Day 50:

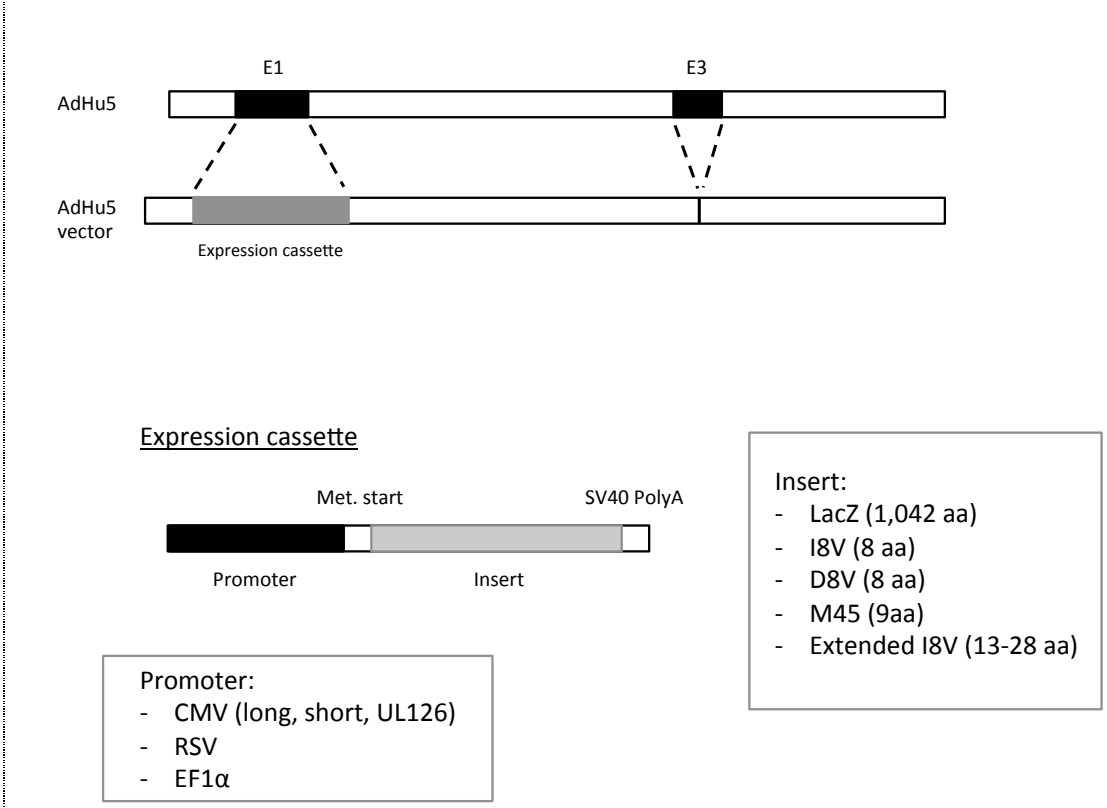
- B6 or LMP7$^{-/-}$ mice
- Groups of 5 typically
- Immunisation:
  - i.v. 200µL virus via tail vein
  - i.p. 200µL virus peritoneal cavity
  - i.m. 20µL virus (5µL per site) tibial muscles
	- Tail vein bleed (5-8 drops)
	- Blood analysed for tetramer^{+} CD8^{+} T cells and Ab panel (CD44/ CD62L/CD127/CD27) on BD LSR II flow cytometer
		- Intermediate time-points as per day 14
		- Additional time-points may be added
			- Final tail vein bleed as per day 14
			- Harvest organs for analysis (liver / lung / spleen)

2.2 Viruses

2.2.1 Adenoviral vectors

All vectors were based upon the same template of an AdHu5 virus, E1/E3 deleted. All expression cassettes are flanked by an initiator methionine, stop codon and a SV40 polyA tail. The promoter and insert regions were then modified according to the question being addressed. This overall template is summarised in figure 2.2 below.

Figure 2.2: AdHu5 template for all vectors used.



Wildtype Ad-LacZ

Recombinant adenovirus expressing the β-gal protein with a short (lacking intron A) HCMV promotor (called Ad-LacZ) and replication deficient (lacking E1) was used, provided through Beatrice Bolinger as previously described (Bolinger et al., 2013).

Virus was grown on HER 911 cells at an MOI of 10 using 10% DMEM (10% FCS) in 150cm^2 culture flasks (Falcon, BD, UK). An adenovirus purification kit (Sartorius, Germany) was then used to purify virus. Viral titre (infectious units/mL) was then determined using a cytopathic effect assay ($TCID_{50}$ assay). Virus was stored at -80°C and used at 2x10^9 infectious units (iu) per mouse.

Constructs made on site

During this work variant Ad-LacZ constructs were developed with The Viral Vectors Core Facility (VVCF), The Jenner Institute (Oxford, UK). These were:

1) Ad-LacZ with an HCMV long (including intron A) promoter (HCMV LP).
2) Ad-LacZ with a mammalian elongation factor 1α (EF1α) promoter.
3) Constructs expressing the minigenes β-gal$_{96\text{-}103}$ (Ad-D8V) and β-gal$_{497\text{-}504}$ (Ad-I8V).
4) Ad-I8V N-terminal extension constructs. These are the Ad-10aa-I8V, Ad-7aa-I8V and Ad-5aa-I8V constructs.

Further details of these can be found in appendix 1.

Table 2.1 gives a summary of all constructs, their promoter and their inserts as well as their viral titres and what concentration they were used at *in vivo*. Table 2.2 shows the specific amino acid sequences for each of the constructs described.

These were all produced using a shuttle vector containing the required promoter and transcription terminator using the inserts described as in table 2.1. These were then recombined into the pAD/PL-DEST vector (Invitrogen, UK) using LR clonase (Invitrogen, UK) to produce whole adenovirus genome plasmids, which were used to transfect TRex HEK 293A cells (Invitrogen, UK). The rescued adenovirus was used to infect a bulk culture (hyperflask) of TRex HEK 293A at 80% confluency. Once CPE had developed throughout the cell monolayer, the cells and media were harvested and the cells isolated by centrifugation. The cell pellet was resuspended in lysis buffer, frozen and thawed three times and treated with benzonase. The sample was then cleared by centrifugation and loaded onto a CsCl column for rate zonal centrifugation followed by isopycnic centrifugation. Finally, the material was dialysed into storage buffer and stored at -80°C. The vaccine antigens in all final products were then sequenced, and viral particles and infectious units determined.

Constructs purchased

Kerafast: Two alternative Ad-LacZ (AdHu5, E1/E3 deleted) constructs were purchased from Kerafast (Boston, USA):

1) Ad-LacZ with an HCMV promoter (UL126).
2) Ad-LacZ with a Rous sarcoma virus (RSV) promoter.

Vector BioLabs: Further minigene and processing constructs (AdHu5, E1/E3 deleted) were purchased from Vector BioLabs (Pennsylvania, USA). These included:

1) A construct expressing the $M45_{985\text{-}993}$ minigene (HGIRNASFI) called Ad-M45.
2) Ad-I8V C-terminal extension constructs with 10aa on the C-terminus alone and with 10aa on both of the N and C-terminus (Ad-I8V-10aa and Ad-10aa-I8V-10aa respectively).
3) Co-expression minigene constructs, with both of the I8V and D8V epitopes within the same vector in both orientations. These are Ad-D8V-I8V (called Ad-DAI) and Ad-I8V-D8V (called Ad-ICD). These vectors contain a glycine-proline linker between the two epitopes.

Again, full details for all of these vectors can be found in table 2.1 and 2.2. All constructs had an HCMV IE promoter unless stated otherwise and were flanked by an initiator methionine, stop codon and a polyA tail.

All AdHu5 constructs held comparable P:I ratios (ratio of virus particles to infectivity), relevant to where direct comparisons are made.

Table 2.1: Summary of the constructs used in this work.

(Optimal viral titres for new constructs were tested *in vivo*. Promoter and minigene constructs data is given in appendix 2.)

Vector	Promoter	Insert	P:I	iu/mL	Used at (iu/mouse)
Ad-LacZ (Wildtype)	HCMV	β-gal	-	$1x10^{10}$	$2x10^{9}$
Ad-LacZ (Jenner)	HCMV (LP)	β-gal	30	$4.9x10^{10}$	$2x10^{9}$
Ad-LacZ (CMV - Kerafast)	HCMV (UL126)	β-gal	23.9	$7.9x10^{10}$	$2x10^{9}$
Ad-LacZ (RSV – Kerafast)	RSV	β-gal	20.4	$3.18x10^{11}$	$2x10^{9}$
Ad-LacZ (EF1α)	EF1α - mammalian	β-gal	23	$4.3x10^{11}$	$2x10^{9}$
Ad-I8V	HCMV (LP)	$\beta\text{-gal}_{497\text{-}504}$	17	$4.11x10^{11}$	$1x10^{8}$
Ad-D8V	HCMV (LP)	$\beta\text{-gal}_{96\text{-}103}$	23	$2.21x10^{11}$	$1x10^{8}$
Ad-7aaI8V	HCMV (LP)	$\beta\text{-gal}_{490\text{-}504}$	20	$8.1x10^{10}$	$1x10^{8}$
Ad-5aaI8V	HCMV (LP)	$\beta\text{-gal}_{492\text{-}504}$	28	$4.6x10^{10}$	$1x10^{8}$
Ad-10aaI8V	HCMV (LP)	$\beta\text{-gal}_{487\text{-}504}$	60	$6.7x10^{10}$	$1x10^{8}$
Ad-10-I8V-10	HCMV	$\beta\text{-gal}_{487\text{-}514}$	42	$1.2x10^{11}$	$1x10^{8}$
Ad-I8V-10aa	HCMV	$\beta\text{-gal}_{497\text{-}514}$	28		
Ad-I8V-linker-D8V	HCMV	$\beta\text{-gal}_{497\text{-}504}$-GGGCCCGGG-$\beta\text{-gal}_{96\text{-}103}$	22	$2.3x10^{11}$	$1x10^{8}$
Ad-D8V-linker-I8V	HCMV	$\beta\text{-gal}_{96\text{-}103}$-GGGCCCGGG-$\beta\text{-gal}_{497\text{-}504}$	21	$2.4x10^{11}$	$1x10^{8}$
Ad-M45	HCMV	$M45_{985\text{-}993}$	25	$2.0\ x10^{11}$	$1x10^{8}$

Table 2.2: Amino acid sequences for extended Ad-I8V minigene vectors and Ad-M45.

Vector	Promoter	Insert (position)	Amino acid sequence
Ad-LacZ	HCMV	β-gal	(full β-gal sequence – 1024aa)
Ad-I8V	HCMV (LP)	β-gal$_{497-504}$	ICPMYARV
Ad-D8V	HCMV (LP)	β-gal$_{96-103}$	DAPIYTNV
Ad-7aaI8V	HCMV (LP)	β-gal$_{490-504}$	DTTATDIICPMYARV
Ad-5aaI8V	HCMV (LP)	β-gal$_{492-504}$	TATDIICPMYARV
Ad-10aaI8V	HCMV (LP)	β-gal$_{487-504}$	GGADTTATDIICPMYARV
Ad-10-I8V-10	HCMV	β-gal$_{487-514}$	GGADTTATDIICPMYARVDEDQPFPAVP
Ad-I8V-10aa	HCMV	β-gal$_{497-514}$	ICPMYARVDEDQPFPAVP
Ad-I8V-linker-D8V	HCMV	β-gal$_{497-504}$-GGGCCCGGG-β-gal$_{96-103}$	ICPMYARVPDAPIYTNV
Ad-D8V-linker-I8V	HCMV	β-gal$_{96-103}$ -GGGCCCGGG-β-gal$_{497-504}$	DAPIYTNVPICPMYARV
Ad-M45	HCMV	M45$_{985-993}$	HGIRNASFI

2.2.2 MCMV

MCMV (Strain Smith, ATCC: VR194) was used, kindly provided by Professor U.H. Koszinoswki, Department of Virology, Max von Pettenkofer Institute, Munich, Germany. MCMV was propagated and titrated on NIH 3T3 cells (ECACC, UK), stored at -80°C and injected intravenously at a dose of $2x10^6$ iu per mouse.

2.2.3 Vaccinia LacZ

A recombinant vaccinia expressing the β-gal protein (VV-LacZ) (Ludewig et al., 2000) was used. VV-LacZ was propagated and titrated on BSC-40 cells, and injected i.p. at a dose of $2x10^6$ iu per mouse.

2.3 Reagents and solutions, tissue culture media, peptides, tetramers and antibodies

2.3.1 Reagents and solutions

Reagents and solutions were sourced from Sigma-Aldrich (USA) unless stated otherwise.

- Phosphate buffered saline 0.01M: NaCl 0.138M, KCL 0.0027M, pH 7.4 was made by dissolving tablets in ddH_20.

- ACK lysing buffer: Working stock solution was purchased from Life Technologies (Invitrogen, UK). (To make this up: 8.29g NH_4CL, 1g $KHCO_3$ and 37.2mg NaEDTA are diluted into 1000mL of ddH_20 and the pH adjusted to between 7.2 and 7.4.)

- FACS lysing buffer: 10x stock solution was purchased from BD (Oxford, UK). A 1x working solution was made up with a dilution 1:10 in ddH_2O.

- FACS buffer: a 10x stock solution was made up of 600mL of 10x PBS, 200mL heat inactivate FCS, 200mL 0.5M EDTA (Promega, USA) and 5g sodium azide. A 1x working solution was then diluted 1:10 in ddH_2O.

- ELISA PBS/T solution: 100mL PBS with 0.5mL Tween 20 (0.05% w/v).

- ELISA coating buffer: 1.59g Na_2CO_3 (15mM Sodium Carbonate), 2.93g $NaHCO_3$ (35mM Sodium Hydrogen Carbonate), 0.2g NaN3 (3mM Sodium Azide) and 1000mL H_20.

- ELISA blocking solution: 0.1g BSA (1% Bovine Serum Albumin) and 10mL PBS/T.

- ELISA stop solution: 2.4g NaOH (3M) and 20mL H_20.

2.3.2 Tissue culture media

DMEM and RPMI media was supplemented with L-glutamine (4mM), penicillin (100U/mL) / streptomycin (100μg/mL) and heat inactivated fetal calf serum (FCS) to the required percentage.

2.3.3 Peptides

All of the 12 Kb-restricted peptides from β-galactosidase (see table 2.3), including the key β-gal$_{96-103}$ (DAPIYTNV) (Overwijk et al., 1997) and β-gal$_{497-504}$ (ICPMYARV) (Oukka et al., 1996) were sourced from Proimmune (Oxford, UK). M45$_{985-993}$ (HGIRNASFI) and M38b$_{316-323}$ (SSPPMFRV) peptides were also purchased from Proimmune.

Table 2.3: Amino acid sequences and β-gal position for 12 Kb-restricted epitopes in β-galactosidase.

No.	Amino Acid Sequence	Abbreviated name	Position within β-gal
1	DAPIYTNV	D8V	β-gal$_{96-103}$
2	TRIIFDGV	T8V	β-gal$_{140-147}$
3	MSGIFRDV	M8V	β-gal$_{205-212}$
4	ELRDYLRV	EV8	β-gal$_{249-256}$
5	IPNLYRAV	IV8	β-gal$_{305-312}$
6	CDVGFREV	C8V	β-gal$_{328-335}$
7	KQNNFNAV	K8V	β-gal$_{380-387}$
8	HDALYRWI	H8I	β-gal$_{468-475}$
9	ICPMYARV	I8V	β-gal$_{497-504}$
10	EDQPFPAV	E8V	β-gal$_{508-515}$
11	AFRQYPRL	A8L	β-gal$_{555-562}$
12	GRYMYQLV	G8V	β-gal$_{1012-1019}$

2.3.4 Tetrameric MHC class I peptide complexes

This work was completed with MHC class I monomers / tetramers complexed with either of β-gal (H-2Kb), M38 (H-2Kb) or M45 (H-2Db) which were kindly provided by the NIH Tetramer Core Facility, Emory University, USA. Recombinant heavy chains (H-2Db and H-2Kb) and β2-microglobulin are produced in *Escherichia coli*, purified from inclusion bodies and refolded in the presence of human β2-microglobulin and the relevant peptide. Refolded complexes are then enzymatically biotinylated, purified by FPLC and tetramerised (Altman et al., 1996). From β-gal these included a D8V (DAPIYTNV) tetramerised with streptavidin-PE, an I8V (ICPMYARV) tetramerised with streptavidin-APC and an E8V (EDQPFPAV) tetramerised with streptavidin-brilliant violet. From MCMV these are an M38b (SSPPMFRV) tetramerised with streptavidin-PE and M45 (HGIRNASFI) tetramerised with streptavidin-APC.

Where NIH biotinylated MHC class I monomers were ordered (these can be stored at -80°C for longer periods of time than the tetramerised versions that are kept at 4°C) these were tetramerised according to the NIH protocol, complexed with streptavidin-PE or streptavidin-APC (Prozyme, USA).

Tetramers were used at a 1:400 dilution (in 50μL of 1x FACs buffer/sample).

2.3.5 Antibodies

Anti-CD8a-eFluoro® 450, anti-CD8a-PerCP-Cy5.5, anti-CD127-PE-Cy7, anti-IFNγ-eFluoro® 450, anti-TNFα-FITC were obtained from eBioscience (San Diego, USA), anti-CD44-FITC, anti-CD62L-alexa700 were obtained from BD Biosciences (Oxford, UK), and anti-CD27-PerCP-Cy5.5 was obtained from Biolegend (San Diego, USA).

All antibodies were diluted at 1:100 (in 50μL of 1x FACs buffer/sample), and live/dead marker was included at 1:50 (from a made up stock of 1:20 dilution).

The anti-mouse TCR Vβ screening panel used with sourced from BD Biosciences, UK. This included FITC-conjugated monoclonal antibodies which recognise mouse Vβ 2, 3, 4, 5.1 and 5.2, 6, 7, 8.1 and 8.2, 8.3, 9, 10^{b}, 11, 12, 13, 14 and 17^{a} T cell receptors. This was used according to the kit protocol, with 20μL of the individual antibodies per $1x10^{6}$ cells.

2.4 Flow cytometry

2.4.1 Preparation of samples

For blood analysis, 5-8 drops (~30-60μL) of blood were collected into 3mL 1x FACs buffer and kept on ice (as previously described). Following centrifugation at 1500rpm for 5mins, the FACs buffer was removed and the blood pellet was then incubated with the applicable tetramer at 37°C for 20mins. The blood pellet was then washed with a further 3mL of 1x FACs buffer, and again spun down at 1500rpm for 5mins. The FACs buffer was removed and the blood pellet was then incubated with the indicated antibodies at 4°C for a further 20 minutes. The blood pellet was then lysed with 1x FACs lysing buffer (BD). This was applied for 1min, before a final wash with 3mL 1x FACs buffer. The sample was spun again at 1500rpm for 5mins. The liquid was then poured off and the tube blotted on paper towel. The remaining cell pellet was then resuspended in 150-200μL of 1x FACs buffer before being run on the LSR II flow cytometer (BD).

For organ analysis, single cell suspensions were generated (see forward) from the indicated organs at a concentration of $1x10^6$. Absolute cell counts were determined by counting leukocytes in an improved Neubauer chamber or by using the Cell Countess (Invitrogen, UK). Cells were then incubated with the indicated tetramer and antibodies as described for blood analysis, before being run on the LSR II flow cytometer (BD).

2.4.2 Gating strategy

Cells were counted/collected using a BD LSR II flow cytometer, gated on viable leukocytes using the live/dead fixable near-IR dead cell stain kit from Invitrogen (Paisley, UK). Tetramer-specific cells were then counted from live $CD8^+$ T cells. The full gating strategy is demonstrated in appendix 3. Results were analysed using Flowjo software, version 9.6.1 (Tree star, USA).

2.5 Isolation of liver, lung and spleen lymphocytes

Perfused livers were smashed through a 40μM cell strainer (BD) using a 10mL syringe plunger end and a phosphate-buffered saline (PBS) wash. The resulting cell suspension was then centrifuged at 1200rpm for 5mins. The excess PBS was poured off and the cell pellet / lymphocytes were then purified by Percoll (GE healthcare, UK) gradient centrifugation. This involved the resuspension of the cell pellet in 5mL of 30% Percoll in a 15mL Falcon tube (100% Percoll was diluted out in PBS to the required percentage). This was then under laid with 3mL 70% Percoll. The samples were then centrifuged at 2000rpm for 25mins with the brake off. The interphase layer (between the 70% and 30% Percoll) was then removed and washed in 10mL PBS. This was then centrifuged at 1200rpm for 5mins. The excess PBS was poured off and the cell pellet was then lysed to remove red blood cells. This was done using 1mL ACK lysing buffer (Invitrogen), applied to each sample for 1min, before being washed in a further 10mL PBS. The sample was then centrifuged for a final time at 1500rpm for 5mins and the cell pellet was then resuspended in 1mL of 3% RPMI (3% FCS), before performing a cell count and further dilution of the samples according to need, moving on to either directly stain for flow cytometry or use in an ICS.

The lungs were cut up with razor blades and incubated in 4mL/sample 3% RPMI containing 60U/mL DNase (ApliChem, Germany) and 170U/mL collagenase II (Life/Gibco) in 6-well plates at 37^{o}C for 45 minutes. Cell

aggregates were then passed through a 40μM cell strainer (BD). Samples were then lysed and resuspended as described for the liver preparation.

Spleens were simply passed through a 40μM cell strainer (BD) into PBS directly. Samples were then lysed and resuspended as described for the liver preparation.

2.6 Intracellular cytokine staining

Single cell suspensions of $2x10^6$ lymphocytes were incubated for 2h at 37°C in 150μL of culture medium (RPMI containing 5% FCS) with 50μL of either the applicable peptide or positive / negative control in 96-well round-bottom plates. Cells were stimulated with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) as a positive control, or left untreated as a negative control. Peptide-specific responses were assessed after stimulation with 10^{-5}M of the relevant peptide (Proimmune, Oxford, UK). After 2h of stimulation, 50μL of GolgiPlug (1μl/1ml final concentration) from BD Biosciences (Oxford, UK) was added to each well, and cells were then incubated for another 4h at 37°C. At this point the plate could be stored overnight at 4°C. A surface stain for CD8a and live/dead was then performed (as previously described) using 25μL/well. This was then washed off using 150μL/well 1x FACs buffer and centrifugation at 1500rpm for 5mins. Cells were then fixed with cytofix/cytoperm solution from BD Biosciences (Oxford, UK), by applying 100μL/well of solution for 10min. The plate is then

centrifuged at 1500rpm for 5mins. The remaining cell pellet was then washed using Perm/Wash (BD Biosciences) solution (1x according to the kit protocol). The intracellular cytokine stain was then performed, typically with a panel of IFNγ and TNFα (25μL/well). This was left on the cells for 25mins at 4°C. The cells were then washed/spun for a further 2 cycles before resuspension in 200μL 1x FACs buffer. $CD8^+$ T cells producing IFNγ and TNFα were then determined using a BD LSR II flow cytometer and Flowjo software.

2.7 β-gal luminescence assay

The β-gal luminescence assay was performed using the FluoReporter® lacZ/Galactosidase quantitation kit (Invitrogen, UK). HER 911 cells were infected with Ad-LacZ (individually with each of the Ad-LacZ viruses with the different promoters of HCMV, RSV and EF1-α) at an MOI of 0.25. Cells and supernatants were collected and frozen down at -20°C. These were then tested according to the kit instructions and luminescence was measured on a microplate reader with an excitation filter centered at 390nm and an emission filter centered at 460nm.

2.8 Adenoviral antibody ELISA

An Ad-GFP virus was used to coat ELISA plates (96-well) (Greiner, Germany) with $5x10^9$ VP/well and was left wrapped in cling-film overnight at room temperature. On the day of use, plates were washed 6x with PBS/T and then

blocked with 1% BSA (100μL/well) for 60mins prior to use. Blood was collected from the applicable mice (EDTA tubes), centrifuged at 2000rpm for 10mins and sera collected. The sera are then diluted 1:200 in PBS/T. Block dilution was flicked off of the plates, and 50μL PBS/T added to rows B-H. 75μL of the diluted sera was then added to row A and serially diluted down the plate by transferring 25μL across A-H. This was then left at room temperature for 2hrs. The plate was then washed 6x with PBS/T. Detecting antibody, goat-anti-mouse IgG alkaline phosphatase conjugate (Sigma), was then added, diluted 1:5000 in PBS/T at 50μL/well. This was left for a further 1hr at room temperature. The plate was then washed 6x with PBS/T. pNPP substrate (Sigma) was then added at 100μL/well and left to develop in the dark for 10mins. 50μL of stop solution was then added to all wells and the plate read at 405nm on a microplate reader.

2.9 Statistical analysis

All data is presented as a mean from within individual groups, and error bars are indicative of the standard error of the mean (SEM).

An unpaired two-tailed Students' test was used. P values <0.05 were considered statistically significant. Statistical data analysis was performed using Graph-Pad Prism version 5.0a for MACs (GraphPad Software, San Diego, CA, USA).

3. Results – The Adenoviral Model

3.1 Introduction

The adenoviral model induces memory inflation, as seen in MCMV. However, there are some features of the adenoviral model that required further investigation prior to moving into the minigene / processing studies. This chapter addresses the role of the HCMV promoter in the vector, the Kb-restricted epitopes available to the model and finally the importance of the route of immunisation.

The Promoter

The balance between the concentration of adenoviral vector used and the choice of promoter can allow for marked differences in expression of the transgene (Arita et al., 2008; Chen et al., 2008; Zheng and Baum, 2005). It is important to note that CMV possesses a very strong promoter, which is highly sensitive to innate signals, such as those derived from LPS (Cook et al., 2006). The CMV promoter, of which there are many variations, is recognised as a ubiquitous promoter. However, RSV (Yamamoto et al., 1980) and EF1α (Kim et al., 1990; Mizushima and Nagata, 1990; Uetsuki et al., 1989) are similarly ubiquitous promoters and their use can be advantageous where a tailored approach is required, according to the target cell type and level of expression. As such, there is a good body of evidence for the use of these promoters in adenoviral vectors.

Given the high transcriptional activity of the HCMV promoter it was first necessary to evaluate the role of this within the Ad-LacZ model, considering this remains the only part of the adenoviral vector that is shared with CMV. The human CMV IE genes include the major IE genes (MIE): UL123 (IE1) and UL122 (IE2) as well as some other auxiliary genes (Isomura and Stinski, 2003). The proteins encoded for by the IE1 and IE2 genes have important roles in the regulation of subsequent viral gene expression. The region upstream of the HCMV MIE promoter is divided into the modulator, the unique region and the enhancer (Meier and Stinski, 1996). This region has important modulatory effects on the MIE. The gene encoding the IE 72-kDA protein of HCMV is transcribed by one of the strongest enhancers/promoters currently known (Boshart et al., 1985) and is able to provide high levels of transgene expression in a wide range of cell types. This is therefore a widely used promoter in vaccine development and gene therapy.

In this Ad-LacZ model a somewhat simplified readout of responses that are essentially present or not present is used. However, it is recognised that the intricacies of promoter choice and the concentration of protein expressed over time hold importance. This will obviously affect the efficacy and toxicity of adenovirus-based therapeutics. Also important to acknowledge are the strong innate and adaptive immune responses elicited by adenoviral vectors, which in turn can affect the efficacy of the promoter (Chen et al., 2008; Schaack et al., 2011). This work does not look to address this in any detail, but acknowledges that future work may need to focus more clearly on this were this to translate into human therapeutics.

Kb-restricted epitopes

Mouse MHC antigen is also called H-2 antigen. Its gene is located on mouse chromosome 17. Mouse MHC is composed of 11 subclasses. These include the "classical MHC class I" (MHC-Ia) which comprises H-2K, H-2D and H-2L subclasses. These are the equivalent allomorphs in mice to that of the human HLA-A, B and C. There are also "non-classical MHC class I" that comprise H-2Q, H-2M and H-2T. The remainder of the 11 subclasses are accounted for by the class II MHCs. Mouse MHC class I molecules consist of a 45kD highly glycosylated heavy chain non-covalently associated with a 12kD β2-microblobulin. Mouse MHC class I antigens are expressed on almost all nucleated cells, playing the same important roles as in humans. These MHC molecules are highly polymorphic. Each laboratory mouse strain is homozygous and has a unique MHC haplotype, this being designated by a small letter in the nomenclature (i.e. the b in H-2Kb). The C57BL/6 mouse class I is H-2Kb and H-2Db (Amadou et al., 1999).

