T CELL BIOLOGY OF RECOMBINANT ADENOVIRUS VACCINES

RECOMBINANT ADENOVIRUS VACCINES, A COMPREHENSIVE INVESTIGATION OF T CELL IMMUNITY

BY

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2009) McMaster University

Medical Sciences Hamilton, Ontario

Title: Recombinant Adenovirus Vaccines, a Comprehensive Investigation of T Cell Immunity

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Number of pages: ix, 225

—ABSTRACT—

Vaccination is arguably the most effective tool at our disposal to prevent the morbidity and mortality associated with infectious disease. However, there are currently several infectious diseases, notably HIV, malaria and tuberculosis, for which we do not posses effective vaccines. Further complicating matters, traditional methods to construct vaccines for these diseases have been unsuccessful. Advances in our understanding of adaptive immunity have demonstrated that vaccines for these diseases likely rely upon potent T cell immunity to be effective. Recombinant adenovirus (rAd) vectors have shown great promise as vaccination platforms since they are easily constructed, stable, well-tolerated and elicit robust T cell responses. The robust activity of rAd vectors based on the human serotype 5 virus (rHuAd5) in murine and simian models merits futher investigation as a prototypic T cell vaccine. To this end, we have undertaken a comprehensive evaluation of T cell immunity following rAd vaccination. Our previous observations determined that the CD8+ T cell response produced by rHuAd5 vaccines displayed a prolonged effector phase that was associated with long-lived antigen presentation. We have further investigated the mechanisms underlying the maintenance of this memory population. Our results have revealed that the memory phenotype is not due to continual recruitment of naïve CD8+ T cells. Rather, the sustained effector phenotype appears to depend upon prolonged expression of the antigen-encoding transgene from the rHuAd5 vector. Interestingly, transgene expression was only required for 60 days after which point the memory population stabilized. Further investigation of the relationship between antigen structure and the CD8+ T cell response revealed that antigens which traffic through the ER produce a CD8+ T cell response that expands more rapidly and displays a more pronounced contraction phase than antigens which are produced within the cytosol. While the