The route of immunisation

Ultimately, vaccine delivery needs to be in a manner that is acceptable and logistically viable for the target population. For a vaccine against an infectious disease, clearly an intravenous route of administration is not one that will be acceptable. Typically such vaccines follow a less invasive route, such as an intramuscular, or better still, an oral route. Immunisation for therapeutic applications, for example oncological treatments, may however be better suited to intravenous routes (given in many cases the target population would already have *in situ* central venous access). The majority of the infectious

disease recombinant adenoviral vaccines currently under development are delivered intramuscularly (Li et al., 2008; Rampling et al., 2015; Swadling et al., 2014). Oral administration of replicating adenoviral vaccines has existed for some time (Takafuji et al., 1979) but there is still some way to go with recombinant replication-deficient adenoviral vaccines via this route, although this would certainly be an effective route if possible (Deal et al., 2013).

The other consideration with regards to the route of administration is the actual delivery of the vector to target sites / cells. Intravenous administration ensures rapid delivery to the liver and lung and potential key target cells. This is something that is likely critical to the Ad-LacZ model and more generally the production of inflationary populations. Intraperitoneal administration results in distribution primarily through the local (mesenteric) draining lymph nodes before reaching the circulation. Intramuscular administration ultimately follows a route similar to that of intravenous administration, but must first disseminate through the muscles and as such demonstrates markedly different kinetics.

3.2 Overview / Aims

- Do inflationary responses in the Ad-LacZ model depend upon the CMV IE promoter?

- Are any other Kb-restricted epitopes detectable within β-gal?

- Are inflationary responses dependent on intravenous immunisation?

3.3 Ad-LacZ remains a good model of memory inflation regardless of the transgene promoter

3.3.1 Comparison of alternative promoters

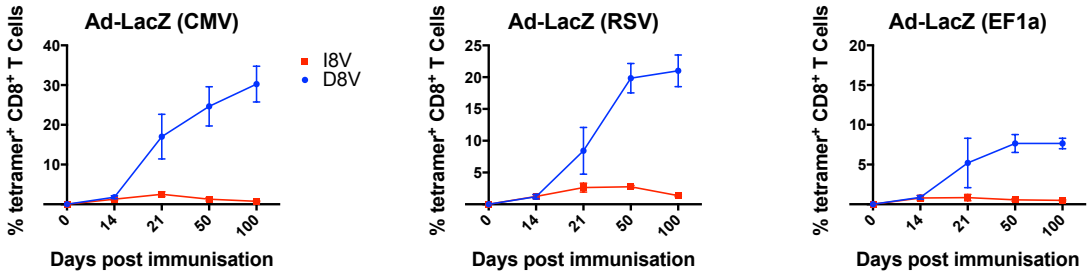
The CMV promoter in Ad-LacZ has been replaced with alternative promoters. A commercial Ad-LacZ vector was purchased from Kerafast (USA), with an RSV (Rous sarcoma virus) promoter and then administered intravenously into mice for comparison alongside the wildtype HCMV promoter Ad-LacZ and a further vector made during this work containing an EF1α (mammalian elongation factor 1α) promoter.

Figure 3.1 shows the immune responses of blood lymphocytes at different time points after immunisation of mice with the three Ad-LacZ constructs with the different promoters. Whilst there is a marked difference in the magnitude of response from the EF1α and RSV promoters, all constructs show inflation. This demonstrates that a CMV promoter is not a prerequisite for the development of inflationary populations.

In order to clearly define these populations as inflationary, the same qualities of phenotype, distribution and functionality are demonstrated. The phenotype of these inflationary cells was assessed using the panel of CD44, CD62L, CD127 and CD27. Figure 3.2 demonstrates results indicative of the typical effector memory phenotype in all of the inflationary responses taken from the constructs with differing promoters.

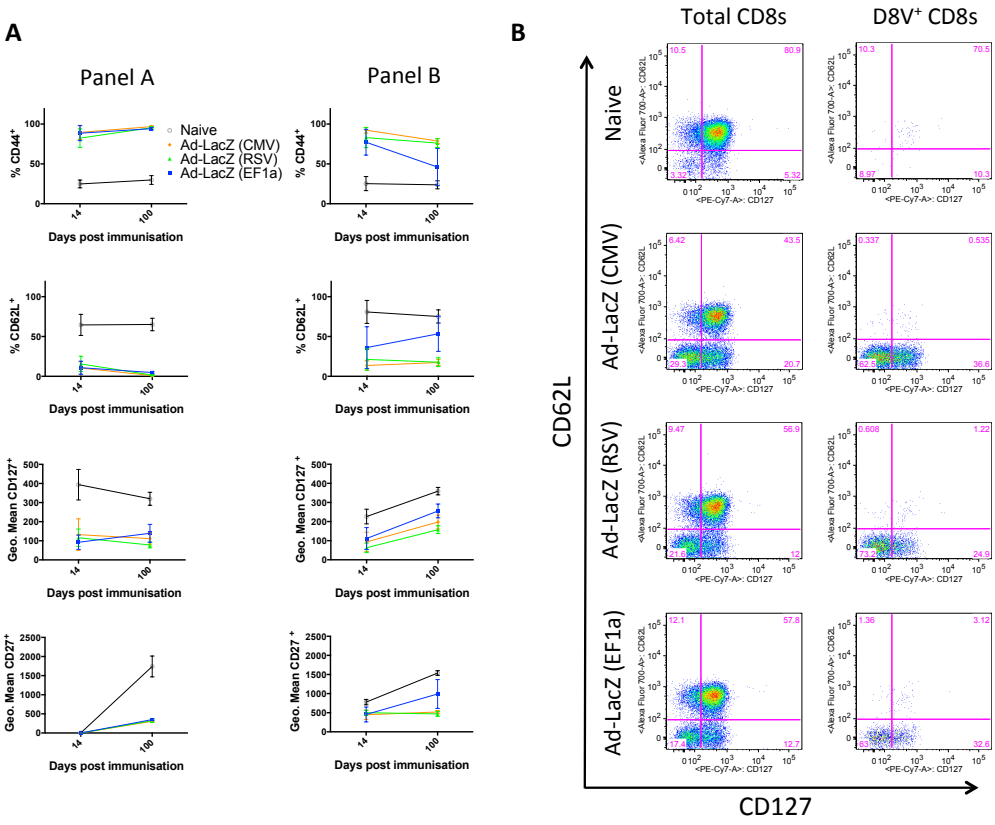
Figure 3.3 shows that the inflationary cells from all constructs were also shown to have the typical pattern of distribution (peripherally) and functionality (as assessed by IFNγ and TNFα production in ICS) as recognised from the wildtype Ad-LacZ (HCMV promoter).

Figure 3.1: Comparison of the immune responses of mice to Ad-LacZ viruses utilising HCMV, RSV and EF1α promoters to drive LacZ expression.



The figure demonstrates a comparison between the three Ad-LacZ constructs containing either of the HCMV (wildtype), RSV or EF1α promoters. A group of naïve mice were also included, but results from this group have been excluded from this figure (there were no tetramer specific responses from this group). C57BL/6 mice were immunised intravenously with $2x10^9$ iu/mouse and blood collected (tail vein) at day 14, 21, 50 and 100-post immunisation. Tetramer-specific responses for both of the D8V/inflationary (blue/circles) and I8V/non-inflationary (red/squares) $CD8^+$ T cells are shown. Groups of 4 mice were used for each vector, with the error bars indicating SEM. These results are representative of 3 separate experiments.

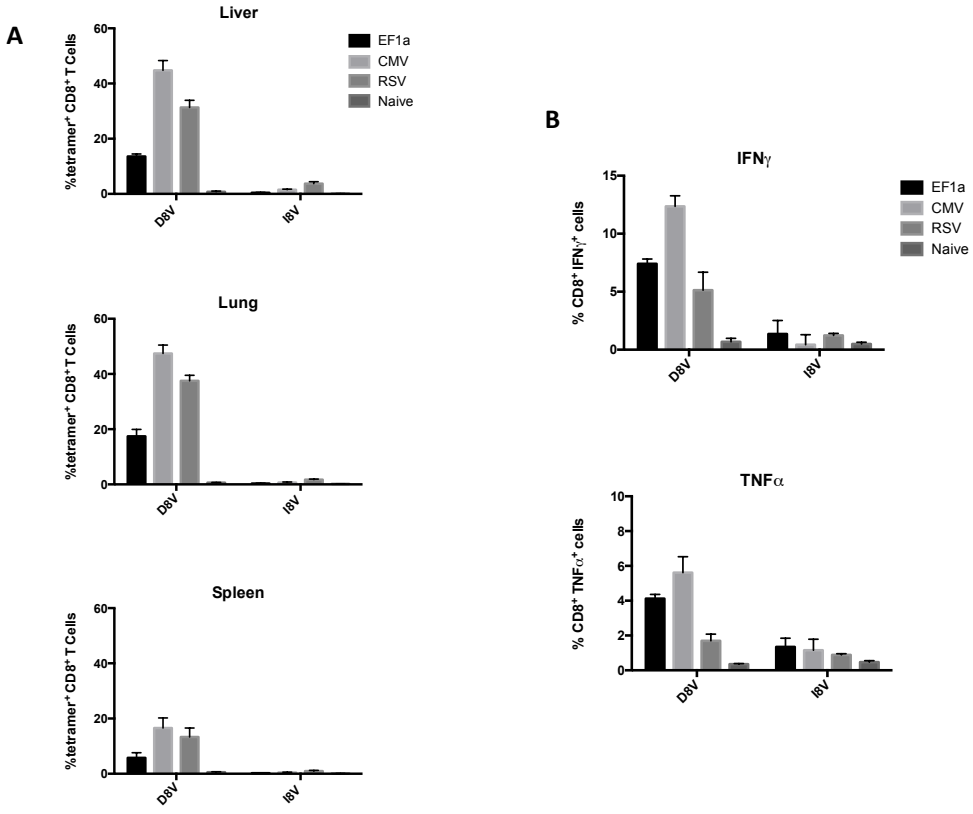
Figure 3.2: Inflationary D8V-specific CD8$^+$ T cells induced by Ad-LacZ viruses with three different promoters have similar T_{EM} phenotypes.



(A) Shows (in blood) the CD44 and CD62L results as a percentage of the tetramer positive CD8$^+$ T cells and the CD27 and CD127 as the geometric mean. Panel A represents results for the D8V (inflationary) specific responses and panel B the I8V (non-inflationary) responses. An early (day 14) and later (day 100) time-point is shown for each antibody marker. Groups of 4 mice were used for each vector, with the error bars indicating SEM. These results are representative of 3 separate experiments.

(B) Shows single representative flow cytometry plots at day 100-post immunisation (gated: left panel CD8$^+$ and right panel D8V$^+$ CD8$^+$ - CD127 vs. CD62L) of the CD62L/CD127 markers for each of a naïve mouse, Ad-LacZ (CMV), Ad-LacZ (RSV) and Ad-LacZ (EF1α), with a significant proportion falling between T_E and T_{EM} compared to naive.

Figure 3.3: Distribution and functionality of the inflationary D8V-specific CD8$^+$ T cells across all three Ad-LacZ viruses with the different promoters.

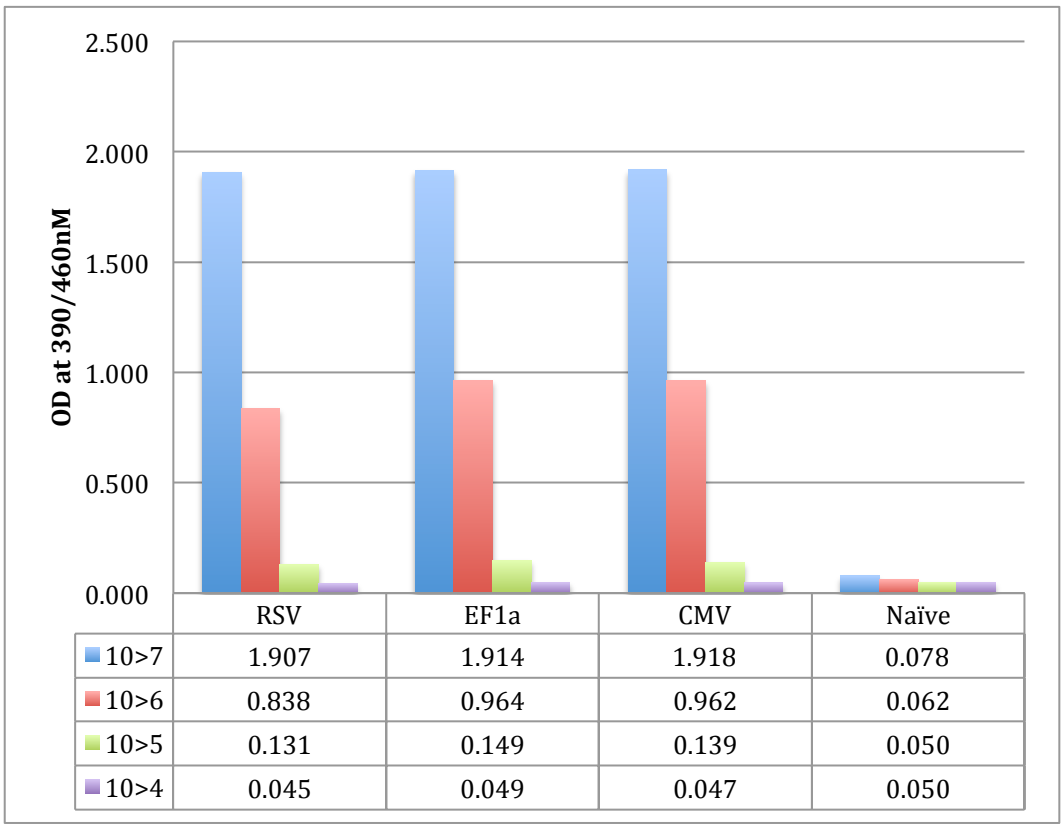


(A) Organs (liver, lung and spleen) were harvested at day 100-post immunisation. Tetramer-specific CD8$^+$ T cells (D8V and I8V) were then counted.

(B) ICS was performed on day 100-post immunisation splenocytes and IFNγ and TNFα production from peptide-specific stimulation with D8V and I8V (alongside a medium negative control and PMA/I positive control – data not shown) was assessed. Groups of 4 mice were used for each vector, with the error bars indicating SEM. These results are representative of 3 separate experiments.

Whilst all three Ad-LacZ constructs with differing promoters produce inflation, the magnitude of the response is noted to be quite different between the vectors. It seems likely that this is down to a combination of promoter strength, target cells and the immune responses to the adenoviral vector, in keeping with the literature (Arita et al., 2008; Qin et al., 2010; Schaack et al., 2011; Zarrin et al., 1999). In order to try and evaluate this further an *in vitro* β-gal luminescence assay was performed to make comparison of β-gal production from HER 911 cells infected with each of the three Ad-LacZ constructs with differing promoters. This assay quantifies β-gal protein through luminescence output. Figure 3.4 demonstrates results from this comparison, and shows that sensitivity across the assay was not sufficient to show a difference between the constructs.

Figure 3.4: β-gal production in vitro from the three Ad-LacZ viruses with different promoters.



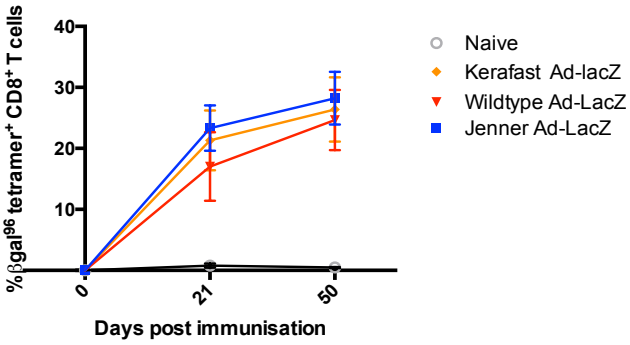
	RSV	EF1a	CMV	Naïve
10>7	1.907	1.914	1.918	0.078
10>6	0.838	0.964	0.962	0.062
10>5	0.131	0.149	0.139	0.050
10>4	0.045	0.049	0.047	0.050

HER 911 cells were infected with 50mL of the three viruses (individually) at a titer of $1x10^7$ iu/mL serially diluted down to $1x10^4$. Cells were at a concentration of $2x10^6$, representing an MOI of 0.25, 0.025, 0.0025 and 0.00025. The FluoReporter® lacZ/Galactosidase quantitation kit (Invitrogen, UK) protocol was then followed. Luminescence was measured with an excitation filter centered at 390nm and an emission filter centered at 460nm. Uninfected / naïve HER 911 cells were also run as a control. Positive controls (provided with kit) gave a read-out of 0.989nm and the reference blank at 0.057nm. All groups were performed in triplicate with the mean result displayed.

3.3.2 Comparison of different HCMV promoters

It was next tested whether the nature of the CMV immediate early promoter utilised in the transgenic expression cassette could influence whether memory T cell responses exhibited inflation. The intron A region of the HCMV promoter has been shown to have a regulatory role on the enhancer region of the IE promoter (Chapman et al., 1991). With both the "short" promoter (lacking intron A) and the "long" or native promoter (containing intron A) (Sridhar et al., 2008), responses to D8V and I8V showed typical memory inflation and classical memory, respectively. Figure 3.5 demonstrates the magnitude of the inflating response induced in B6 mice (frequency of D8V tetramer-specific $CD8^+$ T cells in blood) between Ad-LacZ vectors with the wildtype (short), Jenner (long) and Kerafast (UL126) HCMV promoters. Overall, the response was marginally greater with the "long" promoter over the "short", in agreement with published data (Sridhar et al., 2008), but any differences were not statistically significant.

Figure 3.5: Comparison of Ad-LacZ constructs with differing HCMV promoters: long versus short.



Inflationary/D8V-specific $CD8^+$ T cell responses in blood (at day 21 and 50-post immunisation) are shown for Ad-LacZ vectors containing differing HCMV promoters. C57BL/6 mice were immunised with Ad-LacZ containing either of the wildtype HCMV promoter (in red), Jenner HCMV promoter (in blue) or the Kerafast HCMV promoter (in orange). Groups were of 4 mice per group and virus immunised at $2x10^9$ iu/per mouse. Errors bars indicate SEM. A naïve control group is shown.

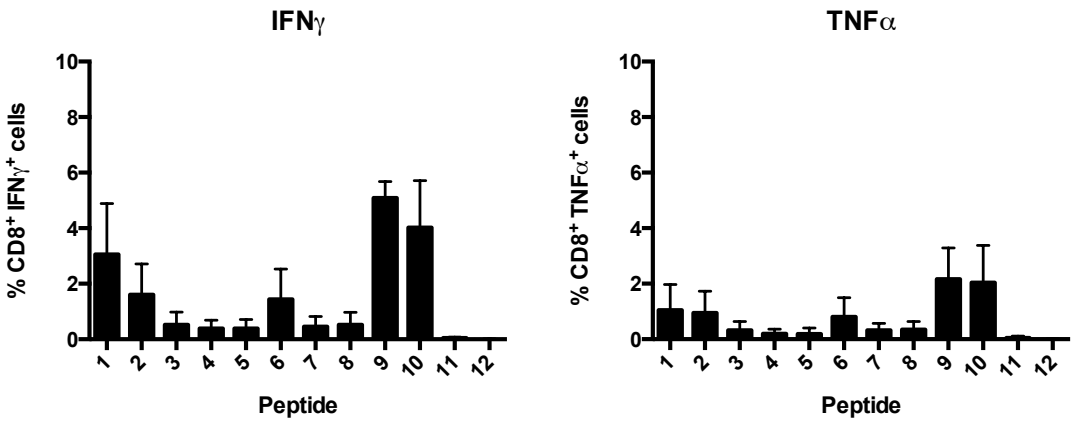
3.4 No other Kb-restricted epitopes can be identified from the Ad-LacZ model

It was of interest to know if any other epitopes were available / relevant in the C57BL/6 / Ad-LacZ model. From the original paper identifying the D8V epitope (Overwijk et al., 1997), 12 key Kb-restricted peptides (table 2.3) were identified and tested. The evaluation of these 12 Kb-restricted epitopes was repeated, by testing IFNγ and TNFα production *ex vivo* from splenocytes harvested from day 50-post Ad-LacZ immunised B6 mice. Figures 3.6 and 3.7 demonstrate these results.

From this (in addition to the D8V – No.1 and I8V – No.9) E8V – No.10, C8V – No.6 and T8V – No.2 were identified as potential candidate relevant epitopes. Figure 3.7 demonstrates the repeat experiments focused on these peptides.

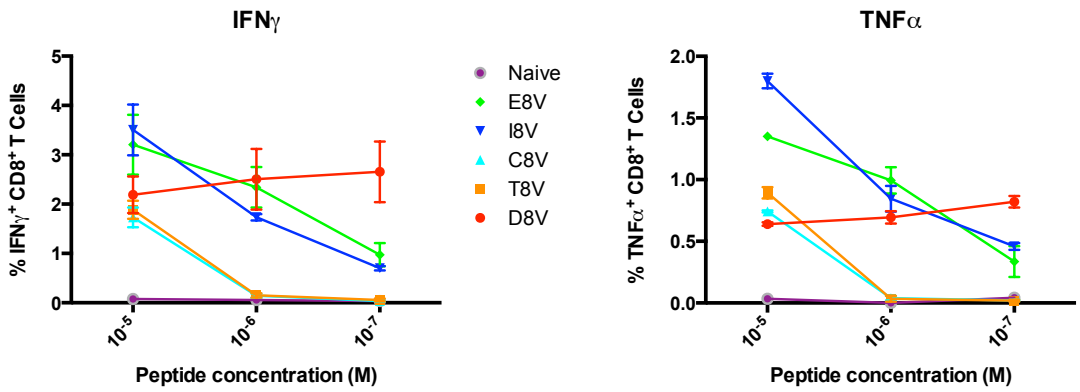
From these data only the EDQPFPAV (E8V) was felt to be of potential significance (other than the D8V and I8V). An E8V tetramer was developed through the NIH tetramer facility and blood lymphocytes from Ad-LacZ immunized B6 mice were then analysed for tetramer-specific responses (figure 3.8). No E8V-specific responses could be tracked *in vivo*. From within the Ad-LacZ model it has therefore not been possible to find any other Kb-restricted epitopes of interest.

Figure 3.6: 12Kb peptide responses ex vivo.



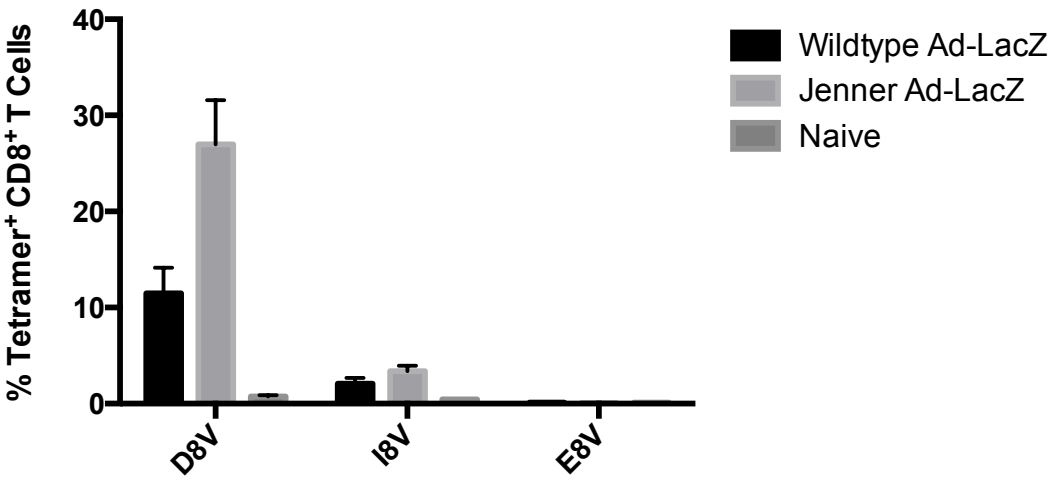
Splenocytes were harvested from B6 mice (n=3) that were day 50-post immunisation with Ad-LacZ (wildtype). An ICS and stimulation with the 12 Kb-restricted peptides was performed. IFNγ and TNFα were then stained for and measured via flow cytometry. Results are expressed as the mean +/- SEM. The 12 Kb-restricted peptides (numbered 1-12) are highlighted in table 2.3. Positive (PMA/I) and negative (medium) controls were included in this experiment, but results have been excluded for presentation.

Figure 3.7: Focused Kb-restricted peptide stimulation ex vivo.



C57BL/6 mice were immunised with $2x10^9$ iu/mouse Ad-LacZ (wildtype) and splenocytes were harvested at day 50-post immunisation. Splenocytes were then stimulated with the Kb-restricted peptides of D8V, T8V, C8V, I8V and E8V. IFNγ and TNFα were then measured via flow cytometry. N=6 and error bars are indicative of the SEM. The peptide β-gal positions are highlighted in table 2.3. Positive (PMA/I) and negative (medium) controls were included in this experiment, with only negative controls indicated here for presentation. Peptides were tested at a concentration of 10^{-5} M, 10^{-6} M and 10^{-7} M.

Figure 3.8: E8V tetramer-specific CD8$^+$ T cell responses in vivo.



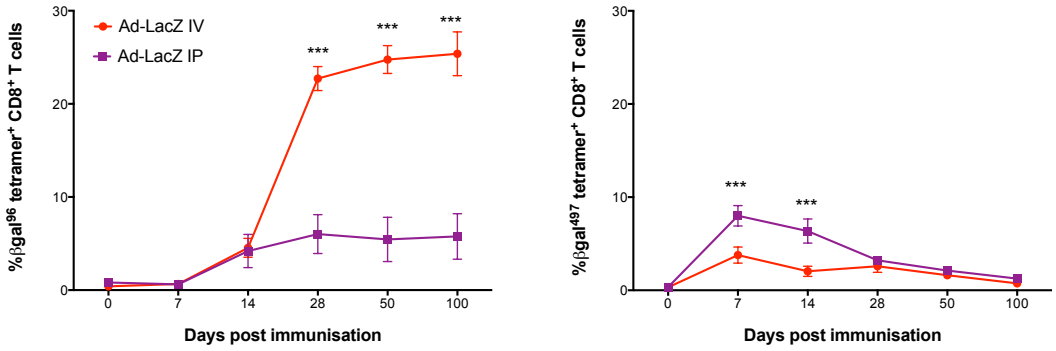
C57BL/6 mice were immunised with Ad-LacZ (both Wildtype and Jenner at 2x10^9 iu/mouse) alongside naïve controls. Blood was tested for tetramer-specific responses at day 28-post immunisation. N=4 per group, error bars indicate the SEM. Results show responses from D8V, I8V and E8V from within the 3 groups. No E8V tetramer-specific CD8$^+$ T cell responses were detectable.

3.5 The importance of the route of immunisation

3.5.1 Intravenous versus intraperitoneal

A direct comparison was made of the inflationary $CD8^+$ T cell responses induced when Ad-LacZ is administered via the intraperitoneal and intravenous routes (figure 3.9). Whilst the non-inflationary I8V-specific response broadly tracked the same path regardless of the route of infection, the inflationary response looked quite different. D8V-specific responses in intravenously immunised mice gradually expanded out to a population that comes to occupy around 20-30% of the total $CD8^+$ T cells. D8V-specific responses from those immunised via the intraperitoneal route clearly plateau at around day 14 and whilst they are maintained as an inflationary pool (with the same features in terms of phenotype, distribution and functionality) the frequency is markedly different.

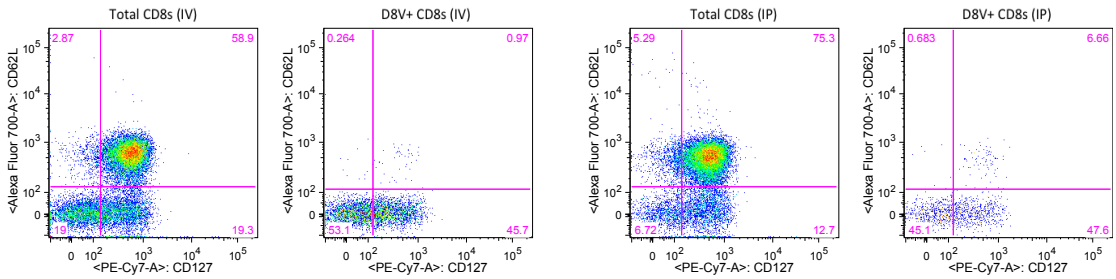
Figure 3.9: Comparison between inflationary responses following intravenous or intraperitoneal Ad-LacZ administration.



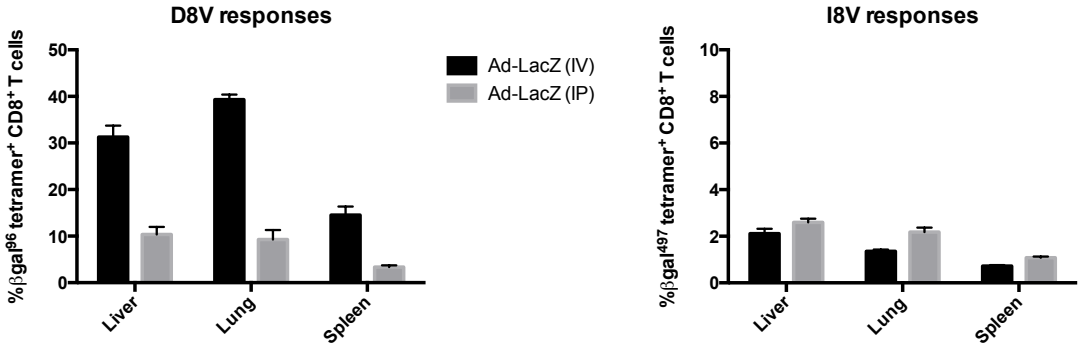
The figure demonstrates a comparison between Ad-LacZ (Jenner) immunised via either of the intravenous or intraperitoneal routes. A group of naïve mice were also included, but results from this group have been excluded from this figure (there were no tetramer-specific responses from this group). C57BL/6 mice were immunised either i.v. or i.p. with $2x10^9$ iu/mouse in all cases and blood was collected at day 7, 14, 28, 50 and 100-post immunisation. Tetramer-specific responses for the D8V (inflationary/β-gal_{96}) and I8V (non-inflationary/β-gal_{497}) $CD8^+$ T cells are shown. N=5 per group with error bars showing the SEM. Statistical analysis (unpaired two-tailed Students' test) ***p <0.0005.

Figure 3.10: Phenotype and distribution of inflationary responses following intravenous or intraperitoneal Ad-LacZ administration.

A



B



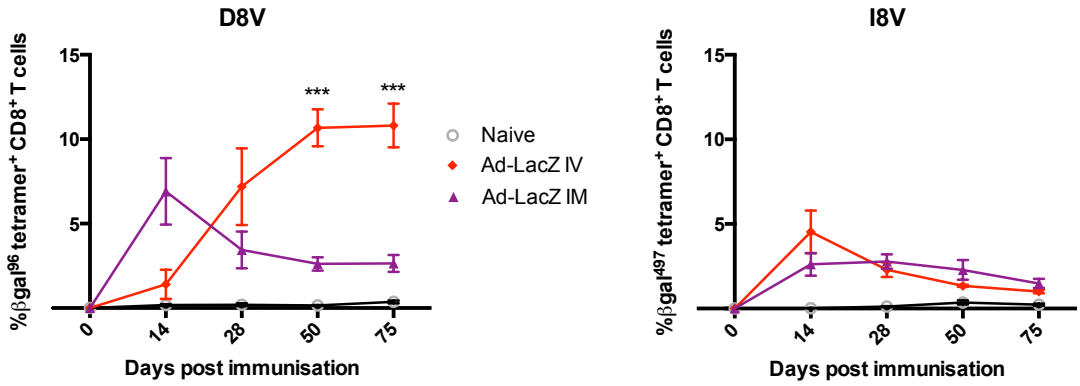
(A) Phenotyping of blood day 100-post immunisation (gated: lymphocytes>live dead>CD8^{+}>$^{+}$/- D8V^{+}>CD127 and CD62L). A representative plot of total CD8^{+} T cells versus D8V tetramer-specific CD8^{+} T cells is shown for one animal from each of the i.v. and i.p. immunised groups.

(B) Distribution of both the D8V and I8V-specific CD8^{+} T cells in organs (liver, lung and spleen) from day 100-post immunisation B6 mice is shown for the i.v. and i.p. groups. N=5 per group, with error bars indicating SEM.

3.5.2 Intravenous versus intramuscular

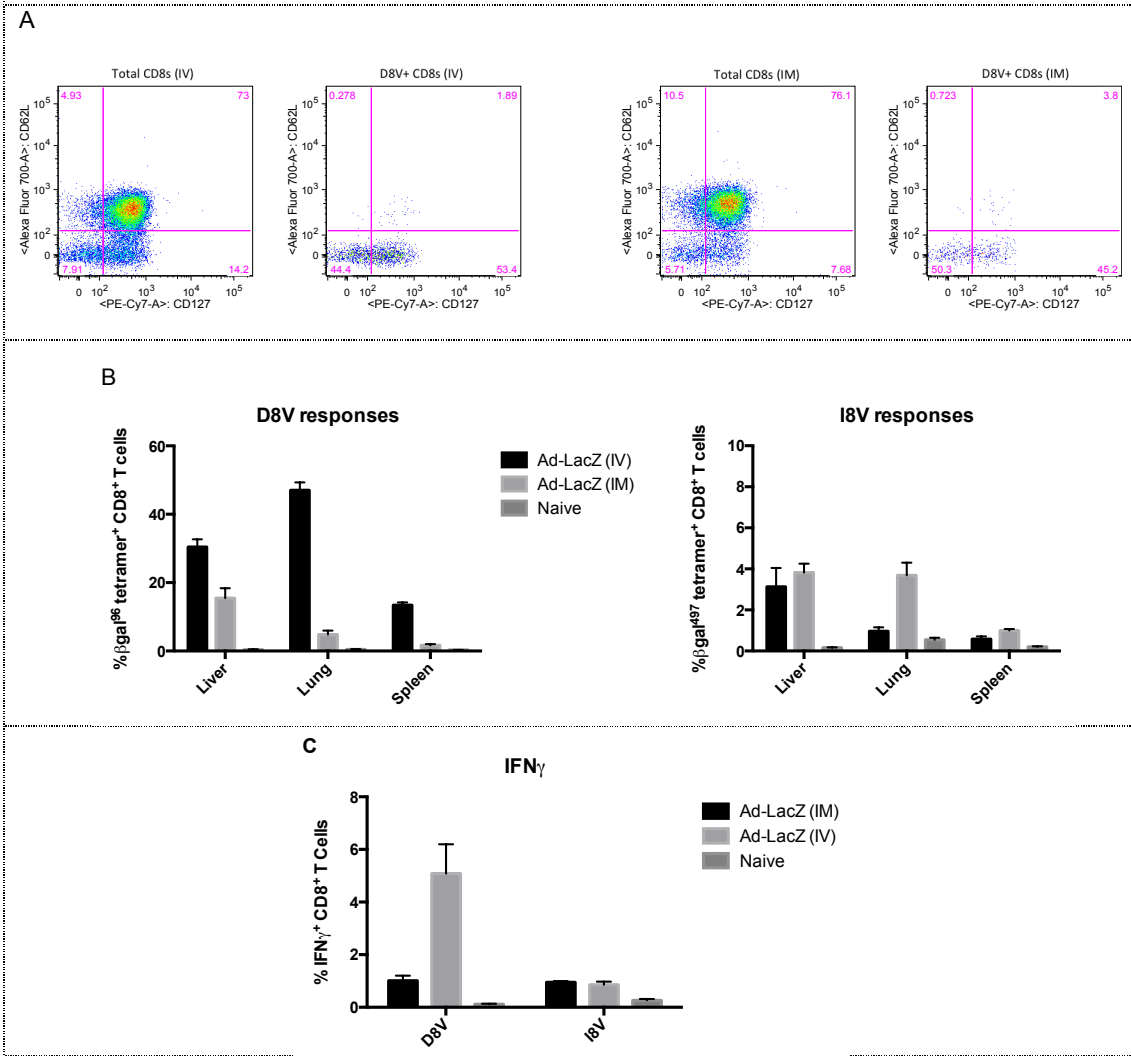
Intramuscular administration demonstrates some markedly different kinetics compared to intravenous delivery. The magnitude of the response from intramuscular administration is several-fold lower (figure 3.11). The inflationary D8V-specific $CD8^+$ T cell populations are only maintained at a low percentage (compared to the initial day 14 response), but this has only been tracked out until day 75-post immunisation. Further work is needed to assess what happens to these cells at later time-points of infection in the host and whether these cells retain the key features of inflationary cells. Of the D8V-specific cells at day 75-post immunisation that do exist, they can still be shown to have the characteristic T_{EM} phenotype and are proportionally distributed in the periphery with the ability to produce IFNγ following peptide stimulation in ICS assays (figure 3.12).

Figure 3.11: Comparison of inflationary responses following intravenous or intramuscular Ad-LacZ administration.



The figure demonstrates a comparison between Ad-LacZ (wildtype) immunised via either of the intravenous or intramuscular routes. Naive controls are included. C57BL/6 mice were immunised either i.v. or i.m. with $2x10^9$ iu/mouse in all cases and blood was collected at day 14, 28, 50 and 75-post immunisation. Tetramer-specific responses for the D8V (inflationary) and I8V (non-inflationary) $CD8^+$ T cells are shown. N=4 per group with error bars showing the SEM. Statistical analysis (unpaired two-tailed Students' test) ***p <0.0005.

Figure 3.12: Phenotype, distribution and functionality of inflationary responses following intravenous or intramuscular Ad-LacZ administration.



(A) Representative CD127/CD62L phenotyping from day 75-post immunisation (gated: lymphocytes>live dead>CD8$^+$>$^+$/- D8V$^+$>CD127 and CD62L) from total CD8$^+$ T cells and D8V$^+$ CD8$^+$ T cells only is shown for each of the i.v. and i.m. routes.
(B) Distribution of D8V and I8V-specific CD8$^+$ T cells in organs (liver, lung and spleen) from day 75-post immunisation B6 mice is shown for the i.v. and i.m. groups.
(C) IFNγ production from splenocytes day 75-post immunisation (Ad-LacZ i.m. or i.v.) was compared. N=4 per group and the error bars indicate SEM.

3.6 Conclusions

The promoter

The adenoviral model remains a good model for memory inflation, regardless of the transgene promoter. It has been shown that the use of alternative promoters, in the form of RSV and EF1α, does not prevent inflationary responses from these Ad-LacZ constructs. This was critical to assert before progressing into the processing studies. The interpretation from these experiments is essentially quite basic – a simple yes or no answer. However, this work has raised some very interesting points. In considering the strengths of individual promoters, it can be advantageous to tailor the type of promoter used and the viral titre of adenovirus immunised to optimise for the best host response (Arita et al., 2008). This may well be reflected in part in the initial studies of Ad-LacZ immunisation and the observation that only a very narrow dose range of the virus *in vivo* would lead to inflationary responses (Krebs et al., 2005). The strength of the HCMV promoter undoubtedly plays a role in the activity of the transgene, where over-activity in turn leads to the tolerisation described (Bolinger et al., 2013; Krebs et al., 2005). This has been a feature that has been appreciated in these studies.

An attempt to try and quantitate what level of transgene expression the different promoters were achieving, by measure β-gal production on HER 911 infected cells *in vitro*, was performed. This work was unsuccessful, given the high readout from the assay, which was not possible to titrate out. This work was not pursued due to time constraints, but an alternative approach to

measure protein production (i.e. Western Blot) would be possible and could be performed in future studies if felt relevant.

Kb-restricted epitopes

C57BL/6 mice hold a H-2Kb and H2-Db class I / haplotype. The original paper identifying the D8V epitope (Overwijk et al., 1997) identified 12 peptides present in β-gal that corresponded to the optimal binding motif for Kb (Rammensee et al., 1995). Overwijk et al. had generated CTLs capable of recognising β-gal-expressing tumour cells of C57BL/6 origin and through these determined that their restriction element was the Kb molecule (and therefore did not look at Db). This was re-tested here using these 12 epitopes and the Ad-LacZ model. Whilst some small responses to the epitopes were found in the *in vitro* assays, only the E8V epitope was taken forward into *in vivo* assays looking for E8V tetramer-specific $CD8^+$ T cells following Ad-LacZ immunisation. None such responses could be found. Db-restricted epitopes do remain unaccounted for.

Route of immunisation

Inflation does occur via other routes of immunisation, but there are some marked variations in the kinetics that warrant further investigation. All other routes of immunisation for Ad-LacZ, other than intravenously, demonstrate a marked reduction in magnitude of response. In all of these studies these responses have only been tracked out to a maximum of 100 days post immunisation. The route of immunisation clearly impacts on the quality of the transgene-specific $CD8^+$ T cell response, as described in the literature (Holst

et al., 2010; Vidy et al., 2013). It seems likely that this impact could relate to the delivery of the vector to specific target cell types (for example a hepatic cell type) more readily when given intravenously in comparison to other routes of immunisation. This in turn may have some effect on the production of inflating $CD8^+$ T cell responses. Whilst the phenotype of the D8V-specific $CD8^+$ T cells in intramuscularly immunised mice is one of T_{EM}, it would be of interest to track these responses after day 100 to assess whether this is maintained. The kinetics of the response look to be more in keeping with a classical central memory response, so it would be of interest to see if these small inflationary responses can be sustained over the entire lifespan of the host.

4. Results – Minigenes

4.1 Introduction

The simple question that underpins these subsequent chapters on processing is “why is it that some epitopes lead to inflationary populations and not others”? Another major question in the field remains as to how such vastly different types of memory can be induced from the same immunogen. This work tests the hypothesis that memory inflation from classical, non-inflationary epitopes can be induced by modulating the antigen context. This idea was based on studies that showed a correlation between independence from immunoproteasome processing and memory inflation in both MCMV and the adenovirus model (Bolinger et al., 2013; Hutchinson et al., 2011).

Minigenes – LacZ

A minigene is essentially a minimal gene fragment (including an exon) with the necessary control regions that allows for the expression of that gene in the same way as the intact antigen. The terminology was first described in the 70s, where cloning of two minigenes was designed to express a peptide (Poonian et al., 1977). These minigenes can then be used as splice reporter vectors and allow for investigation of the factors which hold importance in splicing outcomes (Gaildrat et al., 2010). In the setting of this work, the “minigene” insert is simply that of the recognised epitope for the inflationary or non-inflationary $CD8^+$ T cell populations in the Ad-LacZ (and subsequently the MCMV) model. This is a “minimalist” approach, with just the relevant octamer

or nonamer being expressed, which in turn completely removes the requirements for processing and isolates the epitopes in their own right for presentation.

Minigenes – M45

The acute $CD8^+$ T cell response to MCMV in B6 mice is numerically dominated by lymphocytes responding to an H-2Db-restricted epitope ($M45_{985\text{-}993}$ or HGIRNASFI) of M45 (Munks et al., 2006b). M45 is an early (E) cytoplasmic protein present in the virion. It is expressed early in viral replication and accumulates in the cytoplasm of infected cells at later time-points. It is associated with the virion particle tegument.

4.2 Overview / Aims

- Studies around the Ad-LacZ model: Can minigene constructs of I8V and D8V from β-gal (Ad-I8V and Ad-D8V) allow for inflationary responses?

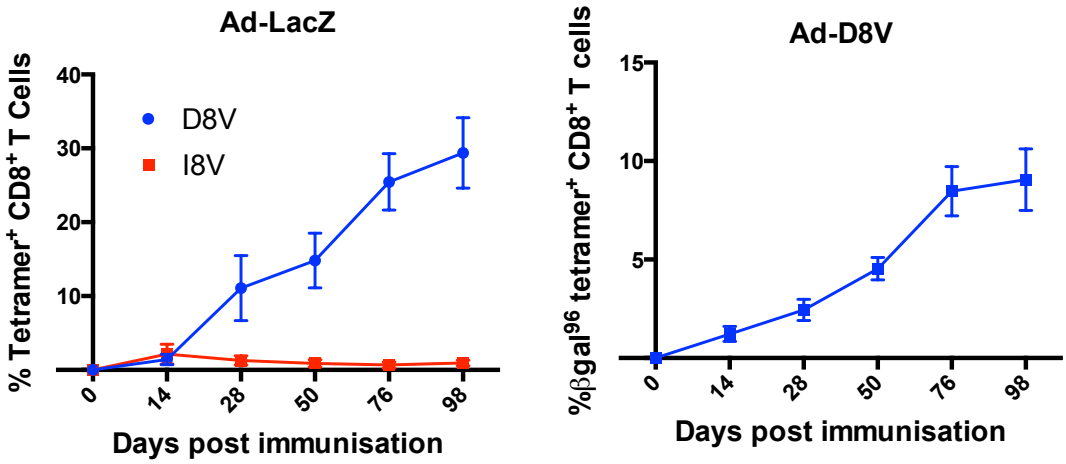
- Studies around the MCMV model: Can other typically "non-inflationary" epitopes produce inflationary responses as minigene constructs? To test this, an Ad-M45 construct was developed, using the immunodominant non-inflationary M45 epitope from MCMV.

4.3 Ad-D8V and Ad-I8V minigenes

4.3.1 The Ad-D8V and Ad-I8V minigenes compared to Ad-LacZ in blood

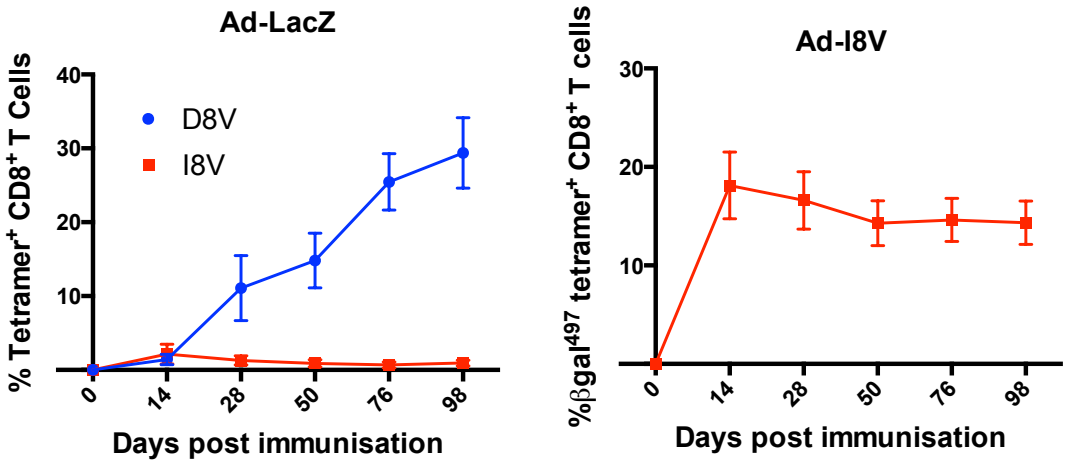
The minigene constructs used in this work are the individual octamers of I8V (β-gal$_{497-504}$) and D8V (β-gal$_{96-103}$) from the full LacZ gene. These are flanked by an initiator methionine, stop codon and a SV40 polyA tail. The insert contains nothing else. The expression cassette includes an HCMV long promoter and this is then cloned into an E1/E3 deleted AdHu5 vector. By removing the surrounding β-galactosidase sequence, it was hypothesised that it may be possible to make the responses induced from the non-inflating epitope inflate, by removing the requirements for processing and/or the competition of the D8V inflating epitope. Figures 4.1 and 4.2 demonstrate the results from the Ad-D8V and Ad-I8V constructs respectively. Critically, I8V tetramer-specific $CD8^+$ T cell responses (in blood) induced from Ad-I8V immunised intravenously into B6 mice show inflation.

Figure 4.1: The Ad-D8V minigene tetramer-specific CD8$^+$ T cell responses in vivo compared to Ad-LacZ.



C57BL/6 mice were immunised with ether of Ad-LacZ or Ad-D8V. A group of naïve mice were also included, but results from this group have been excluded from this figure (there were no tetramer-specific responses from this group). The D8V minigene was initially tested at 4 different doses within a 200μL immunisation: $1x10^7$, $1x10^8$, $1x10^9$ and $1x10^{10}$ iu (see appendices). From here on in all experiments with Ad-D8V were performed at $1x10^8$ iu/mouse. Ad-LacZ was administered at $2x10^9$ iu/mouse. Results show tetramer-specific responses in blood at day 14, 28, 50, 76 and 98-post immunisation. N=5 with error bars showing the SEM. The results shown are representative of multiple repeats.

Figure 4.2: The Ad-I8V minigene tetramer-specific CD8$^+$ T cell responses in vivo compared to Ad-LacZ.



C57BL/6 mice were immunised with ether of Ad-LacZ or Ad-I8V. A group of naïve mice were also included, but results from this group have been excluded from this figure (there were no tetramer-specific responses from this group). The I8V minigene was initially tested at 3 different doses within a 200μL immunisation: $1x10^8$, $1x10^9$ and $1x10^{10}$ iu (see appendices). From here on in all experiments with Ad-I8V were performed at $1x10^8$ iu/mouse. Ad-LacZ was administered at $2x10^9$ iu/mouse. Results show tetramer-specific responses in blood at day 14, 28, 50, 76 and 98-post immunisation. N=5 with error bars showing the SEM. The results shown are representative of multiple repeats.

4.3.2 Phenotype, distribution and functionality of the tetramer-specific CD8$^+$ T cells induced by the Ad-I8V and Ad-D8V minigenes

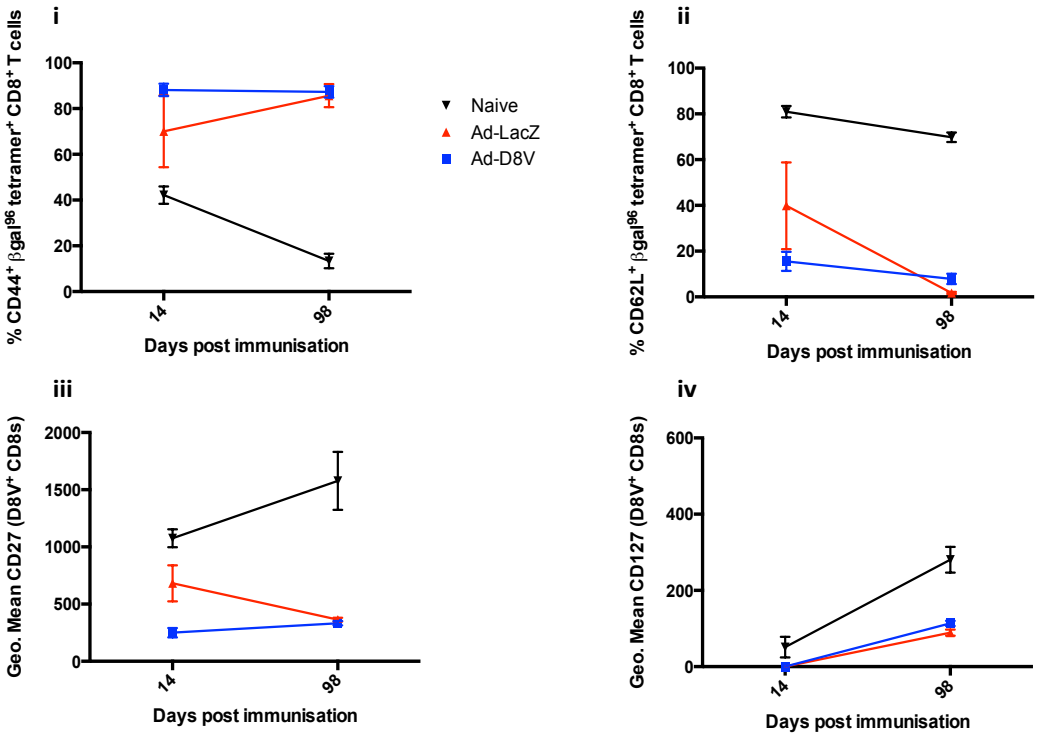
Figures 4.3 and 4.4 show the phenotyping for the tetramer-specific CD8$^+$ T cells induced from the two minigene constructs. These are effector memory cells in both groups, although it is recognised that there is some spread across T_E and T_{EM} pools.

Figures 4.5 and 4.6 show the distribution of the tetramer-specific CD8$^+$ T cells induced from the Ad-D8V and Ad-I8V minigenes (compared to Ad-LacZ) in the peripheral tissues (liver, lung and spleen). The inflating responses induced from Ad-D8V and Ad-I8V are found at high levels in the periphery, as noted with the inflationary responses from Ad-LacZ.

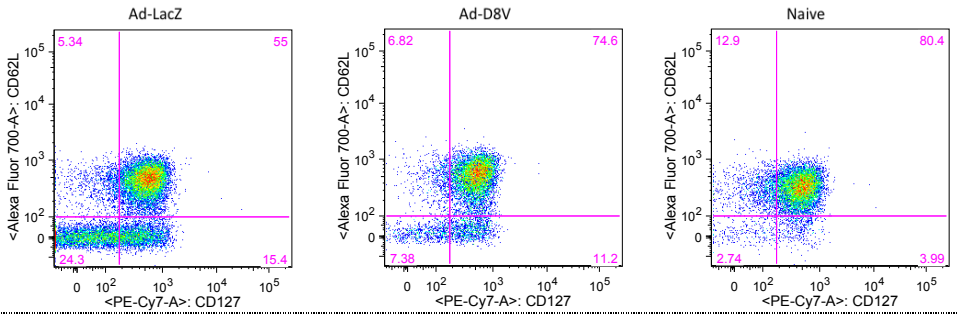
Finally, figure 4.7 demonstrates the functionality of the tetramer-specific CD8$^+$ T cells induced from the Ad-I8V and Ad-D8V minigenes, through intracellular cytokine staining (ICS) assays. These populations show IFNγ production, indicative of retained functionality.

Figure 4.3: The phenotype for the Ad-D8V minigene induced CD8$^+$ T cells.

A



B

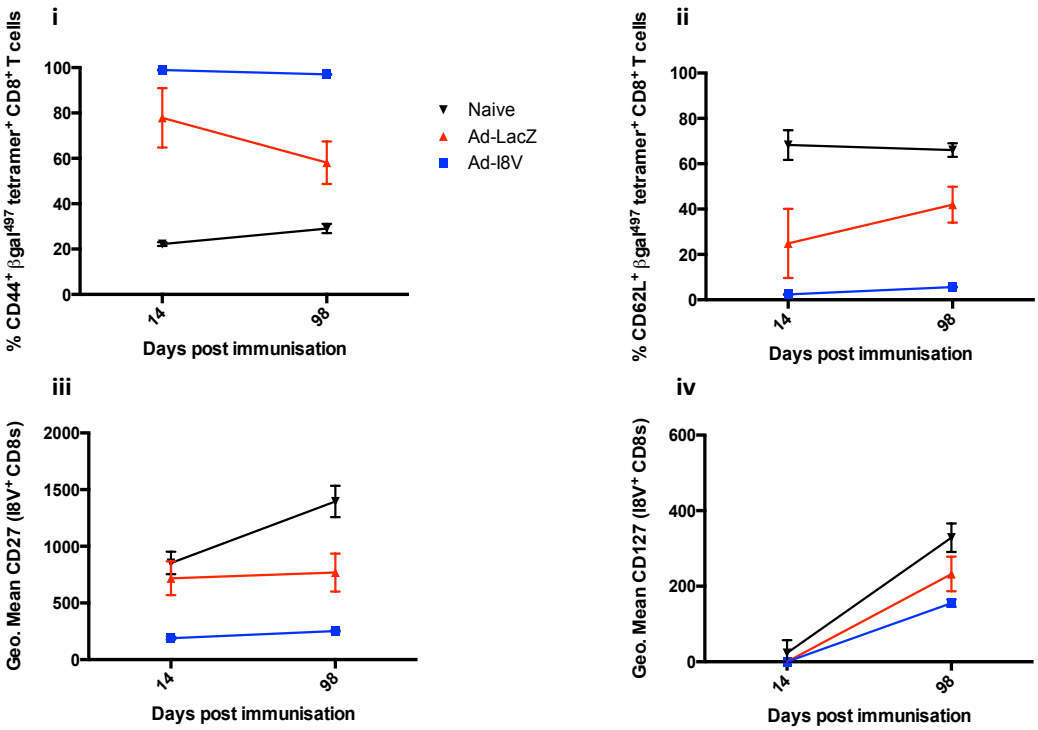


(A) Blood was stained for (i)CD44, (ii)CD62L, (iii)CD27 and (iv)CD127, to assess the CD8$^+$ T cell phenotype. Results show an early (day 14) and late (day 98) time-point after immunisation with either of Ad-D8V or Ad-LacZ, alongside naïve controls.

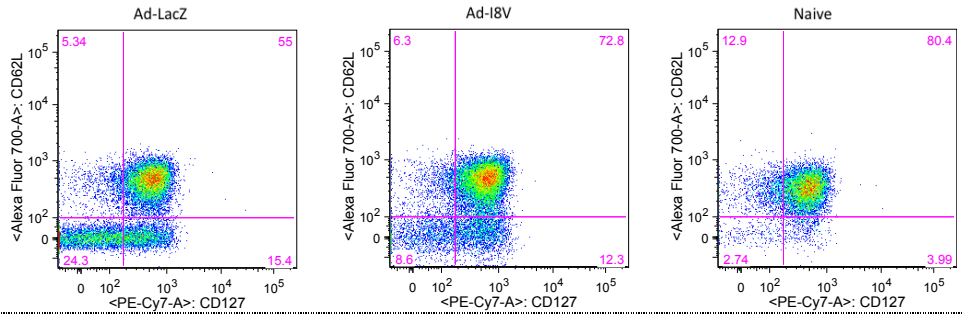
(B) Representative CD127/CD62L plots show the T_{EM} phenotype between Ad-LacZ and Ad-D8V compared to naive (gated: lymphocytes>live dead>CD8$^+$>CD127 and CD62L).

Figure 4.4: The phenotype for the Ad-I8V minigene induced CD8$^+$ T cells.

A



B

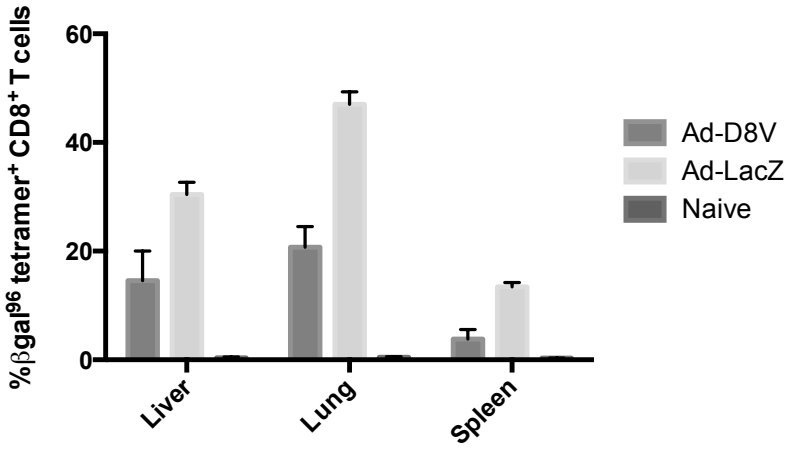


(A) Blood was stained for (i)CD44, (ii)CD62L, (iii)CD27 and (iv)CD127, to assess the CD8$^+$ T cell phenotype. Results show an early (day 14) and late (day 98) time-point after immunisation with either of Ad-I8V or Ad-LacZ, alongside naïve controls.

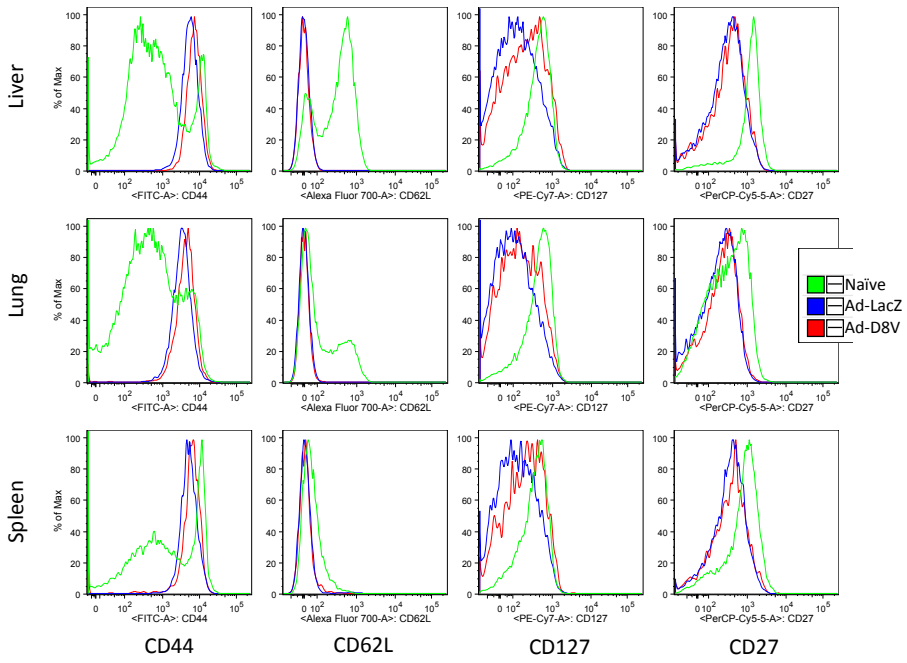
(B) Representative CD127/CD62L plots show the T_{EM} phenotype between Ad-LacZ and Ad-I8V compared to naive (gated: lymphocytes>live dead>CD8$^+$>CD127 and CD62L).

Figure 4.5: Distribution of Ad-D8V induced CD8$^+$ T cells in the peripheral tissues.

A



B

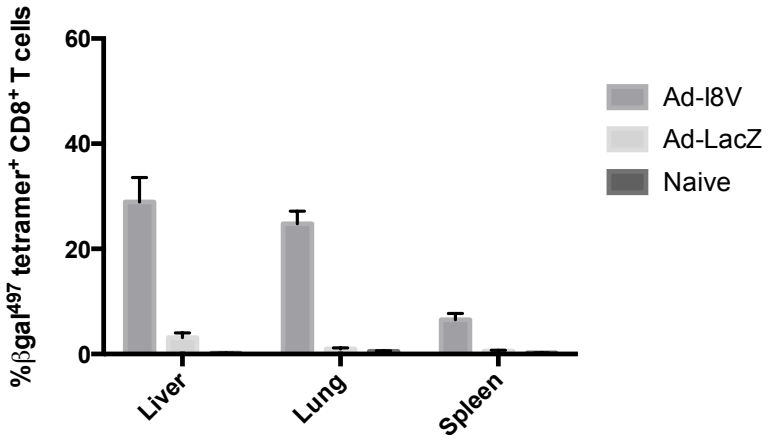


(A) At day 100-post immunisation, organs were harvested and stained for tetramer positive cells (n/groups=5). Levels seen in wildtype compared to Ad-D8V were similar in the lung and liver, comparative to the magnitude of the response in blood.

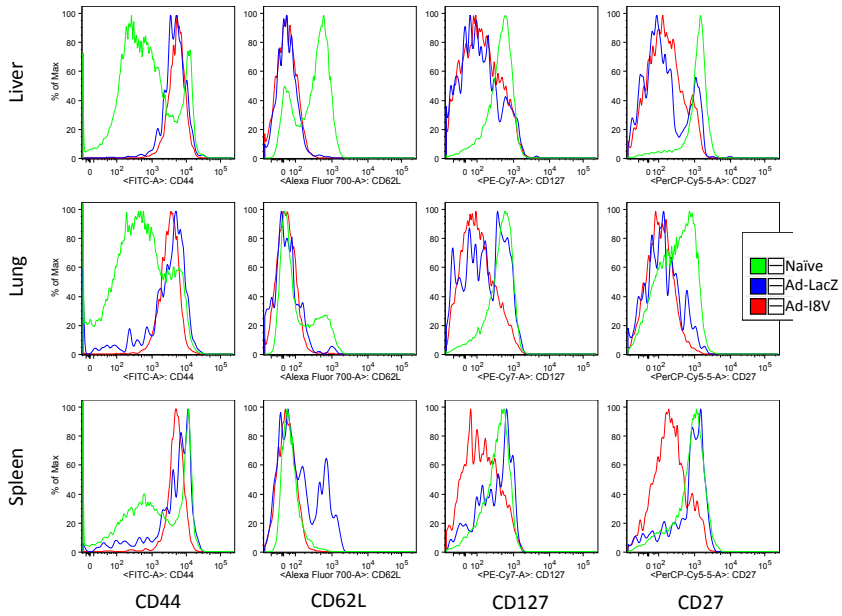
(B) Shows representative phenotypic markers (CD44/CD62L/CD127/CD27) on one sample/animal from each of the organs for Ad-LacZ D8V$^+$ CD8$^+$ T cells, Ad-D8V D8V$^+$ CD8$^+$ T cells and naïve total CD8$^+$ T cells.

Figure 4.6: Distribution of Ad-I8V induced CD8$^+$ T cells in the peripheral tissues.

A



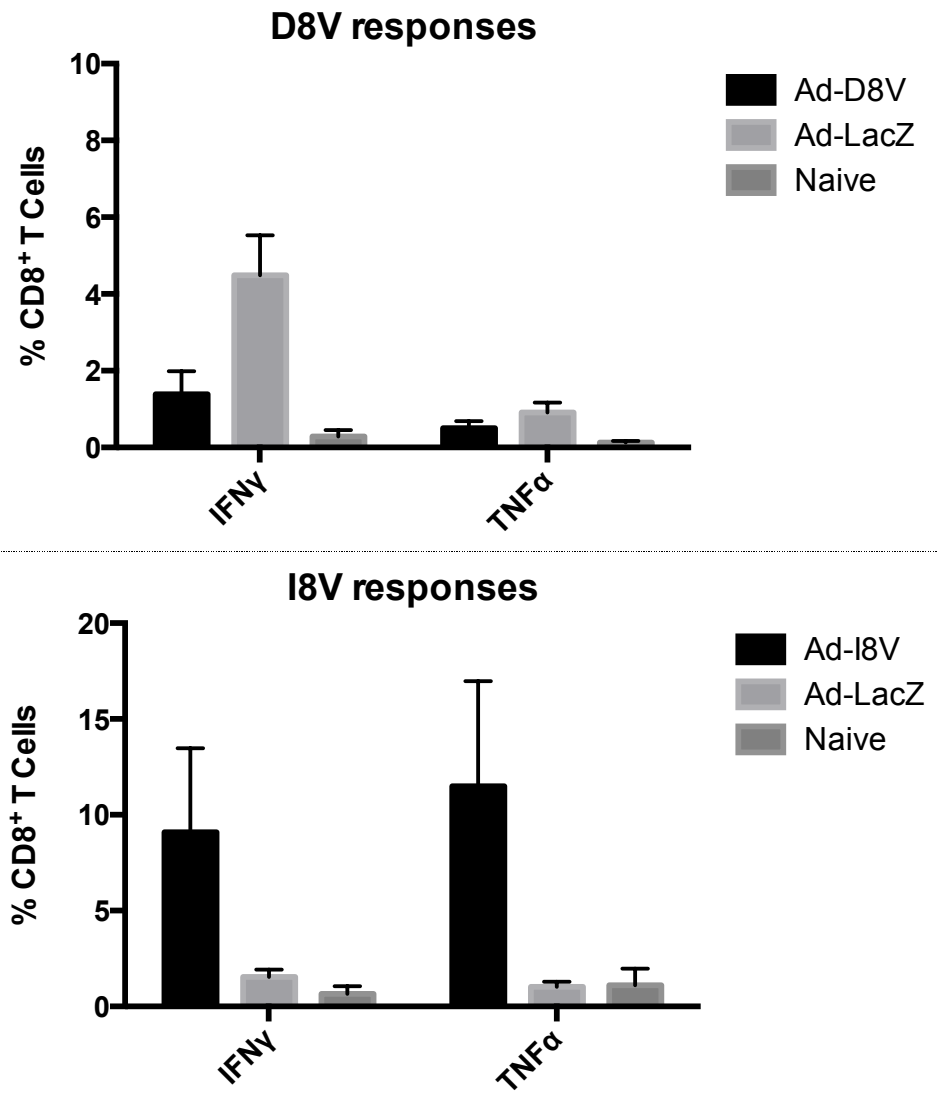
B



(A) At day 100-post immunisation, organs were harvested and stained for tetramer positive cells (n/groups=5). Levels of I8V specific cells seen in wildtype Ad-LacZ are low, in keeping with a classical non-inflating response. This is compared to Ad-I8V, where there are significant numbers distributed in the peripheral tissues, in keeping with an inflationary response.

(B) Shows representative phenotypic markers (CD44/CD62L/CD127/CD27) on one sample/animal from each of the organs for Ad-LacZ I8V$^+$ CD8$^+$ T cells, Ad-I8V I8V$^+$ CD8$^+$ T cells and naïve total CD8$^+$ T cells.

Figure 4.7: Functionality of Ad-D8V and Ad-I8V induced $CD8^+$ T cells in ICS at day 100-post immunisation.



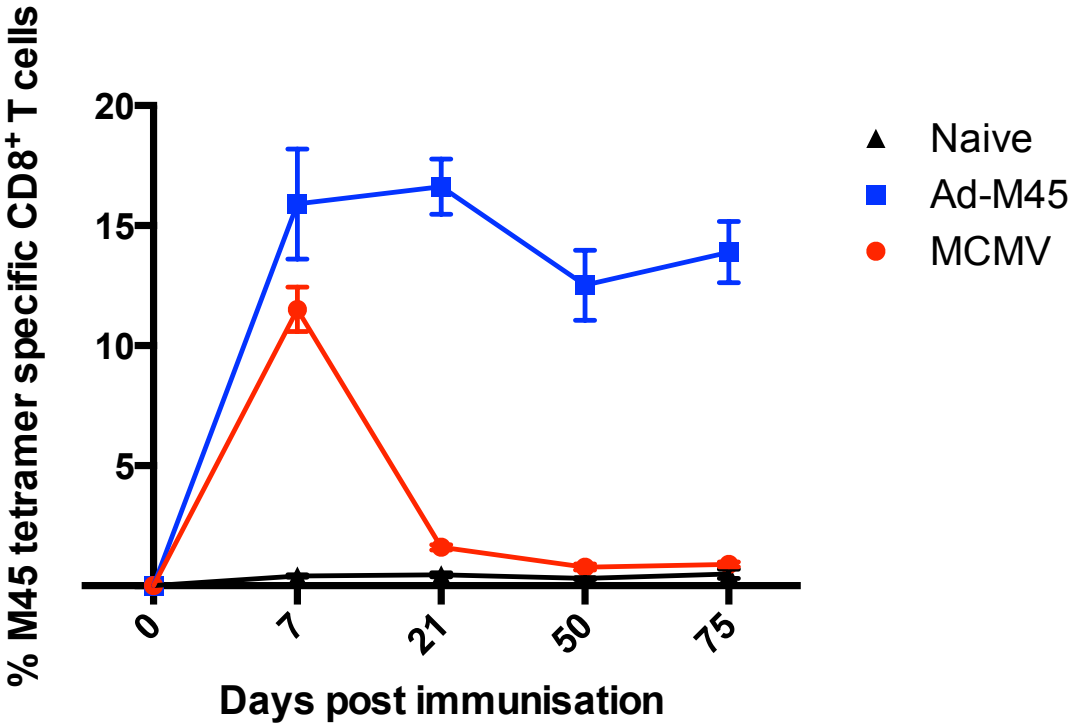
Splenocytes were harvested from day 100-post immunisation B6 mice with Ad-D8V, Ad-I8V, Ad-LacZ and naïve controls (n/group=5). Error bars indicate the SEM. Intracellular cytokine stain (ICS) was performed, looking for IFNγ and TNFα release (through flow cytometry) in response to one of D8V peptide (10^{-5}M), I8V peptide (10^{-5}M), medium alone (negative control – not shown) or PMA and Ionomycin (positive control – not shown). Ad-LacZ immunised mice were included as a control/comparator.

4.4 Ad-M45 minigene

4.4.1 The Ad-M45 minigene compared to MCMV

The minigene construct used in this work is the individual nonamer of M45 ($M45_{985-993}$) from the full M45 gene. This epitope was chosen given that it represents the immunodominant classical "non-inflating" $CD8^+$ T cell response from the MCMV model. This was to allow for direct comparison to the "non-inflating" response (I8V) in Ad-LacZ. The M45 minigene insert is also flanked by an initiator methionine, stop codon and a SV40 polyA tail. The insert contains nothing else. The expression cassette includes an HCMV promoter and this is then cloned into an E1/E3 deleted AdHu5 vector. Through doing this it was hypothesised that it may be possible to make a further "non-inflating" epitope induce inflating responses in this same system. Figure 4.8 demonstrates clear inflation of the tetramer-specific responses (in blood) from B6 mice immunized with Ad-M45, compared to the conventional "non-inflationary" M45 responses from MCMV.

Figure 4.8: The Ad-M45 minigene tetramer-specific CD8$^+$ T cell responses in vivo compared to MCMV.



C57BL/6 mice were immunised i.v. with Ad-M45 alongside control groups of MCMV and naive. Results show the blood time-points (day 7, 21, 50 and 75 p.i.) for M45 tetramer-specific responses in Ad-M45 compared to M45 in the MCMV model. Virus was immunised i.v. at $1x10^8$ iu per mouse for Ad-M45 and MCMV at $2x10^6$ iu per mouse. Groups are representative of 8 mice (pooled from two experiments) with error bars representing the SEM. This experiment has been completed independently 3 times, with these results showing a consensus from all experiments.

4.4.2 Phenotype, distribution and functionality of the tetramer-specific CD8$^+$ T cells induced by the Ad-M45 minigene

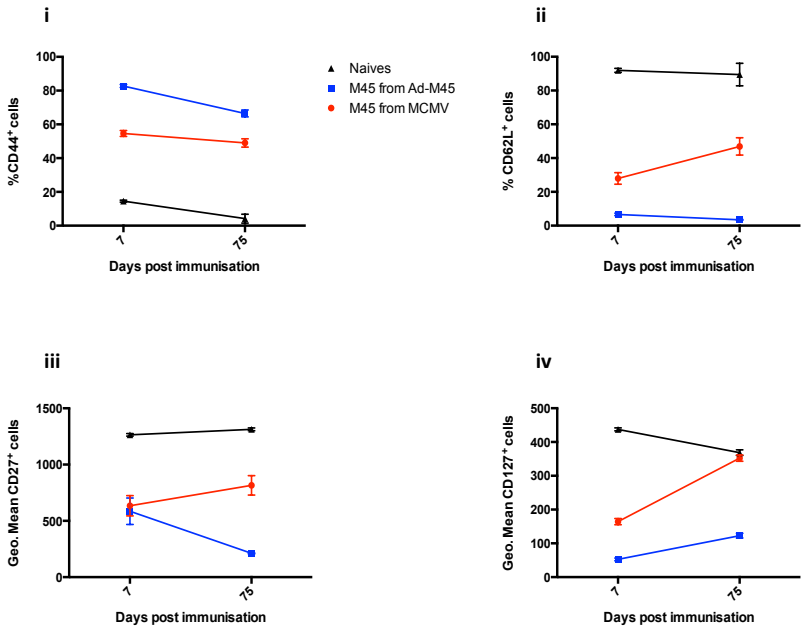
Figure 4.9 shows the phenotype for the tetramer-specific CD8$^+$ T cells induced from the Ad-M45 minigene – these are effector memory cells.

Figure 4.10 looks at the distribution of M45 tetramer-specific CD8$^+$ T cells from the Ad-M45 and MCMV groups in the peripheral tissues (liver, lung and spleen). There are noticeably high levels of M45 tetramer-specific CD8$^+$ T cells from the Ad-M45 group in the peripheral tissues, as seen with M38 in MCMV. This is in stark contrast to the M45 responses from MCMV.

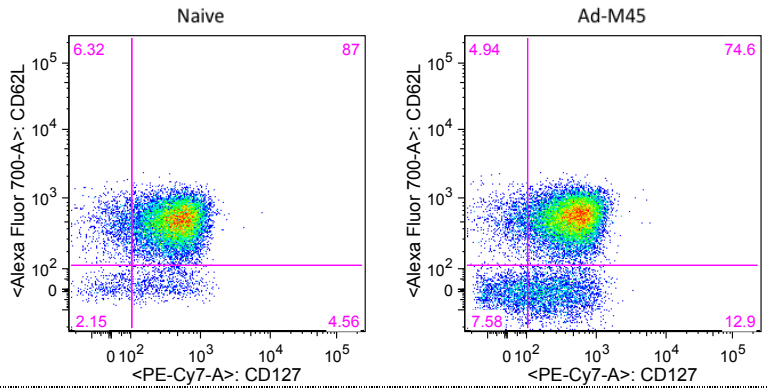
Finally, figure 4.11 demonstrates functionality of the Ad-M45 minigene induced CD8$^+$ T cells, through intracellular cytokine staining (ICS) assays.

Figure 4.9: The phenotype for the Ad-M45 induced CD8$^+$ T cells.

A



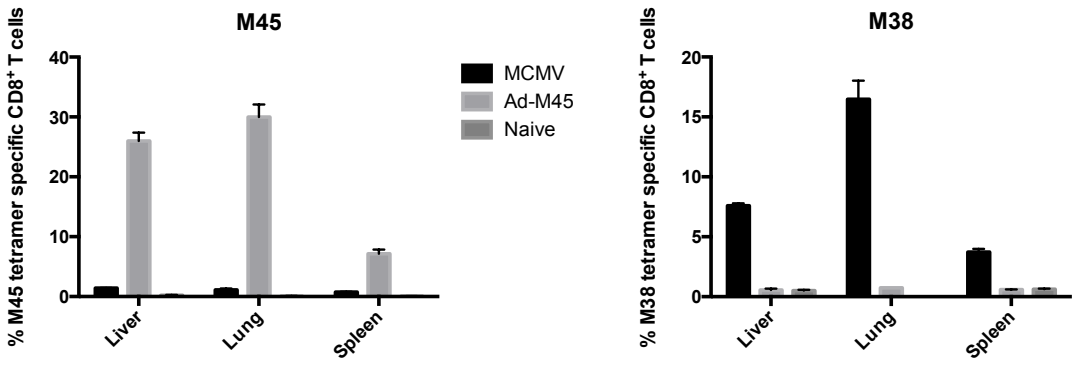
B



(A) Blood was stained for (i)CD44, (ii)CD62L, (iii)CD27 and (iv)CD127, to assess the CD8$^+$ T cell phenotype. Results show an early (day 7) and late (day 75) time-point after immunisation with either of Ad-M45 or MCMV, alongside naïve controls. N=8 with error bars showing the SEM.

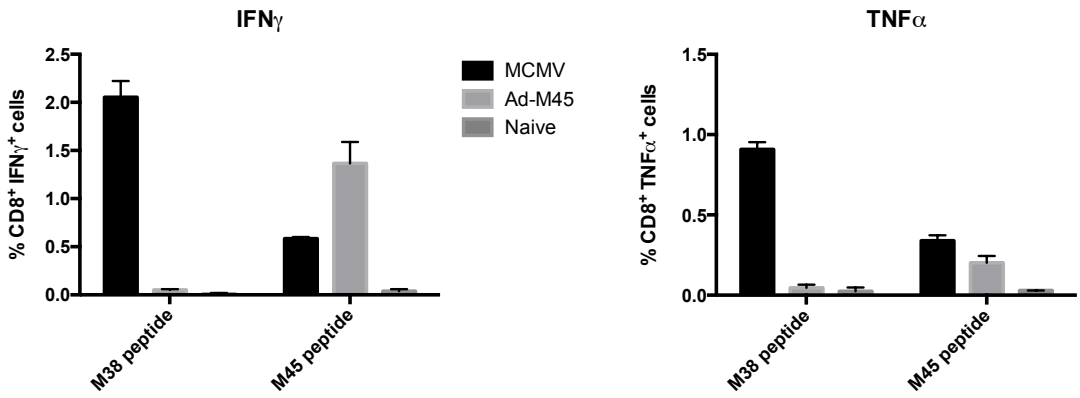
(B) A representative CD127/CD62L plot shows the T_{EM} phenotype for Ad-M45 compared to naive (gated: lymphocytes>live dead>CD8$^+$>CD127 and CD62L).

Figure 4.10: Distribution of Ad-M45 induced $CD8^+$ T cells in the periphery.



Results show the distribution of tetramer-specific cells (M45 and M38) in the liver, lung and spleen at day 75-post immunisation with Ad-M45 or MCMV compared to naïve controls. In all cases groups of 8 mice have been used (pooled from two experiments) with error bars representing the SEM.

Figure 4.11: Functionality of Ad-M45 induced $CD8^+$ T cells in ICS at day 75-post immunisation.



Results show the functionality (IFNγ and TNFα production) of $CD8^+$ T cells in ICS, following stimulation with M45 or M38 peptide. Positive (PMA/I) and negative (medium alone) controls have been excluded for presentation. This is performed on splenocytes at day 75-post immunisation with Ad-M45 or MCMV compared to naïve controls. In all cases groups of 8 mice have been used (pooled from two experiments) with error bars representing the SEM.

4.5 Conclusions

Minigene vectors of Ad-I8V and Ad-M45 allow for the induction of inflationary $CD8^+$ T cell responses from previously "non-inflationary" epitopes. The features of these responses are in keeping with the inflationary responses induced from the Ad-LacZ and MCMV models. In fact, for both Ad-I8V and Ad-M45, very high levels of frequency, distribution and functionality are seen compared to those models. By removing the processing requirements for these epitopes it has been possible to switch their memory phenotype. Throughout this work there has been some fluctuation in the phenotype of these inflationary populations between T_{EM} and T_E noted. This is presumably representative of the fluidity of the system, with $CD8^+$ T cells being captured at different phases in their development. It may also be representative of the fashion in which these populations are maintained, with the potential continuous turnover of inflationary populations (Quinn et al., 2015).

Note is made of the marked differences in the kinetics of the inflationary response curve in blood induced from Ad-I8V and Ad-M45 immunised mice compared to that of Ad-D8V or either of the inflationary responses described from the Ad-LacZ or MCMV models. Specifically, the marked difference at the acute (day 7-14) phase following administration, where the initial inflationary response is extremely high, very much in contrast to what is seen in Ad-D8V, Ad-LacZ and MCMV. This highlights that the acute phase response likely serves as a critical time in the production and maintenance of inflationary populations, but more work is needed in this area.

Modification of antigen context and associated processing requirements does provide a critical tool to modulate the nature of memory induced by adenovirus-vectored vaccines. It is observed that a simple "minimalist" approach in the vaccine construct reproducibly allows for memory inflation in response to previously "non-inflationary" epitopes. It is proposed that this work holds an important translational element. Recent data from human studies do suggest that long-term effector memory $CD8^+$ T cell pools can be induced and sustained following vaccination with adenoviral vectors (Barnes et al., 2012; Swadling et al., 2014). Further, data from CMV-vectored vaccines suggests that such tissue-homing effector memory responses can provide robust protection (Hansen et al., 2011; Karrer et al., 2003). In this context this finding proves that a simple modification of the context of an epitope can allow for a dramatic switch in memory phenotype, and that such responses can be elicited in parallel. Thus this approach holds significant potential for utilisation as a tool in vaccine development.

5. Results – Processing and Presentation

5.1 Introduction

The immunoproteasome

As already introduced, the immunoproteasome is constitutively expressed in haematopoetic cells and induced in non-immune cells during viral infection by interferon signaling. It is specifically constitutively expressed in mature dendritic cells (Guillaume et al., 2010; Macagno et al., 1999). It favours the production of peptides with C-terminal hydrophobic or basic residues, which often act as anchor residues for class I binding (Boes et al., 1994; Driscoll et al., 1993; Gaczynska et al., 1996; Gaczynska et al., 1994; Groettrup et al., 1995). As such, immunoproteasomes likely play an important role in the production of peptides toward $CD8^+$ T cell immune responses (Chen et al., 2001; van Hall et al., 2000). Mice that completely lack immunoproteasomes display major alterations in antigen presentation (Kincaid et al., 2012). It is however of note that some viral antigens and self antigens (particularly) have been found to favour standard proteasome processing, and as such the rules that govern these interactions are likely further convoluted (Basler et al., 2004; Chapiro et al., 2006; Morel et al., 2000; Van den Eynde and Morel, 2001).

The immunoproteasome may also facilitate T cell responses independent of class I presentation. Work with immunoproteasome knock out mouse models has provided evidence of reduced total $CD8^+$ T cell counts, suggestive of a role in T cell development or maturation (Basler et al., 2011; Hensley et al.,

2010; Van Kaer et al., 1994). There are likely numerous other key functions for the immunoproteasome, which as yet remain incompletely described. Overall the immunoproteasome seems to be critically involved in the regulation of $CD8^+$ T cell responses, proinflammatory cytokine production, activation of the NF-κB pathway and management of oxidative stress – reviewed in (McCarthy and Weinberg, 2015).

N- and C-terminal extensions

It is know that proteasomes play a key role in peptide generation, but it is not clear whether they produce these particles of the correct size directly (Dick et al., 1998; Lucchiari-Hartz et al., 2000) or whether they release larger peptides that then require further trimming down (by peptidases) to the required epitope. There is now a body of evidence that supports N-terminal trimming to the specific epitope in the cytosol or endoplasmic reticulum by amino-peptidases, following production of N-extended versions of antigenic peptide from proteasomes (Cascio et al., 2001; Craiu et al., 1997; Falk et al., 1990; Mo et al., 1999; Snyder et al., 1994; Stoltze et al., 2000). Furthermore, one of these aminopeptidases (leucine aminopeptidase) is induced by IFNγ (Beninga et al., 1998).

The C-terminus appears to be of much greater relevance to the processing of peptides. As the vast majority of peptides presented on class I have hydrophobic or basic C-termini, it fits that the immunoproteasome is thought to generate peptides better suited to binding class I compared to that of the

constitutive proteasome, and in turn to be more efficient at generating an immune response (Driscoll et al., 1993; Fruh et al., 1994).

TCR usage

Restricted TCR usage is described in HCMV. The majority of CTL clones specific to the HCMV pp65 peptide from any one seropositive individual use only one or two different TCRs at the level of the nucleotide sequence. For those with the same MHC class I allele, it is noted that CTLs from different seropositive individuals that recognise the same peptide-MHC complex often use the same Vβ segment (Weekes et al., 1999). To some extent this correlates with the type of $CD8^+$ T cell / CD45 isoform (Vargas et al., 2001).

In MCMV, and specific to inflationary populations of $CD8^+$ T cells, oligoclonality has similarly been described within the Vβ TCR usage (Karrer et al., 2003). This work was all carried out in the original BALB/C / MCMV model, with pp89-specific $CD8^+$ T cells. At the later time-points after infection, there is an increase in the proportion of tetramer-positive pp89 $CD8^+$ T cells that use the Vβ8.1 and 8.2 TCR chain. However, at earlier time-points, the TCR usage was more diverse.

5.2 Overview / Aims

- What role does the immunoproteasome play, if any, in our minigene constructs?

- By returning N-terminal β-gal sequence to the I8V minigene, will this affect the way in which it is processed?

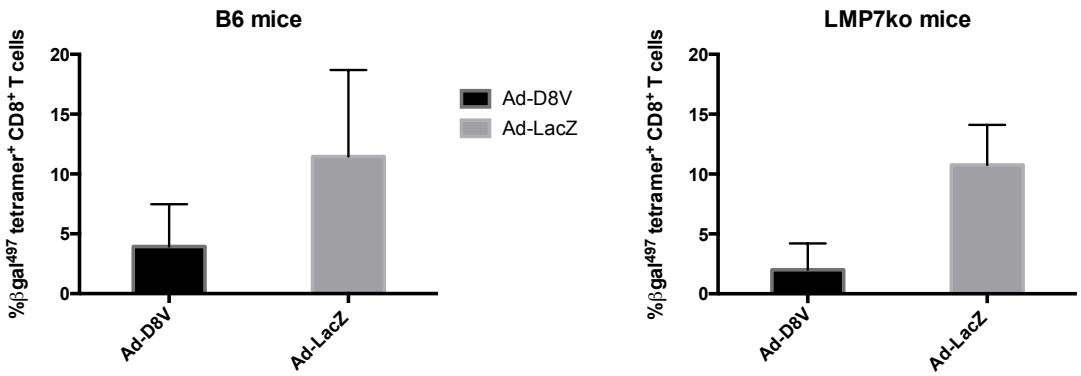
- By returning C-terminal β-gal sequence to the I8V minigene, will this affect the way in which it is processed?

- Is there restricted TCR usage within the Ad-LacZ model, as seen within the MCMV model and in HCMV?

5.3 Immunoproteasome (LMP7$^{-/-}$) studies

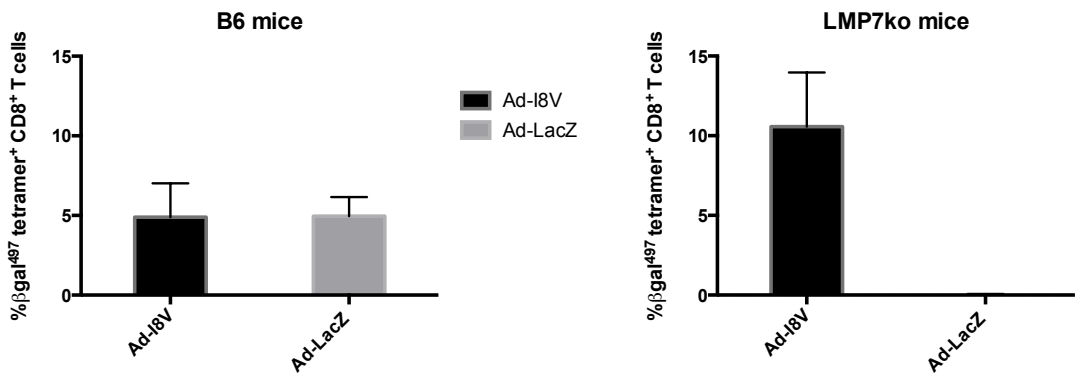
As discussed, we know that inflationary response epitopes demonstrate independence from the immunoproteasome in both the Ad-LacZ and MCMV models. An LMP7 (β5i) knock out mouse model (LMP7ko or LMP7$^{-/-}$) allows for the tracking of these responses *in vivo*. Of note, it seems probable that the minigene inserts are likely devoid of any requirement of proteasome / immunoproteasome processing as they are just octamers/nonamers, which could be loaded directly onto class I presumably. None-the-less, when testing the minigene constructs in this LMP7$^{-/-}$ mouse model, the tetramer-specific CD8^{+} T cell responses are found to be independent of the immunoproteasome. Figure 5.1 and 5.2 presents this data alongside the wildtype Ad-LacZ responses, which shows the published immunoproteasome independence of the inflating D8V-specific CD8^{+} T cell response and the dependent non-inflating I8V-specific response (Bolinger et al., 2013).

Figure 5.1: Immunoproteasome studies and Ad-D8V.



Ad-LacZ and Ad-D8V tetramer-specific responses (D8V) are shown in B6 mice and LMP7$^{-/-}$ mice at day 14-post immunisation. Groups are of 4 mice and the error bars indicate the SEM. There is detectable D8V tetramer-specific response from LMP7$^{-/-}$ mice immunised with Ad-LacZ (as previously published). There is also a clear Ad-D8V response, albeit at a reduced magnitude.

Figure 5.2: Immunoproteasome studies and Ad-I8V.

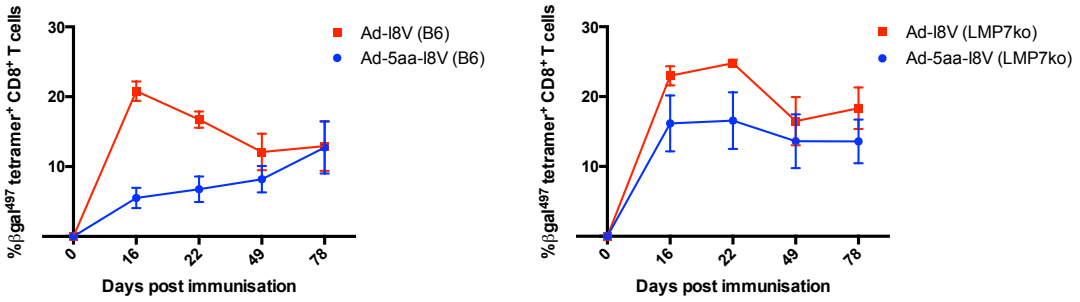


Ad-LacZ and Ad-I8V tetramer-specific responses (I8V) are shown in B6 mice and LMP7$^{-/-}$ mice at day 14-post immunisation. Groups are of 4 mice and the error bars indicate the SEM. There is no detectable I8V tetramer-specific response from LMP7$^{-/-}$ mice immunised with Ad-LacZ (as previously published), but there is a clear Ad-I8V response.

5.4 N-terminal extensions

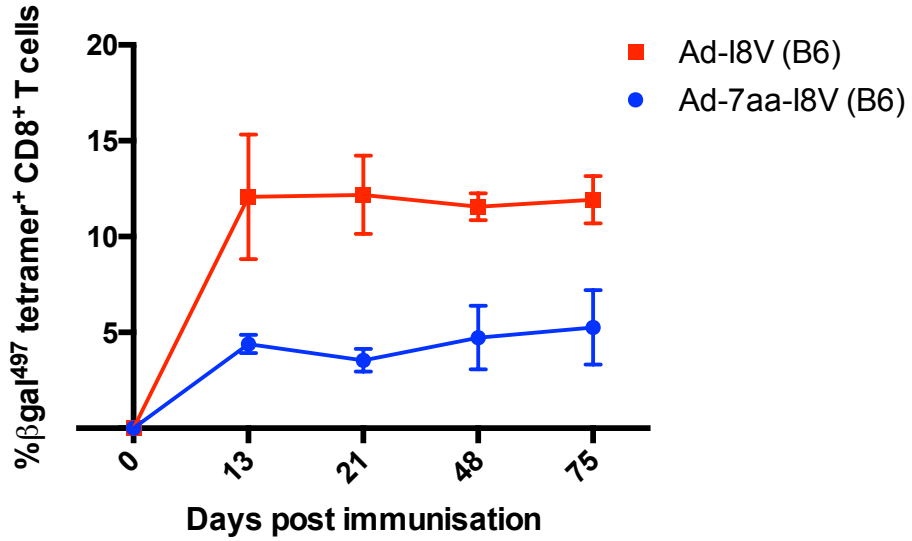
Determining the "critical number" of N-terminal amino acids to add back onto the Ad-I8V construct was difficult, but evidence from work by another group on memory inflation in MCMV provided some guidance (Farrington et al., 2013; Turula et al., 2013). This work suggested a critical number of 7 amino acids added back onto the N-terminus. Constructs with 5,7 and 10 amino acid N-terminal extensions were therefore developed. Amino acids were added back as they are in the native full-length β-galactosidase. These constructs were administered intravenously into B6 and LMP7$^{-/-}$ mice. The LMP7$^{-/-}$ group was set up to act as an early read out in these experiments. If the I8V-specific CD8^{+} T cell responses had been returned to a classical memory response through the addition of flanking sequence, it would follow that these responses will now be immunoproteasome dependent again. If this had been achieved, it would therefore be expected to see no I8V-specific responses at day 14-post immunisation (and vice versa) in the LMP7$^{-/-}$ mouse. None of 5 (figure 5.3), 7 (figure 5.4) or 10 (figure 5.5) amino acid extensions had any significant impact on the I8V response. Ultimately all constructs still showed inflation from the I8V-specific CD8^{+} T cells. The quality of the inflationary responses from all three constructs was one in keeping with inflationary responses. There was no difference found in the phenotype of the tetramer-specific CD8^{+} T cells isolated compared to those in Ad-I8V. Figure 5.6 shows the phenotypic data for the Ad-10aa-I8V construct only.

Figure 5.3: Ad-5aa-I8V.



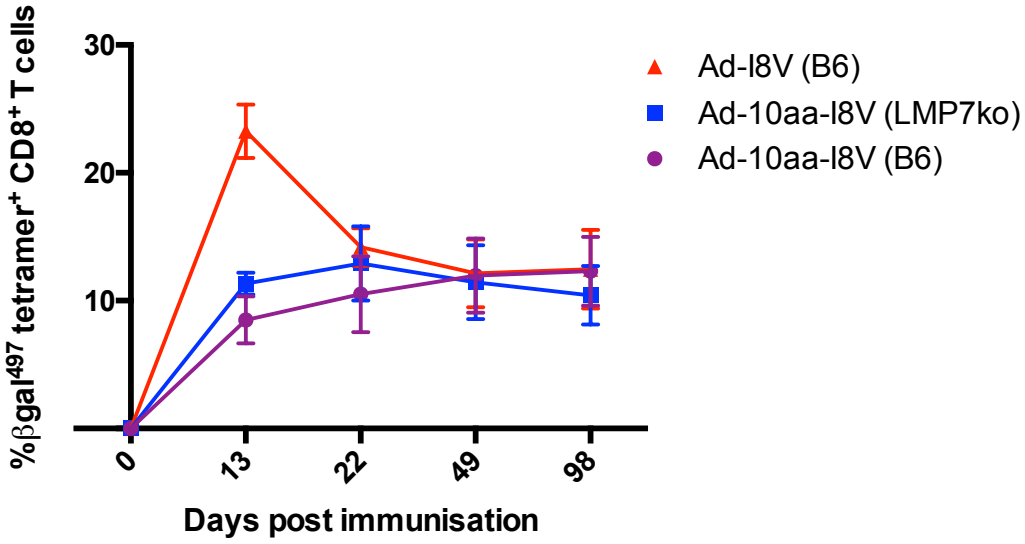
C57BL/6 and LMP7$^{-/-}$ mice were immunised i.v. with Ad-5aa-I8V alongside Ad-I8V and naive controls. The data for the naïve group has been omitted (no tetramer-specific responses were detected in this group). These were all in groups of 4 mice. Error bars indicate the SEM. Results are presented separately for the B6 and LMP7$^{-/-}$ mice. The graphs demonstrate the I8V tetramer-specific CD8^{+} T cell responses from Ad-I8V and Ad-5aa-I8V responses in blood at day 16, 22, 49 and 78-post immunisation.

Figure 5.4: Ad-7aa-I8V.



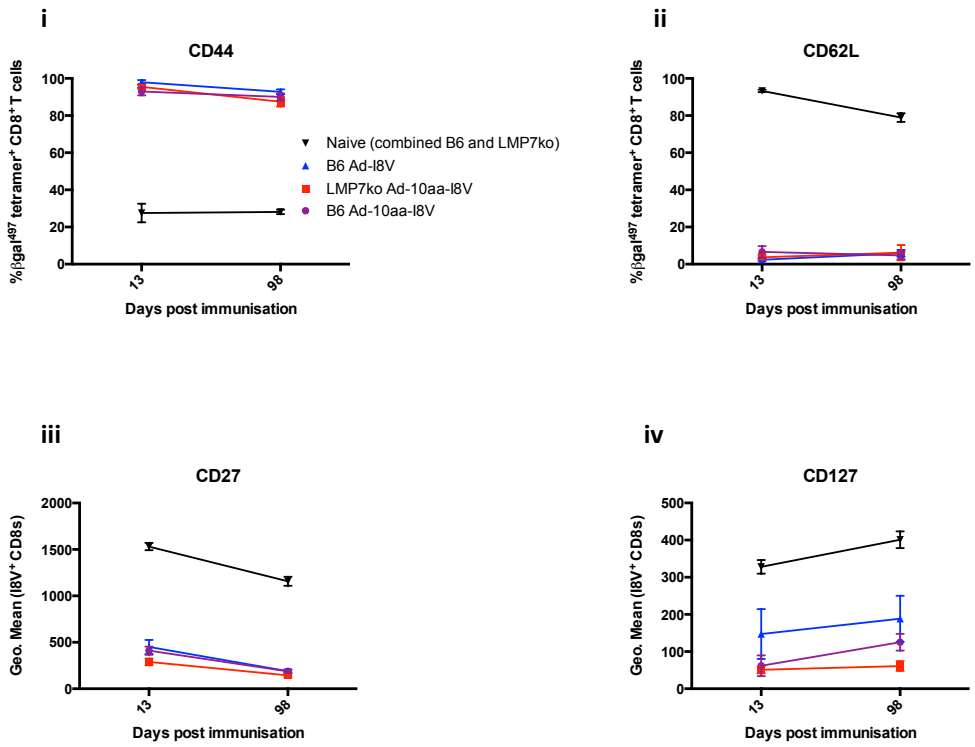
C57BL/6 mice were immunised i.v. with Ad-7aa-I8V alongside Ad-I8V and naive controls. The data for the naïve group has been omitted (no tetramer-specific responses were detected in this group). These were all in groups of 5 mice. Error bars indicate the SEM. The graph demonstrates the I8V tetramer-specific $CD8^+$ T cell responses from Ad-I8V and Ad-7aa-I8V responses in blood at day 13, 21, 48 and 75-post immunisation.

Figure 5.5: Ad-10aa-I8V.



C57BL/6 and LMP7$^{-/-}$ mice were immunised i.v. with Ad-10aa-I8V alongside Ad-I8V and naive controls. The data for the naïve group has been omitted (no tetramer-specific responses were detected in this group). These were all in groups of 5 mice. Error bars indicate the SEM. Results are presented for the control Ad-I8V group in B6 only, alongside the Ad-10aa-I8V in B6 and LMP7$^{-/-}$ mice. The graphs demonstrate the I8V tetramer-specific CD8^{+} T cell responses in blood at day 13, 22, 49 and 98-post immunisation.

Figure 5.6: Ad-10aa-I8V phenotype.

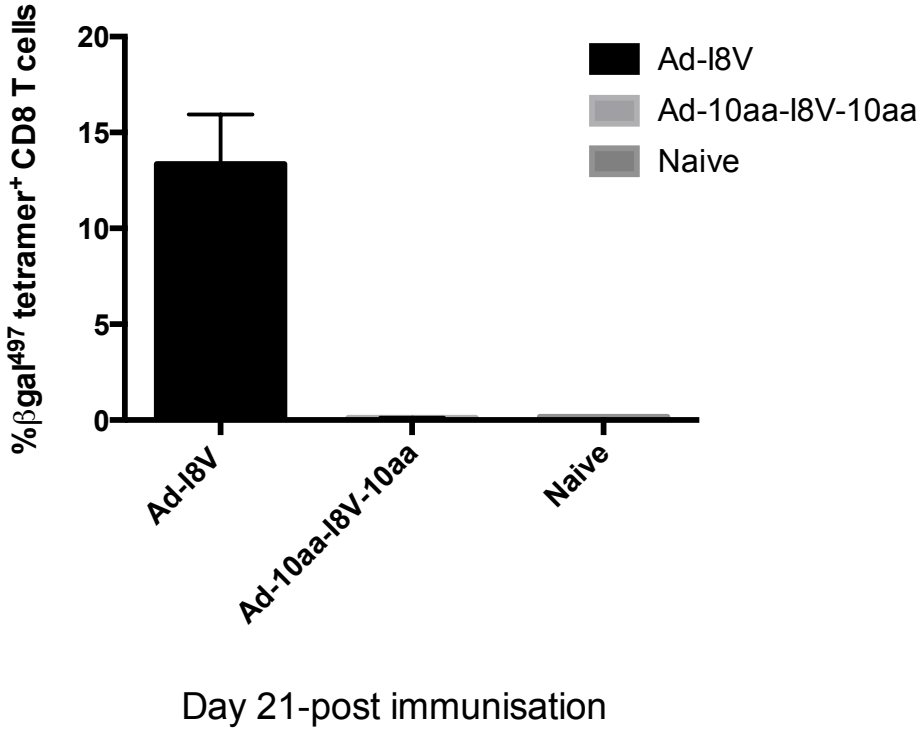


C57BL/6 and LMP7$^{-/-}$ mice were immunised i.v. with Ad-10aa-I8V alongside Ad-I8V and naive controls. These were all in groups of 5 mice. Error bars indicate the SEM. Results are presented for the control Ad-I8V group in B6 only, alongside the naives and Ad-10aa-I8V in B6 and LMP7$^{-/-}$ mice. The graphs demonstrate the I8V tetramer-specific CD8^{+} T cell phenotype for (i)CD44, (ii)CD62L, (iii)CD27 and (iv)CD127 in blood at day 13 and 98-post immunisation. CD44 and CD62L are presented as the percentage of tetramer-positive CD8^{+} T cells. CD127 and CD27 are presented as the geometric mean.

5.5 C-terminal extensions

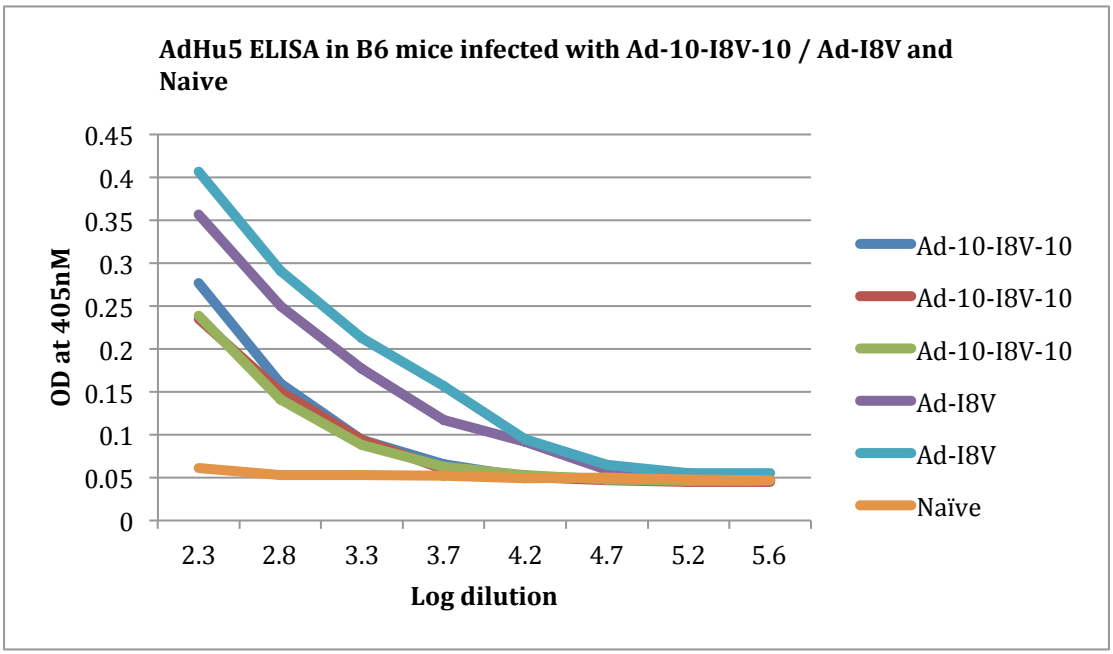
The effect of C-terminal extensions was next evaluated. Additions of 10 amino acids long on the C-terminus (with and without 10aa N-terminal extensions) do not allow for an I8V peptide-specific response *in vivo;* figure 5.7 shows day 21 results in blood for Ad-10aa-I8V-10aa and figure 5.9 day 14 results in blood for Ad-I8V-10aa. It was not possible to track any I8V tetramer-specific $CD8^+$ T cell responses. Given the lack of impact of N-terminal extensions, it suggests that this is likely due to aberrant expression and/or processing in the context of the C-terminus extension and one may hypothesise that longer C-terminal extensions, or even a full length LacZ insert, might be required for natural processing of this epitope.

Figure 5.7: Ad-10aa-I8V-10aa.



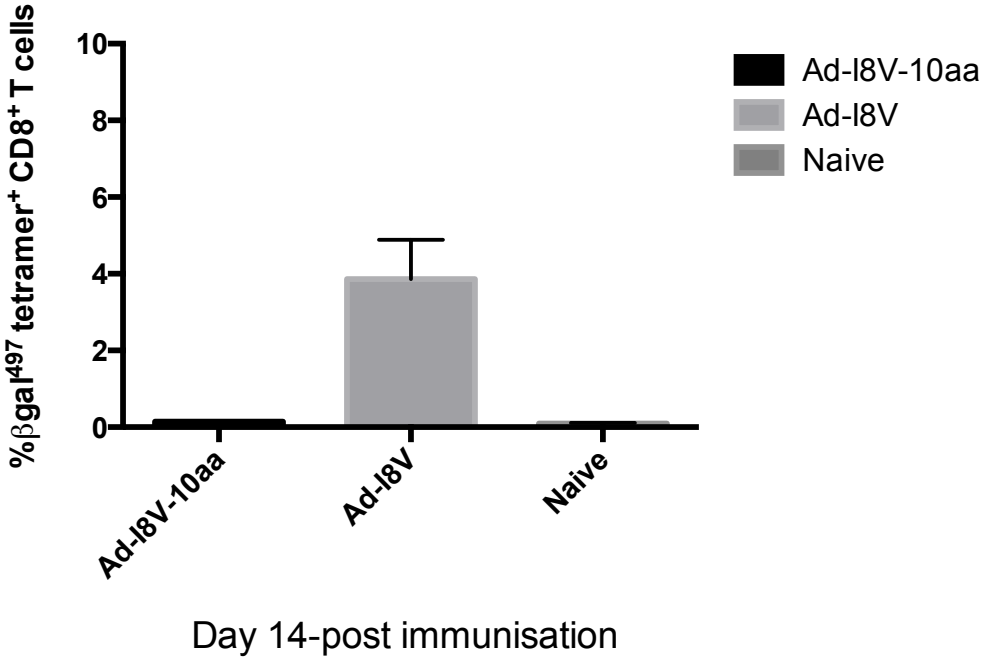
C57BL/6 mice were immunised i.v. with Ad-10aa-I8V-10aa alongside Ad-I8V and naive controls. Tetramer-specific $CD8^+$ T cell responses in blood are shown for day 21-post immunisation only. These were all in groups of 4 mice. Error bars indicate the SEM. There were no I8V tetramer-specific responses in the Ad-10aa-I8V-10aa or naïve groups.

Figure 5.8: Ad-10aa-I8V-10aa adenovirus antibody ELISA.



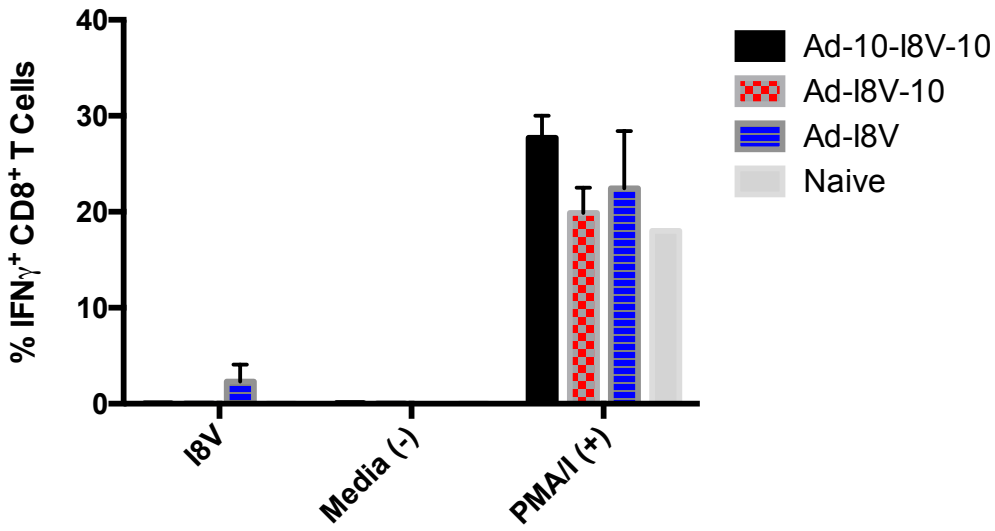
C57BL/6 mice were immunised i.v. with Ad-10aa-I8V-10aa alongside Ad-I8V and naive controls. Blood was analysed for adenovirus antibody responses at day 28-post immunisation in a standard ELISA, and optical density measured at 405nm. Ad-10aa-I8V-10aa and Ad-I8V showed an antibody response, compared to a naïve control.

Figure 5.9: Ad-I8V-10aa.



C57BL/6 mice were immunised i.v. with Ad-I8V-10aa alongside Ad-I8V and naive controls. Tetramer-specific $CD8^+$ T cell responses in blood are shown for day 14-post immunisation only. These were all in groups of 4 mice. Error bars indicate the SEM. There were no I8V tetramer-specific responses in the Ad-I8V-10aa or naïve groups.

Figure 5.10: Ad-I8V-10aa and Ad-10aa-I8V-10aa induced $CD8^+$ T cell responses following peptide stimulation in an ICS assay.

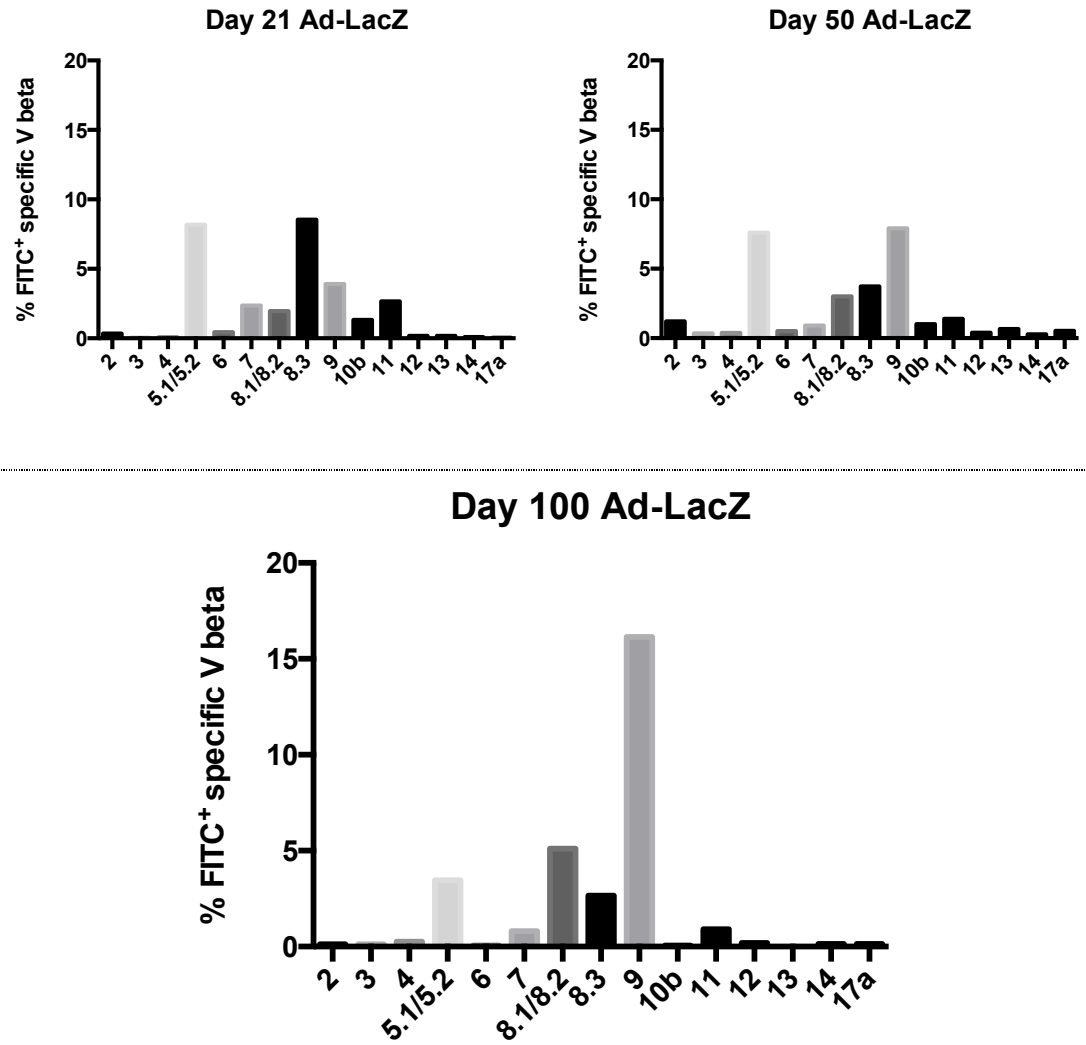


B6 mice that were day 14-post immunisation with either of Ad-I8V-10aa, Ad-10aa-I8V-10aa, Ad-I8V or naïve were compared in an ICS assay. Splenocytes were stimulated with I8V peptide and IFNγ production measured. There was no response from any of the Ad-I8V-10aa, Ad-10aa-I8V-10aa and naïve control group. Positive (PMA/I) and negative (medium alone) controls are shown. Groups were of 4 mice, with error bars indicating the SEM.

5.6 TCR usage

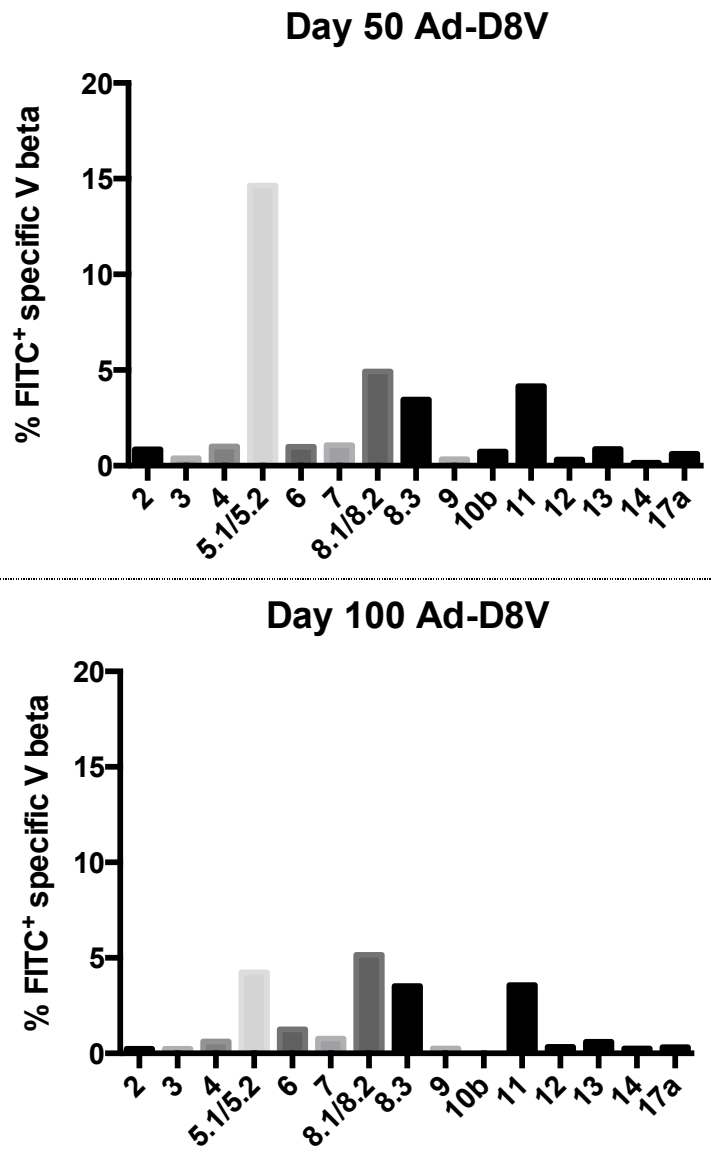
A Vβ TCR antibody panel was used to assess the TCR usage across the Ad-LacZ model, in blood. A caveat of this work for the smaller populations of tetramer-specific cells at either early or later time-points (i.e. the D8V response from Ad-D8V at day 21 or the I8V responses from Ad-LacZ) is the difficulty in gaining enough cells to work with. As such, just the D8V-specific responses from Ad-LacZ (day 21, 50 and 100) and Ad-D8V (day 50 and 100) are shown. Figure 5.11 demonstrates the development of some TCR oligoclonality at the later time-points of infection following Ad-LacZ administration. Similarly, figure 5.12 demonstrates the results for Ad-D8V. Between the two constructs there are some shared TCRs, but also some subtle differences.

Figure 5.11: Comparison of Vβ TCR usage following Ad-LacZ immunisation.



C57BL/6 mice were immunised with Ad-LacZ in groups of 4. Naïve controls were included, but results are not shown (these showed a range of responses across the TCR panel on total $CD8^+$ T cells). Blood was then pooled from all animals into one representative sample and staining preformed for Vβ TCR usage. D8V tetramer-specific responses are shown in blood at day 21, 50 and 100-post immunisation for each of the Vβ TCRs.

Figure 5.12: Comparison of Vβ TCR usage following Ad-D8V immunisation.



C57BL/6 mice were immunised with Ad-D8V in groups of 4. Naïve controls were included, but results are not shown (these showed a range of responses across the TCR panel on total $CD8^+$ T cells). Blood was then pooled from all animals into one representative sample and staining preformed for Vβ TCR usage. D8V tetramer-specific responses are shown in blood at day 50 and 100-post immunisation for each of the Vβ TCRs.

5.7 Conclusions

The Immunoproteasome

The immunoproteasome is not critical to the Ad-D8V and Ad-I8V minigenes, but it seems likely that processing is bypassed with the minigenes, given they are presented as optimal 8 amino acid peptides. The studies performed reassert the findings of immunoproteasome independence for the inflating D8V-specific responses in Ad-LacZ. What this means to the model and inflationary populations more widely remains a topic of interest. The fact that inflating populations are independent of the immunoproteasome suggests the possibility of a non-professional APC being involved. Alternatively, it may suggest a preference for proteasome processing in pAPCs, something that would equally require more investigation.

The I8V and D8V epitopes fit more widely into the context of the β-gal sequence with some conformity as to what we do know about immunoproteasome preference. The I8V epitope has an aspartic acid (an acidic amino acid) immediately at position $\beta\text{-gal}_{505}$ on its C-terminus. As discussed, we know immunoproteasomes favour basic and hydrophobic C-terminal amino acids. D8V has a threonine, a neutral amino acid, on its C-terminus. This may be one quite simple explanation as to the epitope hierarchy in the system.

N- and C-terminal extensions

N-terminal extensions up to 10 amino acids are effectively trimmed / have no impact on the Ad-I8V construct. This is demonstrated in both B6 and LMP7$^{-/-}$ mice, from a maximum peptide sequence of 18 amino acids. This seems to be in conformity with the literature, and suggests a much less significant role for the N-terminus in peptide processing.

A 10 amino acid C-terminal extension, with or without an N-terminal extension, is unable to produce any tetramer-specific CD8^{+} T cell responses for I8V. The context of the C-terminus, as discussed in the immunoproteasome studies, is critical to the peptide processing. By adding back small amino acid lengths of native β-gal sequence, this drastically changes the processing. Exactly how this is occurring remains unanswered. One explanation could be due to aberrant expression and/or processing in the context of the C-terminus extension. Were there a longer C-terminal extension, or even a full-length LacZ insert, natural processing of this epitope may be permitted.

TCR usage

In the early time-points of infection no appreciable oligoclonality is recognised for TCR usage. Of note, this is somewhat confounded by there being insufficient numbers of tetramer positive cells available at earlier and/or later time-points (for this reason the day 21 Ad-D8V results are not shown and I8V results are not shown at all, given later I8V responses from Ad-LacZ are barely detectable). TCR usage at later points of infection, similar to that seen

in MCMV, shows some movement towards oligoclonality. In both Ad-LacZ and Ad-D8V, there is an increased use of the Vβ 5.1/5.2 and 8.3 TCRs early on and Vβ 8.1/8.2 later. The Vβ 9 TCR becomes the most dominant at day 100-post immunisation in the Ad-LacZ model, whilst Vβ 11 is additionally present in the Ad-D8V samples. Overall, there seems to be common ground between the Ad-LacZ and Ad-D8V constructs with regards TCR usage, with just some subtle variation noted. However, the antibody panel used has some clear limitations. For example, some of the antibodies used are restricted to reacting with TCRs of strain-specific haplotypes. The 5.1/5.2, 8.1/82, 8.3, 9 and 11 antibodies are all restricted to react with TCRs of strains having the *b* haplotype (e.g., C57BL/6) of the *Tcrb* gene complex (Behlke et al., 1986; Haqqi et al., 1989). Maybe this allows for some bias in these assays. The 17a antibody is restricted to that of mice with an *a* haplotype (Wade et al., 1988), meaning this would not stain at all in the C57BL/6 mice used. The antibody panel used gives an impression of the TCR usage within the Ad-LacZ model (and is directly comparable to work done in the MCMV model). Ultimately, it would be beneficial to look at TCR usage via TCR sequencing, allowing for a more accurate and sensitive approach.

6. Results – Co-inflation and Boosting

6.1 Introduction

Co-inflation / Competition

An alternative or contributory explanation as to why some epitopes inflate and not others could be due to competition. A major contributing factor to the final depth and breadth of the $CD8^+$ T cell responses to complex antigens is immunodominance, where $CD8^+$ T cells recognising their cognate ligand then inhibit the proliferation of other $CD8^+$ T cells engaged with the same APC. This phenomenon was first described in the context of $CD4^+$ T cells (Sercarz et al., 1993). Immunodominance represents a critical hurdle to developing vaccines that generate effective $CD8^+$ T cell responses (Yewdell and Bennink, 1999). There are a number of reasons as to why immunodominance occurs, one of which relates to the abundance of a particular epitope. In memory inflation, it is has been previously reported that competition for, or availability of, antigen at the level of the APC is critical to immunodominance and is a determinant of the inflationary response to MCMV (Farrington et al., 2013).

A further critical question around inflating populations of $CD8^+$ T cells is how many kinds of these populations can an individual host sustain? It has been previously shown with MCMV that through modification of the immunodominance hierarchy, co-infection with viruses can allow for co-inflation of responses (Farrington et al., 2013). This in particular is of relevance in the context of immune senescence (and the association of

increased morbidity and mortality in individuals with large populations of inflationary cells), but also in translational settings, where it may be beneficial to string together multiple epitopes into a vector.

Boosting

Many of the effective vaccines currently in use require more than one single immunisation, in the form of a prime-boost vaccine strategy. In the setting of childhood vaccinations for example, these are homologous boosts, where the same vaccine is given at set time-points on repeated occasions. In human adenoviral vaccine trials, the prime-boost approach is well recognised as a method to improve on initial antibody and T cell responses (Barnes et al., 2012; Hill et al., 2010; Ogwang et al., 2015; Swadling et al., 2014). This works best in the setting of a heterologous boost (different types of vaccines) (Lu, 2009). The ordering of the vaccines used also proves critical, and there is now good evidence as to the increased immunogenicity of an adenovirus prime and MVA boost approach (Barnes et al., 2012; Swadling et al., 2014).

6.2 Overview / Aims

- Can a single host mount an inflating response to both Ad-I8V and Ad-D8V (when combined) at a comparable level to that seen in a mouse immunised with just the individual constructs?

- Is there a ceiling as to how many inflating responses one host can accommodate?

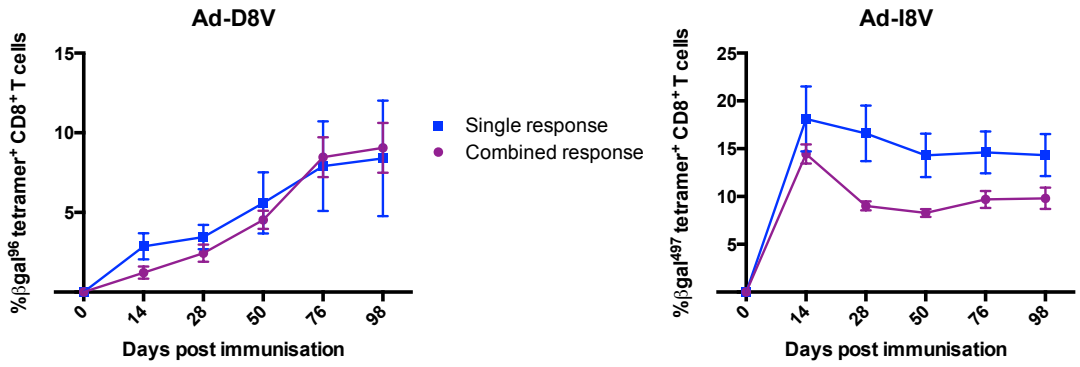
- Can an Ad-LacZ response be further boosted, despite the already high levels of inflationary specific responses?

6.3 Minigenes combined – co-inflation / competition

6.3.1 Co-inflation between Ad-I8V and Ad-D8V

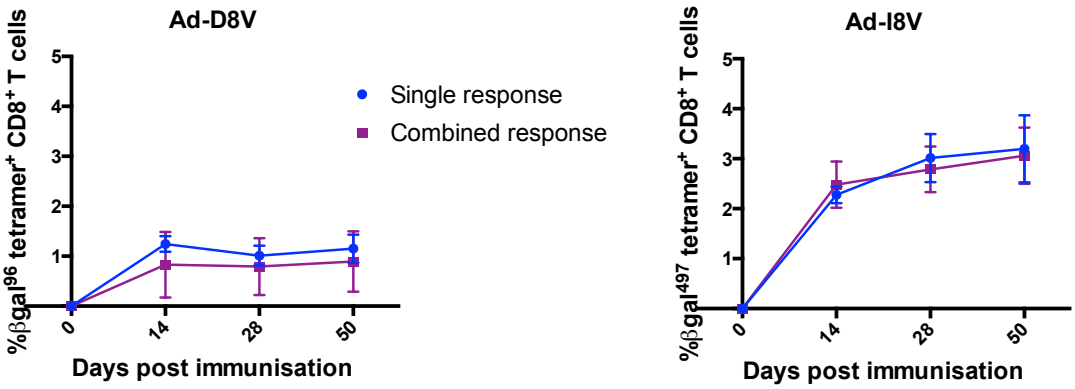
The mechanism through which the Ad-I8V minigene is able to induce inflating populations is likely critically related to removal of the processing requirements, and to the removal of the impact of the D8V epitope in the full β-gal protein. The question is as to the balance of processing versus competition. Initial attempts to develop a construct with the full β-gal insert, but with the inflating (D8V) epitope mutated were difficult, and this work remains ongoing. However, as an initial simple experiment, the two minigene vectors were administered in a combined immunisation. This was initially done via intravenous immunisation. Figure 6.1 shows that Ad-I8V and Ad-D8V induce a similar level of inflationary $CD8^+$ T cell responses in mice receiving either one or both vaccines. This experiment has been repeated with intramuscular immunisation and shown to give the same results (figure 6.2). This route of immunisation allows for co-administration in the same host, but at separate sites (Ad-I8V into the left thigh and Ad-D8V into the right thigh).

Figure 6.1: Ad-I8V and Ad-D8V administered intravenously in the same immunisation.



C57BL/6 mice were co-immunised with Ad-I8V and Ad-D8V in the same host and compared to responses in hosts with a single immunisation (individual Ad-I8V or Ad-D8V). The results show tetramer-specific responses for D8V in Ad-D8V and then I8V in Ad-I8V in mice immunised i.v. The naïve control background responses are undetectable and have been omitted from the graphs. Data is representative of one experiment with groups of 5 mice and the error bars indicate the SEM. A repeat experiment showed the same results.

Figure 6.2: Ad-I8V and Ad-D8V administered intramuscularly in the same immunisation.

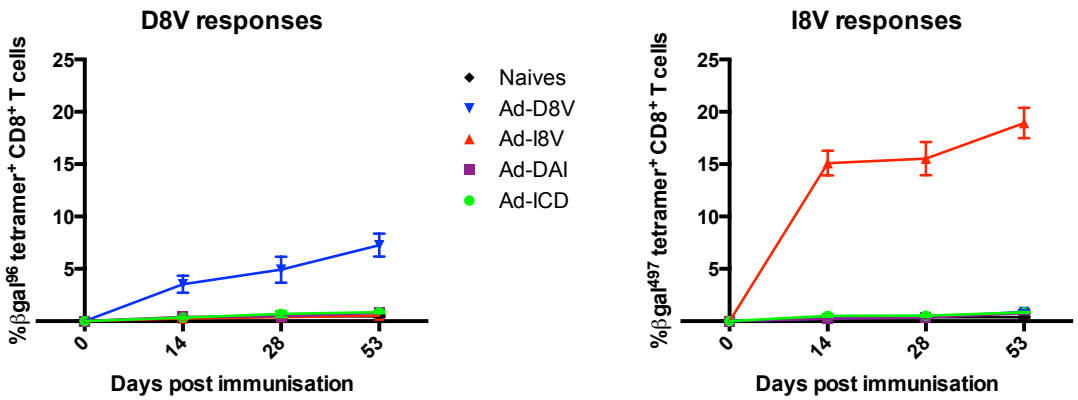


C57BL/6 mice were immunised i.m. In this experiment Ad-I8V and Ad-D8V were combined in the same mouse, but administered (at the same time) at separate sites (Ad-I8V left thigh, Ad-D8V right thigh) compared to mice singularly immunised with either of Ad-D8V or Ad-I8V. Each time point is representative of the tetramer-specific responses in blood from groups of 5 mice and the error bars indicate the SEM.

6.3.2 Co-inflation between I8V and D8V tetramer-specific responses from the same insert on the vector

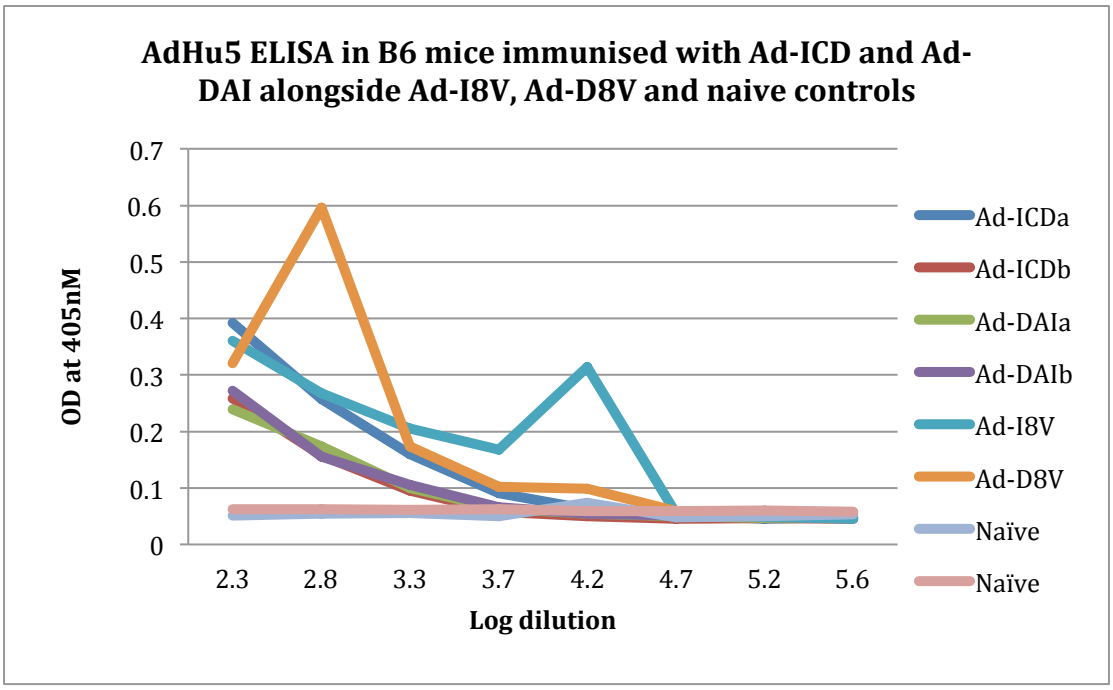
Expression of the two epitopes within the same vector seemed to be a cleaner approach to the question of competition. Vectors were made containing the epitopes in both orientations (first or second) with a glycine-proline linker. That is an Ad-I8V-linker-D8V (called Ad-ICD) and an Ad-D8V-linker-I8V vector (called Ad-DAI). This linker was chosen based upon experience within malaria vaccine development (Draper et al., 2010). In this setting, both epitopes would be delivered to the same cell and processed simultaneously, allowing for an actual readout of competition between these two epitopes, and not just the observation of co-inflation. Figure 6.3 demonstrates results, in which unfortunately no tetramer-specific responses could be tracked *in vivo*, despite of the evidence of the vector being present through the adenoviral antibodies induced (figure 6.4) and thorough checks around the insert sequence and design.

Figure 6.3: Vectors containing an insert of both epitopes with a glycine-proline linker with either epitope first.



C57BL/6 mice were immunised i.v. with Ad-ICD and Ad-DAI ($1x10^8$ iu/mouse) alongside controls of Ad-I8V, Ad-D8V and naïve controls. Each time point is representative of the tetramer-specific responses in blood from groups of 4 mice and the error bars indicate the SEM. Blood was sampled at day 14, 28 and 53-post immunisation. No tetramer-specific responses could be detected from the Ad-ICD and Ad-DAI constructs. Naïve controls are included and show the same level of background to the Ad-ICD and Ad-DAI constructs.

Figure 6.4: Ad-ICD and Ad-DAI adenovirus antibody ELISA.

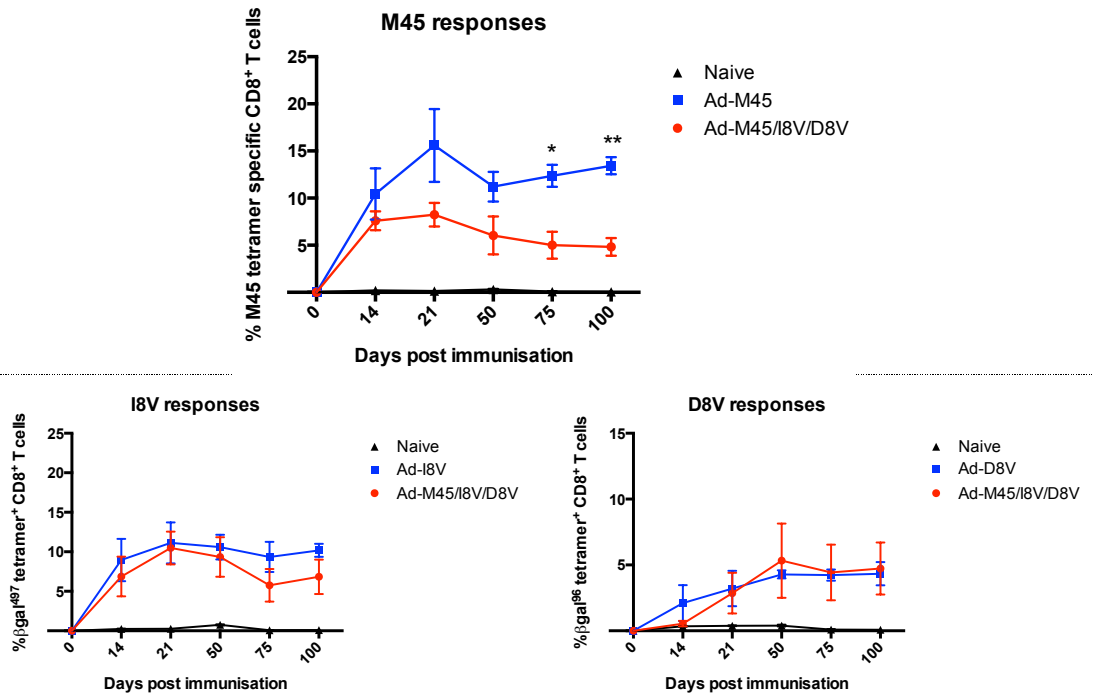


C57BL/6 mice were immunised i.v. with Ad-ICD and Ad-DAI, alongside Ad-I8V, Ad-D8V and naive controls. Blood was analysed for adenovirus antibody responses at day 21-post immunisation in a standard ELISA, and optical density measured at 405nm. All of the mice immunised with an adenoviral construct showed detectable levels of antibody response, compared to naïve controls.

6.3.3 Co-inflation between Ad-I8V, Ad-D8V and Ad-M45

All three minigene constructs were combined in a further co-inflation experiment. This was performed via i.v. immunisation and alongside singularly immunised mouse controls for each of Ad-I8V, Ad-D8V and Ad-M45. This work has currently only been performed in a pilot experiment with groups of three mice and will be repeated with groups of 5. Whilst this work is incomplete, it was felt that this was of some worth presenting, given the possibility of some variations in the frequencies of inflating cells induced from the co-immunisations shown here (figure 6.5). It suggests a possible limit to co-inflation numbers, but further work is needed before any conclusions can be drawn from this work.

Figure 6.5: Ad-M45, Ad-I8V and Ad-D8V administered intravenously in the same immunisation.



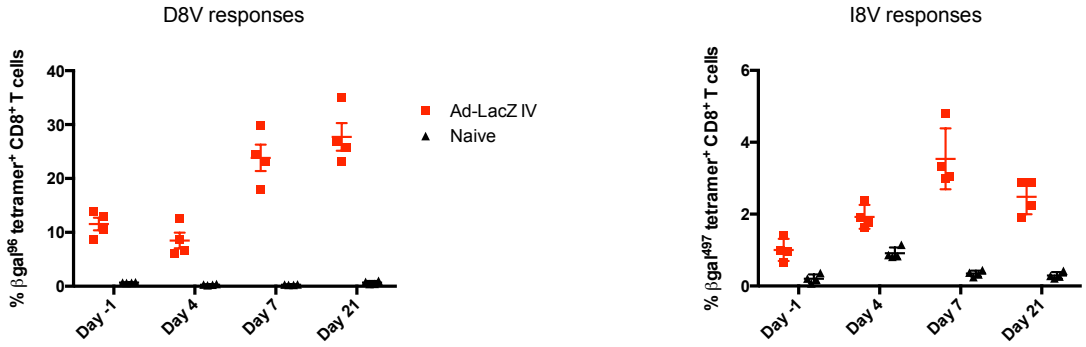
C57BL/6 mice were co-immunised intravenously with Ad-M45, Ad-I8V and Ad-D8V (all $1x10^8$ iu) in the same host and compared to responses in hosts with a single immunisation (individual Ad-M45, Ad-I8V or Ad-D8V). The results show tetramer-specific responses for M45 in combined Ad-M45/I8V/D8V and single Ad-M45 immunised mice, and then I8V and D8V in the same fashion. The naïve control background responses are included. Data is representative of one experiment with groups of 3 mice and the error bars indicate the SEM. Statistical analysis on M45 - *p 0.05, **p <0.005.

6.4 Boosting

Key to the translation of this work will be proof of protection from these minigene vectors. Within the Ad-LacZ model there are limited tools for assessing this. A vaccinia virus with a LacZ insert (VV-LacZ) was available for use in challenge studies, which was dependent on naïve controls and the ability to demonstrate levels of vaccinia virus control in plaque assays between the two groups (previously infected with Ad-LacZ and naïve). After 3 attempts, no meaningful results from the VV plaque assay work were obtained (due to technical difficulties with the plaque assay and likely issues with the VV-LacZ virus itself), and this will form the main focus of future studies using a LCMV challenge model.

However, previously Ad-LacZ immunised mice did show a boosted D8V (and early I8V) response following later VV-LacZ administration (figure 6.6). This was tracked out as far as day 21 following the boost, and at this time was still seemingly increasing.

Figure 6.6: D8V and I8V-specific responses in previously Ad-LacZ immunised mice, following challenge with VV-LacZ.



B6 mice were immunised with Ad-LacZ i.v. ($2x10^9$ iu/mouse). At day 80-post immunisation blood was tested for tetramer-specific responses (labeled as day -1). Mice were then challenged with VV-LacZ i.p. ($2x10^6$ iu/mouse). Blood was then tested at day 4, 7 and 21-post VV-LacZ challenge for I8V and D8V tetramer-specific $CD8^+$ T cell responses. Groups were of 4 mice each. Individual values can be seen alongside the mean and error bars indicating the SEM. This was repeated 3 times (repeats only went to day 7-post VV-LacZ challenge) with all experiments showing the same boosted responses.

6.5 Conclusions

Co-inflation

Co-administration of the Ad-I8V and Ad-D8V minigenes seems to allow for co-inflation of these responses. From these experiments, where there is presumably sufficient antigen (and sufficient APCs) for each epitope, it is concluded that there is no competitive process between the two constructs and both responses can be accommodated in a single host. Note is made of previously published data indicating later inflation of epitopes, which are initially “subdominant”, including recombinant epitopes (Karrer et al., 2003), as well as similar experiments in MCMV with co-administration of viruses in a modified antigenic context and subsequent co-inflation (Farrington et al., 2013). Co-inflation between the two minigenes (Ad-I8V and Ad-D8V) is possible. It is not possible to draw any real conclusion as to the role of competition between the two epitopes (in the context of Ad-LacZ) from this work. The individual viruses (minigenes) are presumably meeting different cells in the host.

The more relevant experiment would look to put both epitopes into the same cell, allowing for simultaneous processing. However, attempts to express the I8V and D8V epitopes in a single insert did not elicit any tetramer-specific responses *in vivo*, despite of detectable antibody responses to the AdHu5 vector. Whether this relates to the use of a linker, or again is an intricacy of processing that has not been fully understood remains unknown. Potential

future work would look to redesign this vector allowing for separate expression of the I8V and D8V in the same insert, but avoiding the need for side-by-side expression. It may be possible to use a CMV bidirectional promoter for this (such as the Clontech Laboratory Inc. specific vectors), allowing for an equivalent level of expression of both of the epitopes. Alternatively, a CMV IRES method could be adopted: CMV-Epitope1-IRES-Epitope2 (Jackson et al., 1990; Jang et al., 1988; Rees et al., 1996). The disadvantage of this method would be that levels of expression of the two epitopes would not be equivalent. A further potential alternative approach would be to create a full LacZ insert with a mutated D8V, as originally planned. However, it is unknown what impact the mutated D8V may have on the structure of the LacZ, and in itself may throw up more uncertainties.

Work with the co-administration of all 3 minigenes (Ad-I8V, Ad-D8V and Ad-M45) is currently incomplete, but has been included in this report to allow for the possible caveat that there may be a ceiling as to the number/quality of inflationary responses that a single host can accommodate. This again will prove critical to the translation of this work, where presumably the ability to string together multiple peptides would be of relevance.

Challenge / Boosting

The relevance of inflationary populations towards protection is of clear importance. Previous work has shown that immune protection from a CMV / HSV-1 model did not require inflationary $CD8^+$ T cell responses for the protection seen (Dekhtiarenko et al., 2013). Data from CMV-vectored vaccines suggests that such tissue-homing effector memory responses can provide robust protection (Hansen et al., 2011; Karrer et al., 2003). It is possible that inflationary responses from Ad-LacZ and the minigene constructs are not protective, and as discussed one cannot correlate the magnitude of a response with the quality / protective capacity of that response (Jeyanathan et al., 2013). Demonstrating the ability of Ad-LacZ and the minigene vectors to protect will form the basis of critical future work. It has not been possible to address this fully during the progress of this work. Ad-LacZ chronically immunised mice (day 50+ post immunisation) were challenged with VV-LacZ, alongside naïve controls. Attempts to show vaccinia virus control between the Ad-LacZ and naïve groups were then made in VV plaque assays. Technical difficulties were encountered with the plaque assays, and it was not possible to demonstrate any meaningful results (genuine plaques) from either group on repeat experiments. This is possibly due to issues with the VV-lacZ virus itself, or more likely the plaque assay. These studies were not pursued further, given that it was felt that this could be tested more rigorously in future experiments using an LCMV model (with a Gp_{33-41} minigene prime and a LCMV infectious challenge). This work is fully described in the future work section reading forward.

Following administration of VV-LacZ to previously Ad-LacZ immunised mice, it does seem that boosting of what is already a significant response is possible in the Ad-LacZ model. The results presented here demonstrate that in an inflationary response that is already present at a very high frequency, there is additional room for further expansion of that response.

In all of this work, it is noted that a comparison of absolute numbers as opposed to percentage of $CD8^+$ T cells may have proved a more insightful measure with regards the differences in the populations produced from the minigenes, in both blood and organs. Future work will look to make these comparisons.

7. Discussion and Future Work

7.1 Ad-LacZ: The HCMV promoter and the role of the AdHu5 vector

It is clear from these studies that the HCMV promoter is not critical to the development of inflating populations from the Ad-LacZ model (this being the only part that had any genetic component of CMV). The Ad-LacZ constructs using RSV and EF1-α as the promoter were able to induce inflating $CD8^+$ T cell responses. The quality of the inflationary cells across the different vectors was comparable. Whilst all vectors have shown the development of a robust inflating response, it is noted that this occurs at very different frequencies (percentage D8V tetramer-positive cells from total $CD8^+$ T cells) across the vectors: CMV>RSV> EF1-α. It seems most likely this is representative of the differing "strengths" between the promoters, as is well described in the literature (Arita et al., 2008; Chen et al., 2008; Qin et al., 2010; Schaack et al., 2011; Zarrin et al., 1999). Note is made of the variation between differing CMV promoters themselves. The wildtype Ad-LacZ contains a short HCMV promoter. Comparison between this and an Ad-LacZ with an HCMV (long) promoter with the intron A included (Sridhar et al., 2008) as well as the commercially sourced Ad-LacZ with a UL126 HCMV promoter has been made. Within the subtleties of the varying HCMV promoters, small (but not significant) differences are seen. All of these constructs show retention of the overall "inflating" profile. Overall, there is an observed variation of frequency of response dependent on the transgene promoter, but from the relatively clear-cut perspective of the ability to produce memory inflation, the type of

promoter does not matter, and retention of a CMV promoter is not the reason as to why it is possible to show inflation from the Ad-LacZ model. This remains dependent on the AdHu5 vector and LacZ insert.

When LacZ is delivered in a vaccinia virus (VV-LacZ) inflationary responses are not seen (Bolinger et al., 2013). The AdHu5 vector clearly plays a significant role within the model. Some critical features of the Ad-LacZ model that require future investigation are:

1) The narrow dose range noted at administration to allow for inflationary responses (an issue previously highlighted with regards the magnitude of the inflationary response in the MCMV model as well (Redeker et al., 2014),
2) The marked variations in the magnitude of response seen via alternative routes of immunisation (possibly linked to point 1), and
3) The sustenance of an inflating response despite of the AdHu5 being a non-replicating vector.

The narrow dose range has been previously highlighted for the Ad-LacZ model (Krebs et al., 2005), and in this work it has also been noted. When the Ad-LacZ, and to some extent the minigene vectors, are administered intravenously at anything greater than a log either side of the optimum dose ($2x10^9$ iu for Ad-lacZ), this impacts upon the inflationary responses, with ultimate loss of the classic inflationary responses. At the higher doses this

could presumably be the consequence of tolerisation, but this has not been explored further in this work.

The later point is of particular interest, given the body of work that would support the maintenance of inflation being associated with viral antigen and persistent exposure (in the context of MCMV and latent infection). It may well be that adenoviral vectors do persist *in vivo* at some level (Tatsis et al., 2007), and certainly within the Ad-LacZ model it has been previously shown that viral DNA can be tracked out to the very later time-points post immunisation (Bolinger et al., 2013). It may alternatively and/or additionally be that some other mechanism is responsible for the sustenance of inflating populations, as alluded to in the introduction with regards $CD8^+$ T cell stemness and also Trm populations. These remain key areas of future research in the field.

Finally, AdHu5 clearly translates poorly into future potential therapies or vaccines, given the body of evidence associated with adverse affects and pre-existing antibodies in the vast majority of people. Vaccine platforms are now consistently using non-human adenoviruses to avert these issues. It will be key to test the Ad-LacZ model on a background of alternative adenovirus vectors, and this will form part of planned future work. It may be that constructs that utilise a B species vector (and in turn different receptor recognition) perform differently, which would add to understanding around target cells. For translational purposes, Chimpanzee adenoviral vectors will also be tested, given their current wide spread use within the field.

7.2 The impact of antigen processing on CD8$^+$ T cell memory inflation

7.2.1 Minigenes

The adenoviral model has proved an efficient approach to try and address why it is that some epitopes lead to inflationary responses and not others. Using this model, it has been possible to demonstrate a key role for antigen processing and the presentation of "dominant" epitopes to CD8$^+$ T cells, rather than this being an inherent property of the epitope itself. Through removal of the surrounding β-galactosidase sequence, it is possible to clearly and reproducibly show inflation from a minigene vector containing "non-inflating" epitopes. In doing so, the processing step has been removed. This has been shown to be consistent amongst dominant "non-inflating" responses, having demonstrated this for both Ad-I8V (Ad-LacZ model) and Ad-M45 (MCMV model). As to the distribution, functionality and the phenotype of these responses induced from the minigene constructs, this mirrors what is seen in the inflating populations from both the Ad-LacZ and the MCMV models. In terms of potential translation for therapeutic or prophylactic vaccines based on adenoviral vectors, it will be important to see whether this principal can be extended to epitopes found in other natural pathogens or carcinomas, and this is the focus of future work.

To address more specifically whether the surrounding β-gal sequence is critical to why an I8V-specific response is non-inflationary, flanking native β-gal sequence was returned to the I8V epitope. Very clear N-terminal trimming on constructs of I8V extended out to up to 10 amino acids on the N-terminus

was observed. These constructs act much the same as the Ad-I8V construct and still show induction of robust inflationary responses. Short C-terminal extensions did not demonstrate any I8V tetramer-specific responses in these constructs (Ad-10aa-I8V-10aa and Ad-I8V-10aa). It is possible that this is due to aberrant expression and/or processing and it is hypothesised that longer C-terminal extensions, or even a full length LacZ insert, might be required for natural processing of this epitope.

Previous work in this field has demonstrated a critical role for both processing and presentation on non-professional APCs in the development of inflating $CD8^+$ T cells (Smith et al., 2014; Torti et al., 2011a). It is know from work in the $LMP7^{-/-}$ mouse model that inflating populations show relative independence from the immunoproteasome (Bolinger et al., 2013; Hutchinson et al., 2011). This likely supports this hypothesis, but it is also possible that pAPCs are involved, but there is a favouring of processing through the constitutive proteasome. There is additionally some description of destructive cleavage of antigenic peptides (by immunoproteasomes or proteasomes) resulting in differential antigen presentation (Chapiro et al., 2006), which may also be of relevance to the question as to why certain epitopes inflate and not others. Overall, the questions around APCs and processing are critical to understanding into the mechanisms of memory inflation. Future work will look at the APCs involved in more detail, using a number of Cre-mouse models.

7.2.2 Co-inflation, boosting and protection

An alternative hypothesis as to why some epitopes inflate and not others could be through competition. This work has been unable to properly address this question. However, through simple experiments involving co-administration of the Ad-I8V and Ad-D8V minigenes into the same host, it has been possible to show that responses induced by the individual vectors are comparable to those seen in a singly immunised mouse. This observation was confirmed using both intravenous and intramuscular administration. From these experiments, where there is presumably sufficient antigen (and sufficient APCs) for each epitope, it is concluded that there is no competitive process between the two constructs and that both responses can be accommodated in a single host. This is consistent with previously published data indicating later inflation of epitopes that are initially "subdominant", including recombinant epitopes (Karrer et al., 2003). This work is not sufficient to draw any significant conclusions on the role of competition. However, what it does demonstrate is the capacity for co-inflation within the host. Work with co-administration of the 3 minigene vectors (Ad-I8V, Ad-D8V and Ad-M45) combined in one host is ongoing, but at later time-points (day 50) after immunisation it is apparent that there is some limit to the level of co-inflation achieved within one host (with the reduction in the Ad-M45 specific responses).

Finally, comment on the functionality and likely protective capacity of the $CD8^+$ T cells produced from Ad-LacZ and the minigene vectors. There are

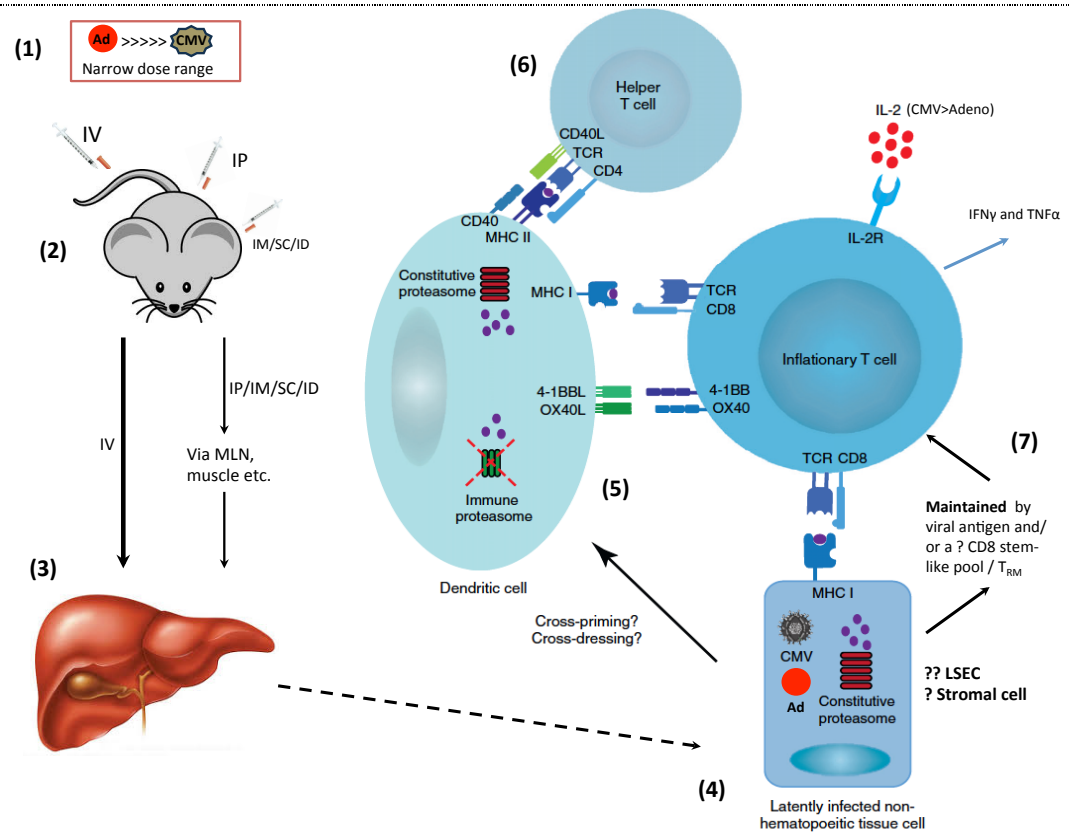
notable differences in the frequency of tetramer-positive cells induced from Ad-D8V and Ad-I8V, along with their IFNγ production *ex vivo*. The magnitude of response is seen to be much higher in the Ad-I8V construct compared to Ad-D8V. Ad-M45 holds a similar pattern of inflation and IFNγ production to that of Ad-I8V. In this work, functionality was assessed by ICS, where total $CD8^+$ T cells were stimulated with the relevant peptide in all cases. A more accurate experiment would have been to stain the $CD8^+$ T cells going into the ICS for the relevant tetramer first. That way the number of tetramer-positive cells could be correlated to the production of IFNγ and provide a clearer picture of functionality of those cells and therefore a better comparison.

It has not been possible to show whether Ad-LacZ or the minigene constructs confer any protection, this being critical to future translational applications of this work. Using a VV-LacZ virus, it was possible to boost D8V and I8V specific responses in previously Ad-LacZ immunised mice, but it was not possible to show any difference in vaccinia virus control. The system did not work well to address the question. It is critical not to infer protection based upon the high frequency of responses seen from the minigene constructs (Jeyanathan et al., 2013). Future work will focus on the ability of minigene constructs to protect using an LCMV model, as described reading forward.

Key findings:

1) Ad-LacZ is a good model of memory inflation, regardless of the transgene promoter,
2) Inflationary responses can be elicited following alternative routes (i.m. and i.p.) of immunisation, albeit at a reduced frequency,
3) Ad-D8V induces inflationary $CD8^+$ T cell responses,
4) Ad-I8V induces inflationary $CD8^+$ T cell responses, suggesting that the polypeptide context of a $CD8^+$ T cell epitope may determine whether classical or inflating memory responses are induced,
5) N-terminal extended Ad-I8V minigene constructs show N-terminal trimming,
6) No tetramer-specific responses can be induced from short C-terminal extended Ad-I8V minigene constructs,
7) Co-inflation of both I8V and D8V tetramer-specific $CD8^+$ T cell responses occurs when both minigenes (Ad-I8V and Ad-D8V) are administered concomitantly within the same host,
8) A further minigene vector, Ad-M45, induces inflationary $CD8^+$ T cell responses.

Figure 7.1: Summative model of the mechanisms of memory inflation.



(1) There is a narrow dose range for administration of Ad-LacZ and production of inflationary responses. This remains the same for the minigenes. **(2)** Immunisation via the i.v. route results in significantly greater populations of inflationary responses compared to other routes. **(3)** Delivery directly to the liver and/or lung may be a key feature. **(4)** Inflationary responses are independent of the immunoproteasome and this may suggest the involvement of a non-professional APC. **(5)** It may also be that pAPCs are involved, but inflationary responses favour a constitutive proteasome. Minigene constructs likely bypass the proteasome all together. **(6)** CD4-help likely remains critical (this is the subject of ongoing work with in the Klenerman laboratory). **(7)** How inflationary populations are maintained is unknown. Viral antigen is clearly present in the MCMV model, but this remains less clear for the Ad-LacZ model.

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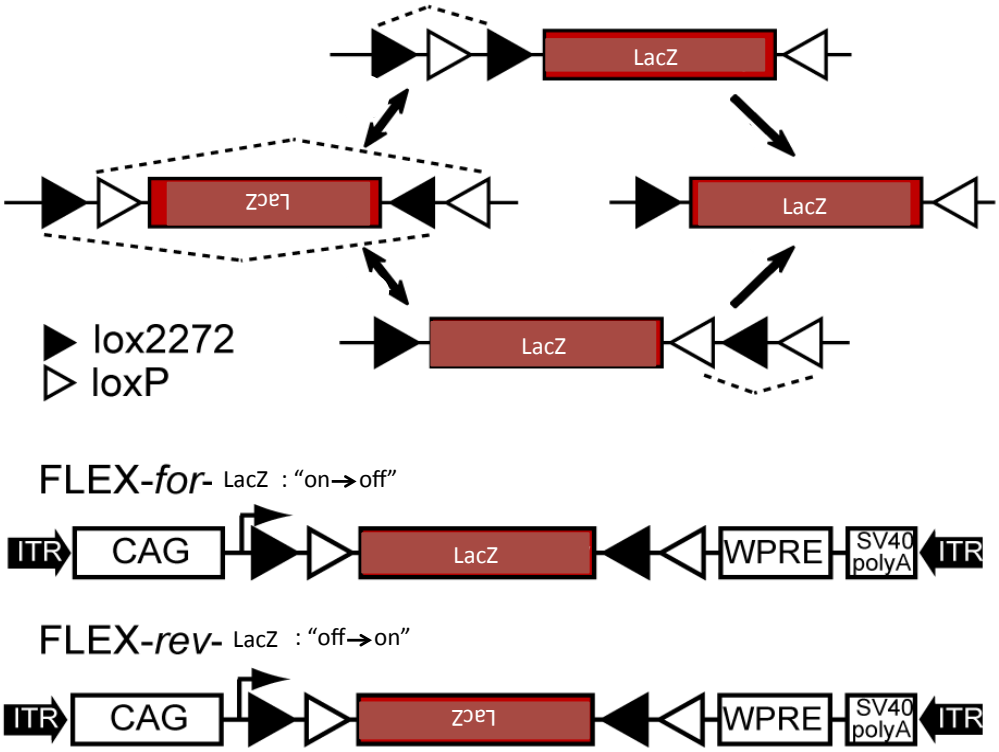
7.3 Future Work

Which antigen presenting cells (APCs) prime inflating $CD8^+$ T cells?

A set of Ad-LacZ vectors, which have been designed with a FLEX ON and OFF insert that can be used in Cre-mouse models (figure 7.2) has been developed. With these tools, it will be possible to turn on and off expression of LacZ at key stages post immunisation, in key knock out models (i.e. control the vaccination process in time and space). This unique set up will enable the definition of the role of specific cell types, with a view to identifying the APCs involved in priming and maintaining these robust immune responses. This is critical to further understanding the processing environment.

The Cre mouse models available for use in this work are: Ccl19-cre (targeting lymph node fibroblastic reticular cells), VE-cadherin-cre (for lineage analysis and gene deletion in endothelial cells), CD11c-cre (for dendritic cell homeostasis and function), LysM-cre (for generating myeloid cell-specific targeted mutants) and a CMV-cre (as in control). The FLEX ON/OFF constructs will be immunised into these mice and the $CD8^+$ T cell memory responses induced in blood and tissues analysed using *ex vivo* tetramer staining. Functional analyses will be performed using FACs based analysis of cytokine release (IFNγ/TNFα) following peptide stimulation.

Figure 7.2: Representation of the FLEX ON/OFF system.



In the FLEX/ON system the transgene of interest (LacZ) is inserted in reverse orientation relative to the 5' promoter and is flanked by oppositely oriented loxP and lox2272 sites. In the absence of Cre expression, the transgene will not be produced. In the presence of Cre expression, the transgene will be "Flip-exchanged" or FLEXed, leading to the expression of the transgene. This is due to a permanent Cre-mediated recombination/inversion of the flanked transgene. The FLEX/OFF system is the same except with the gene of interest cloned in the right orientation. **This is illustrated here, adapted from the original article in which this technology was used (Atasoy et al., 2008).**

What is special about adenoviruses? Do they interact more readily with certain cell types?

The adenovirus backbone is key to the Ad-LacZ model. It is known that inflation is not possible through a VV-LacZ virus (Bolinger et al., 2013). This suggests an important role as to the delivery of the transgene by the virus. A number of Ad-LacZ vectors have been developed in conjunction with Okairos (a biotech company involved in the development of a number of alternative adenovirus vector platforms), utilising differing adenoviral serotypes. These are as highlighted in the table below;

Table 7.1: Summary of Ad-LacZ vectors to be tested according to serotype.

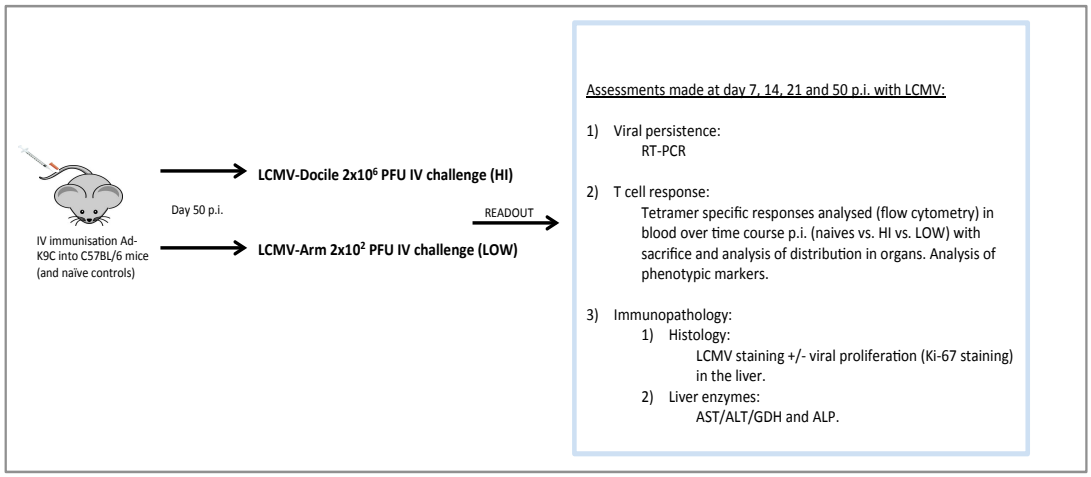
	Species	Serotype	Primary attachment molecule	Human/ Chimp
Wildtype – AdLacZ	C	5	CAR	Hu
AdLacZ	C	3	CAR	Ch
AdLacZ	B	35 (B:2)	CD46/CD80/CD86	Hu
AdLacZ	E	63	CAR	Ch

This will allow comparison between the different classes of adenovirus: human versus chimpanzee and those with differing primary attachment molecules. This is also of relevance given the current literature around the complications of AdHu5 vectors in human vaccine trials, with pre-existing immunity being associated with reduced immunogenicity (Buchbinder et al., 2008; Geisbert et al., 2011; Gray et al., 2011; Kobinger et al., 2006; McElrath et al., 2008; Sumida et al., 2005). If there were a translational approach to be taken with minigene adjuncts in vaccines, it would be important to know that the same effect can be induced from the non-AdHu5 platforms.

Can minigene induced inflationary CD8$^+$ T cells afford protection?

It has been difficult to assess protection from the minigene constructs with the current tools available. This has led to the development of an AdHu5 vector with a lymphocytic choriomeningitis virus (LCMV) Gp_{33-41} insert (minigene – Ad-K9C) as well as a full Gp protein construct (Ad-Gp). This will enable future studies to take advantage of the well-established LCMV model. Using this natural mouse challenge it is intended to show clear protection outcomes with these minigene constructs (summarised in figure 7.3 below). This is of vital importance to the translation of this work. It is felt that the LCMV model will provide a widely recognised and biologically relevant readout to answer this question.

Figure 7.3: Schematic of the LCMV challenge/protection studies.



7.4 Potential benefits and application of the current findings and future work

Basic immunobiology

Ultimately, this work retains a heavy basic science component. A mechanistic description as to the development and maintenance of inflating $CD8^+$ T cells remains important to the field. It seems possible that understanding the Ad-LacZ model may provide translational opportunities, but in the short term, it is of critical importance just to understand the immunobiology involved. This work has shown that modification of antigen context and associated processing requirements does allow for modulation in the nature of memory induced by adenovirus-vectored vaccines. A simple "minimalist" approach in the vaccine construct reproducibly allows for memory inflation in response to previously "non-inflationary" epitopes and this inflation is not dependent on the presence of a specific promoter.

Understanding into CMV infection

Understanding into the APCs involved in priming an inflationary response through the adenoviral model will also aid the field of CMV research. CMV is a very real problem in immunosuppressed cohorts, especially in transplant recipients. The contribution of memory inflation towards immune senescence is also of increasing importance. It is hard to dissect all of the immunology *in vivo* using CMVs and this adenoviral model, by inducing a highly related T cell response, provides an important opportunity to define this further.

Understanding into adenoviral vaccines

What is special about the adenoviral model? As mentioned, it is known that a VV-LacZ virus is not able to produce memory inflation, despite the same transgene. The adenovirus provides a critical platform in the form of APC interaction, transgene delivery and maintenance. Given the increased use of adenoviruses as vaccine platforms (Antrobus et al., 2014; Churchyard et al., 2015; Ewer et al., 2013; Ledgerwood et al., 2014; Omosa-Manyonyi et al., 2015; Rampling et al., 2015; Swadling et al., 2014) this work is of increased relevance. Understanding into which APCs are involved, and the cellular context of antigen presentation should provide unique insight into how it is that adenovirus vaccine strategies show such good priming in man.

Can inflationary epitopes be used in vaccines / immunotherapies?

There is an important translational element to this work. Recent data from human studies do suggest that long-term effector memory $CD8^+$ T cell pools can be induced and sustained following vaccination with adenoviral vectors (Barnes et al., 2012; Swadling et al., 2014). Further, data from CMV-vectored vaccines suggests that such tissue-homing effector memory responses can provide robust protection (Hansen et al., 2011; Karrer et al., 2003). In this context these findings prove that a simple modification of the context of an epitope can allow for a dramatic switch in memory phenotype, and that such responses can be elicited in parallel. Thus this approach holds significant potential for utilisation as a tool in vaccine development.

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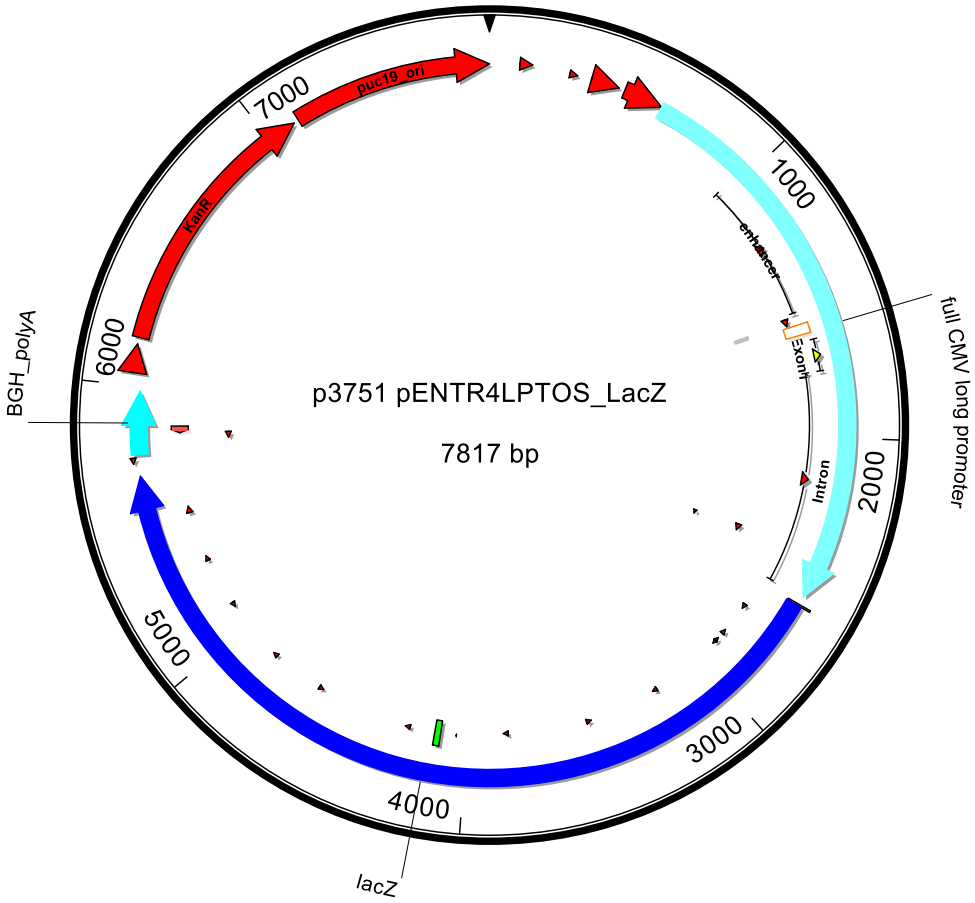
Appendices

Appendix 1

Ad-LacZ (Jenner) vector diagram



LacZ amino acid sequence for Wildtype, Jenner and Kerafast constructs

Wildtype (1,042)

MASWGSRSYPYDVPDYAGTGSITNSLAVVLQRRDWENPGVTQLNRLAAHPPFASWRNSEEAR
TDRPSQQLRSLNGEWRFAWFPAPEAVPESWLECDLPEADTVVVPSNWQMHGYDAPIYTNVTY
PITVNPPFVPTENPTGCYSLTFNVDESWLQEGQTRIIFDGVNSAFHLWCNGRWVGYGQDSRL
PSEFDLSAFLRAGENRLAVMVLRWSDGSYLEDQDMWRMSGIFRDVSLLHKPTTQISDFHVAT
RFNDDFSRAVLEAEVQMCGELRDYLRVTVSLWQGETQVASGTAPFGGEIIDERGGYADRVTL
RLNVENPKLWSAEIPNLYRAVVELHTADGTLIEAEACDVGFREVRIENGLLLLNGKPLLIRG
VNRHEHHPLHGQVMDEQTMVQDILLMKQNNFNAVRCSHYPNHPLWYTLCDRYGLYVVDEANI
ETHGMVPMNRLTDDPRWLPAMSERVTRMVQRDRNHPSVIIWSLGNESGHGANHDALYRWIKS
VDPSRPVQYEGGGADTTATDIICPMYARVDEDQPFPAVPKWSIKKWLSLPGETRPLILCEYA
HAMGNSLGGFAKYWQAFRQYPRLQGGFVWDWVDQSLIKYDENGNPWSAYGGDFGDTPNDRQF
CMNGLVFADRTPHPALTEAKHQQQFFQFRLSGQTIEVTSEYLFRHSDNELLHWMVALDGKPL
ASGEVPLDVAPQGKQLIELPELPQPESAGQLWLTVRVVQPNATAWSEAGHISAWQQWRLAEN
LSVTLPAASHAIPHLTTSEMDFCIELGNKRWQFNRQSGFLSQMWIGDKKQLLTPLRDQFTRA
PLDNDIGVSEATRIDPNAWVERWKAAGHYQAEAALLQCTADTLADAVLITTAHAWQHQGKTL
FISRKTYRIDGSGQMAITVDVEVASDTPHPARIGLNCQLAQVAERVNWLGLGPQENYPDRLT
AACFDRWDLPLSDMYTPYVFPSENGLRCGTRELNYGPHQWRGDFQFNISRYSQQQLMETSHR
HLLHAEEGTWLNIDGFHMGIGGDDSWSPSVSAEFQLSAGRYHYQLVWCQK

Jenner (1,022)

MNSSRDPVVLQRRDWENPGVTQLNRLAAHPPFASWRNSEEARTDRPSQQLRSLNGEWRFAWF
PAPEAVPESWLECDLPEADTVVVPSNWQMHGYDAPIYTNVTYPITVNPPFVPTENPTGCYSL
TFNVDESWLQEGQTRIIFDGVNSAFHLWCNGRWVGYGQDSRLPSEFDLSAFLRAGENRLAVM
VLRWSDGSYLEDQDMWRMSGIFRDVSLLHKPTTQISDFHVATRFNDDFSRAVLEAEVQMCGE
LRDYLRVTVSLWQGETQVASGTAPFGGEIIDERGGYADRVTLRLNVENPKLWSAEIPNLYRA
VVELHTADGTLIEAEACDVGFREVRIENGLLLLNGKPLLIRGVNRHEHHPLHGQVMDEQTMV
QDILLMKQNNFNAVRCSHYPNHPLWYTLCDRYGLYVVDEANIETHGMVPMNRLTDDPRWLPA
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Kerafast (1,244)

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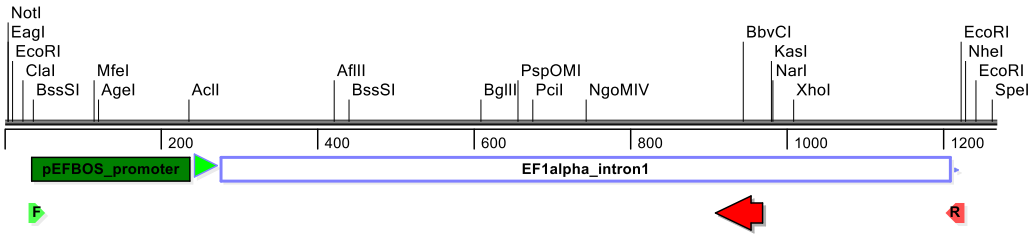
EF1α promoter details



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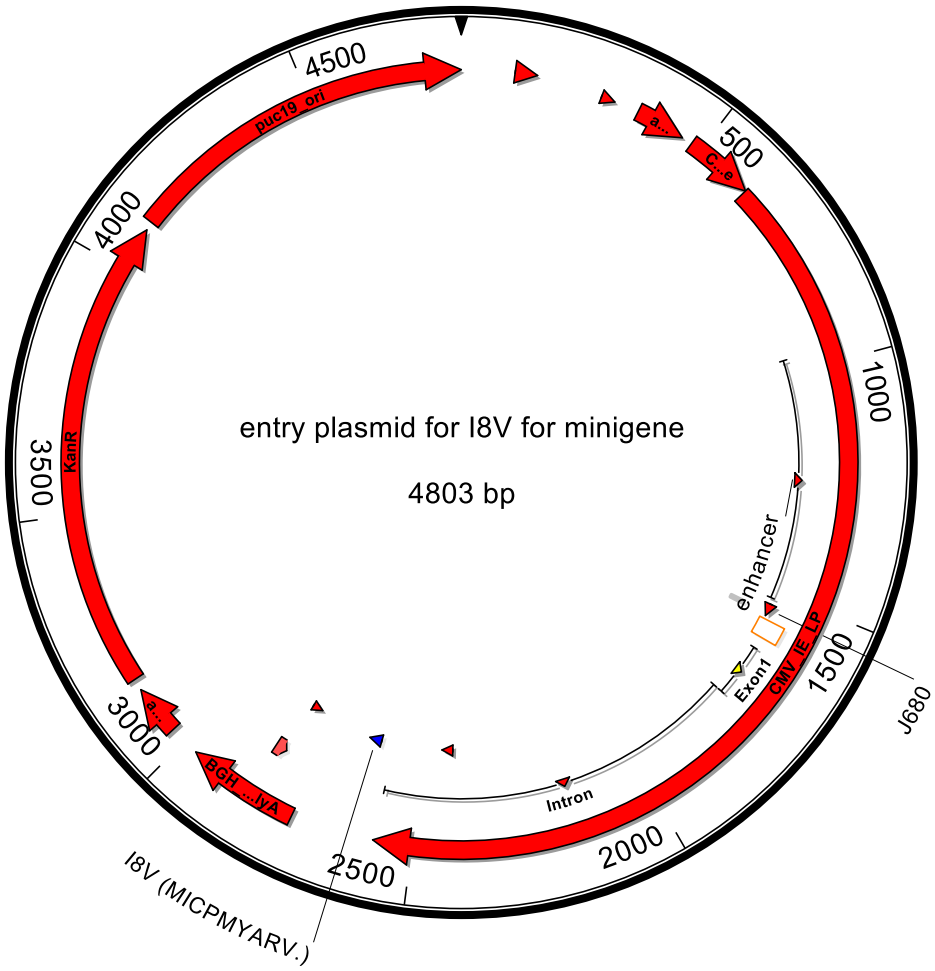
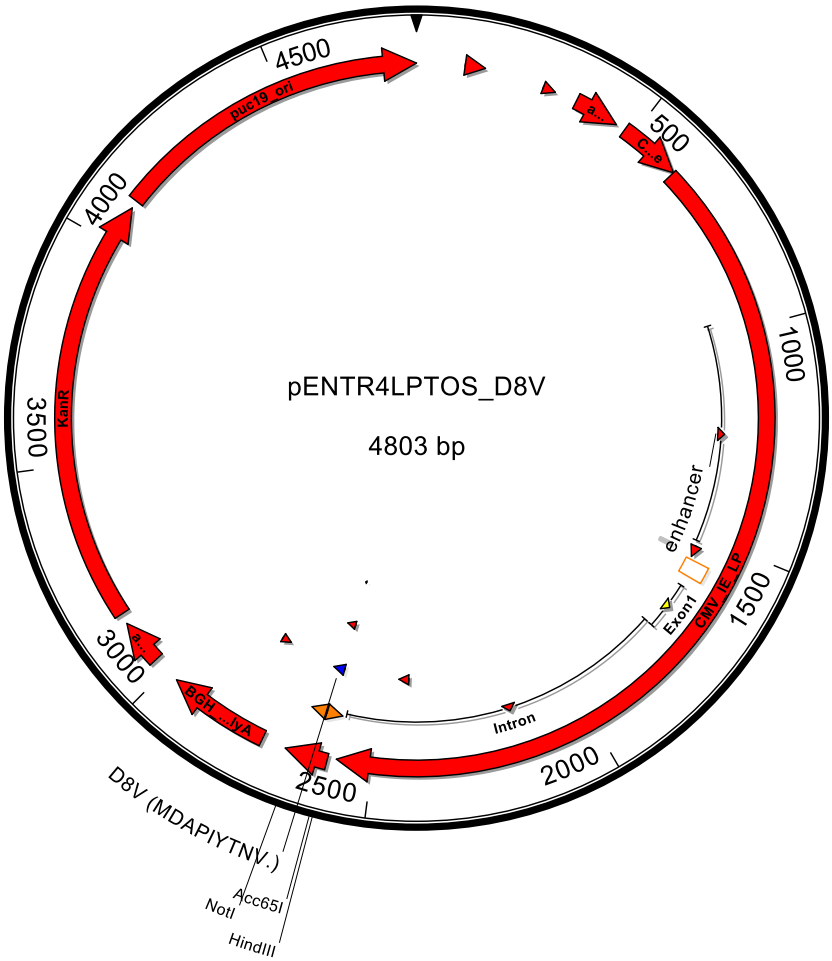
(The purple highlighted area represents the start of promoter and yellow the end.)

The minigenes: Ad-I8V entry vector



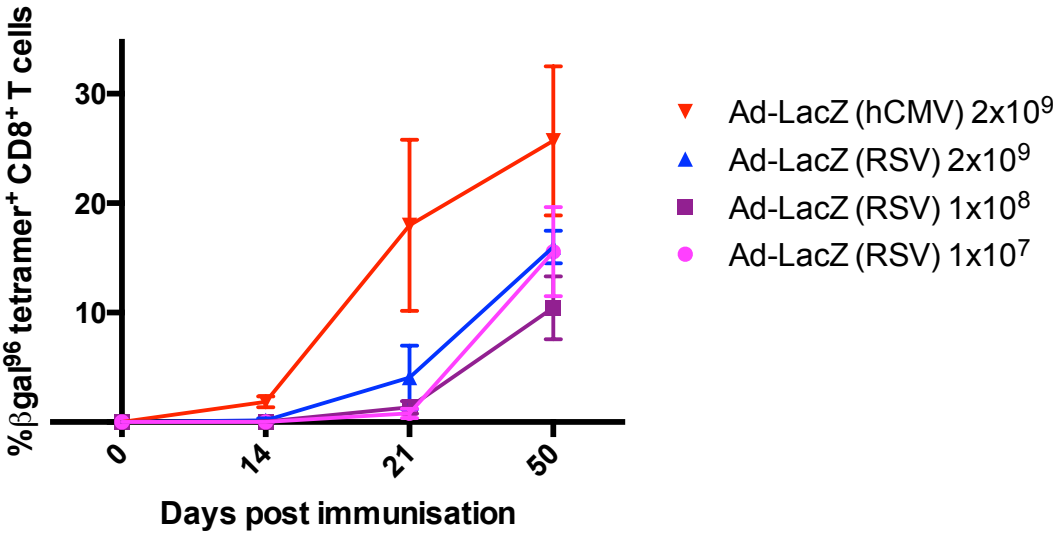
The minigenes: Ad-D8V entry vector



Appendix 2

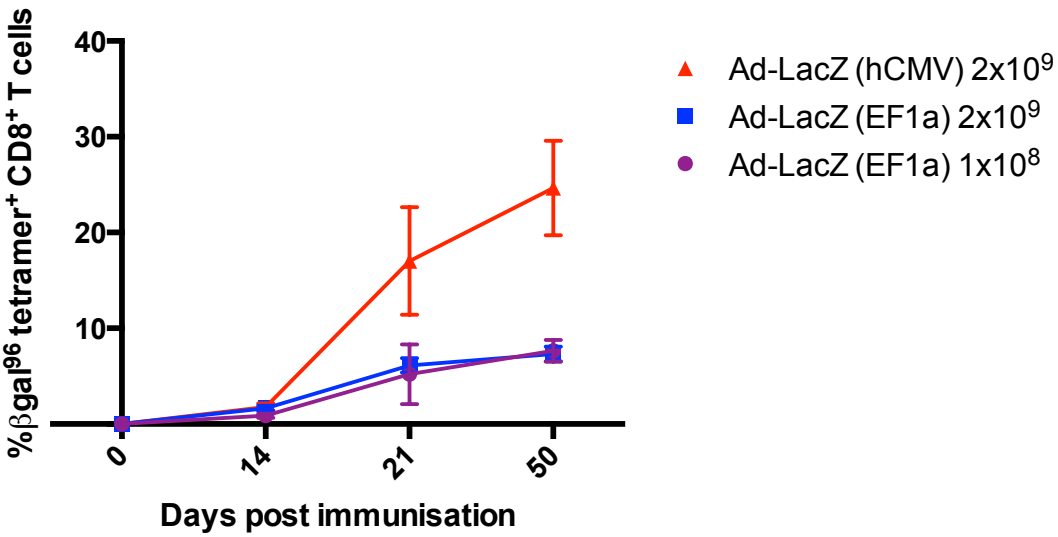
Determination of appropriate iu / immunisation dose within the new constructs

Figure a2.1: Ad-LacZ (RSV) titrated in B6 mice.



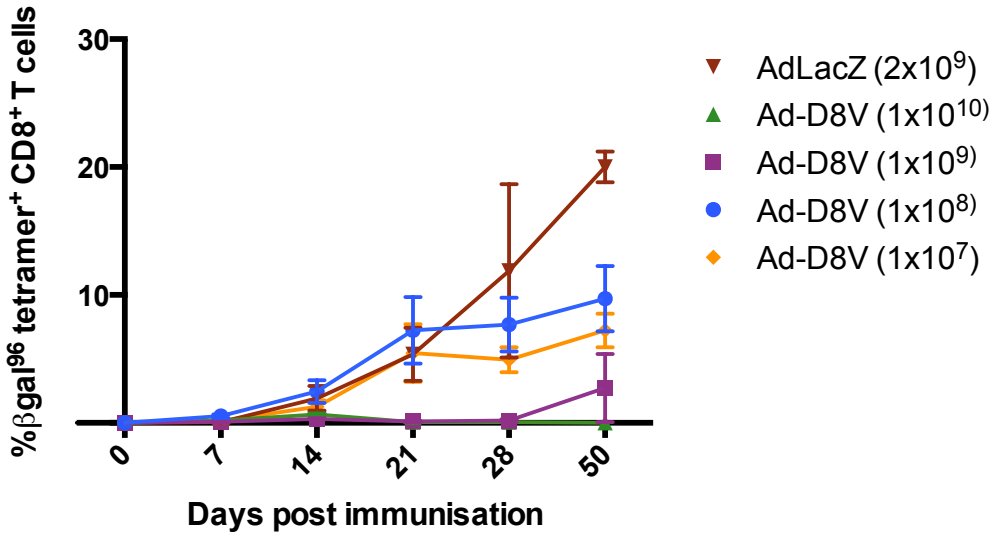
This shows D8V tetramer-specific results in Ad-LacZ (RSV) compared to Ad-LacZ (HCMV). B6 mice (n/group=3) were used to test each concentration, alongside naïve controls (data for naives excluded, no tetramer-specific responses seen). Results show tetramer-specific responses in blood at day 14, 21 and 50-post immunisation. Error bars indicate the SEM.

Figure a2.2: Ad-LacZ (EF1α) titrated in B6 mice.



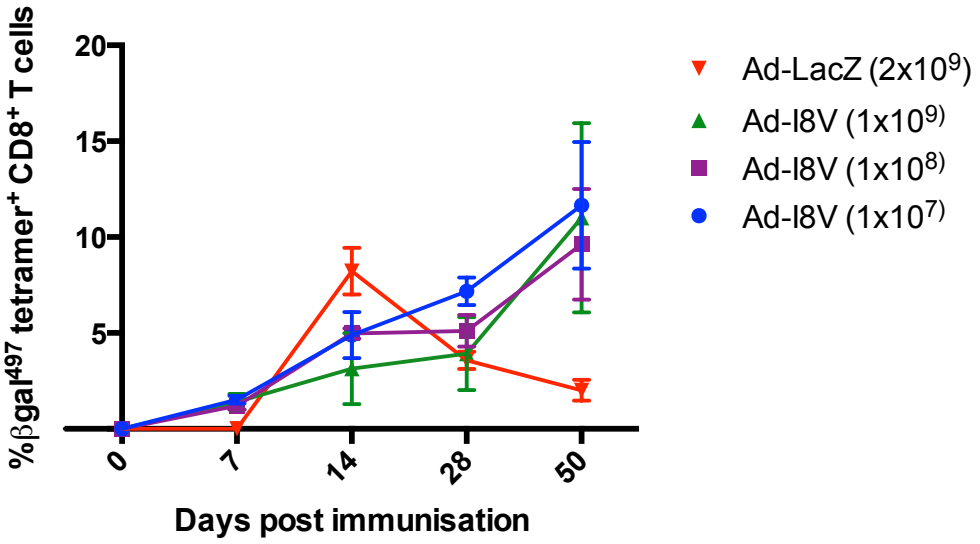
This shows D8V tetramer-specific results in Ad-LacZ (EF1α) compared to Ad-LacZ (HCMV). B6 mice (n/group=4) were used to test each concentration, alongside naïve controls (data for naives excluded, no tetramer-specific responses seen). Results show tetramer-specific responses in blood at day 14, 21 and 50-post immunisation. Error bars indicate the SEM.

Figure a2.3: Ad-D8V titrated in B6 mice.



Ad-LacZ has been previously shown to produce inflationary responses within a fairly narrow dose range. It was important to optimise the dosage for the minigenes *in vivo*. This shows Ad-D8V results. B6 mice (n/group=3) were used to test each concentration, alongside Ad-LacZ as a control and naives (data for naives excluded, no tetramer-specific responses seen). Results show tetramer-specific responses in blood at day 7, 14, 21, 28 and 50-post immunisation. Error bars indicate the SEM.

Figure a2.4: Ad-I8V titrated in B6 mice.

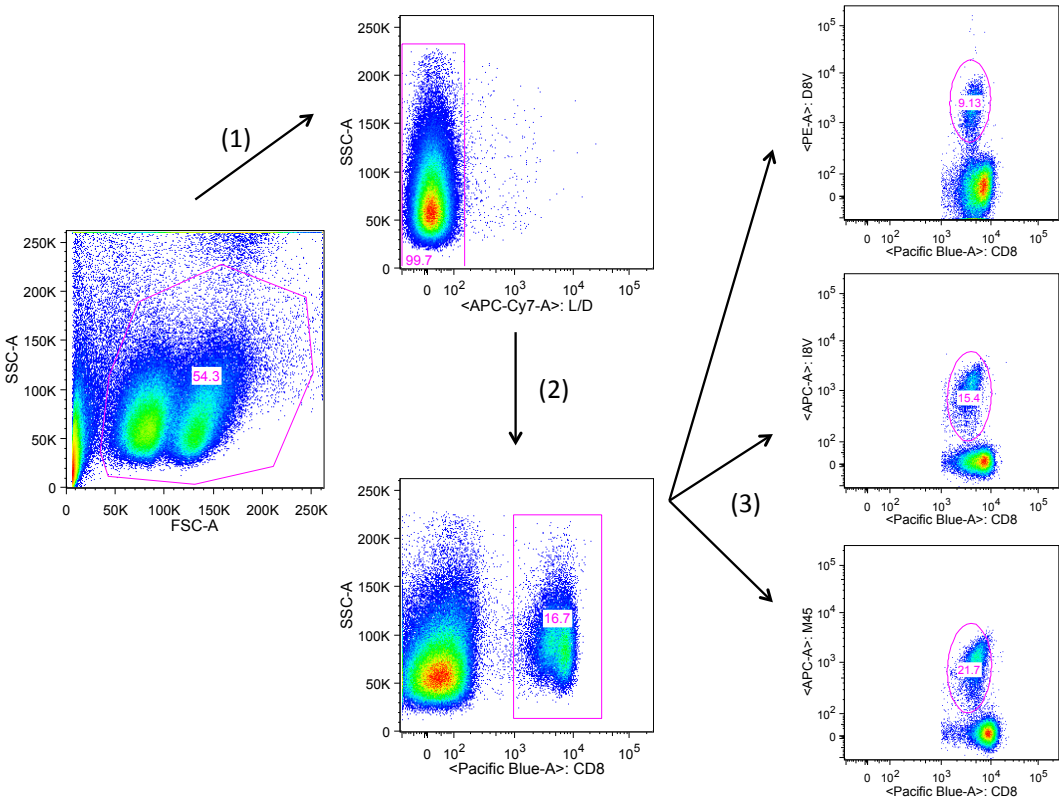


This shows Ad-I8V results. B6 mice (n/group=3) were used to test each concentration, alongside Ad-LacZ as a control and naives (data for naives excluded, no tetramer-specific responses seen). Results show tetramer-specific responses in blood at day 7, 14, 28 and 50-post immunisation. Error bars indicate the SEM.

Appendix 3

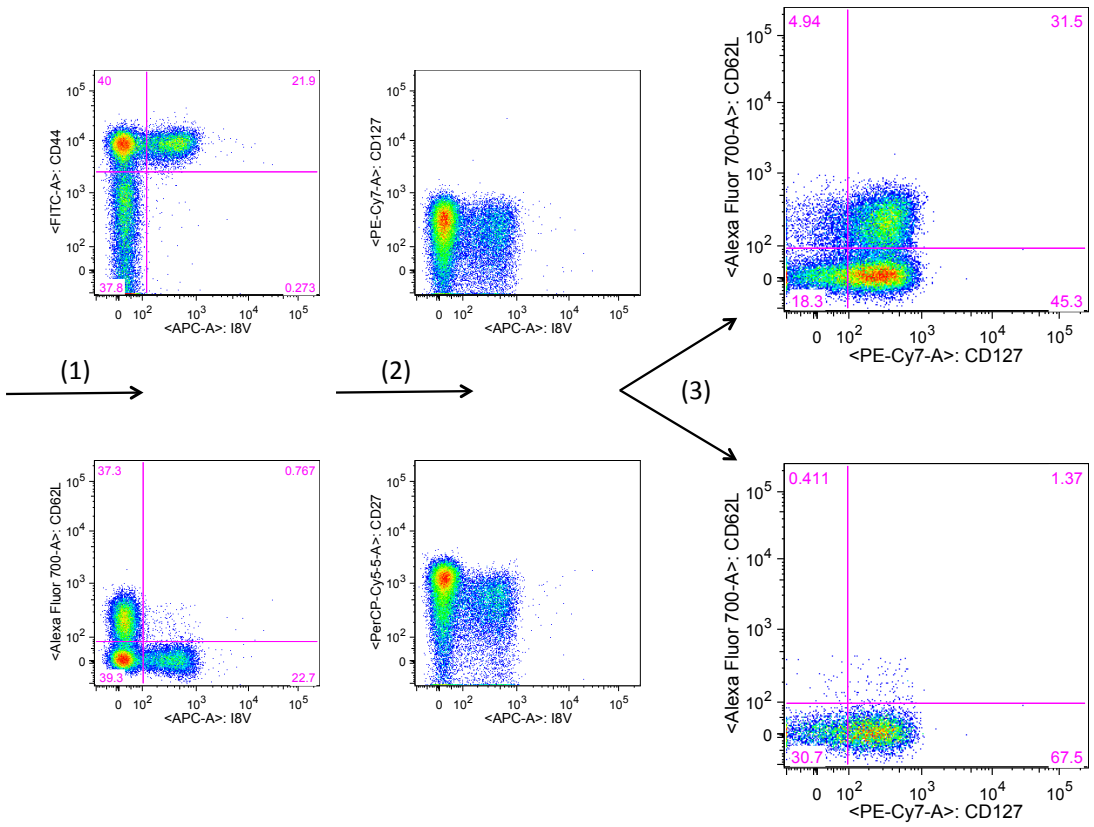
Gating strategies

Figure a2.5: Gating strategy – tetramer specific populations



All flow cytometry was performed on the BD LSR II machine. Events were initially collected for lymphocytes on FSC/SSC. **(1)** A live dead marker was then used to gate onto the live lymphocytes. **(2)** A CD8a-PB (eFluro450) stain was then used to gate on total $CD8^+$ T cells. Events recorded on each experiment were collected on the $CD8^+$ T cells gate (30,000 events). **(3)** Gates could then be applied to count the individual tetramer-specific populations, with I8V-APC, D8V-PE and M45-APC shown. Not shown are M38-PE and E8V-brilliant violet (in the E8V experiments CD8a-PerCP-Cy5.5 was used and the CD27 stain was excluded).

Figure a2.6: Gating strategy – antibody panel



Starting from total CD8$^+$ T cells, individual antibody stains could then be assessed on whole CD8$^+$ T cells and / or tetramer-specific populations of CD8$^+$ T cells. **(1)** Total CD8$^+$ T cells are shown with the antibody stain on the y-axis and the applicable tetramer stain (I8V in this case) on the x-axis. CD44 and CD62L were analysed as the percentage of total CD8$^+$ T cells / total tetramer-specific CD8$^+$ T cells that were CD44 or CD62L positive. **(2)** CD27 and CD127 were analysed according to the geometric mean of total CD8$^+$ T cells. **(3)** On a plot of total CD8$^+$ T cells (top right) or tetramer-specific CD8$^+$ T cells (bottom right – I8V in this case) a comparison of CD127 vs. CD62L could be made and the phenotype of these CD8$^+$ T cells inferred from that stain, as described in the introduction (Bachmann et al., 2005).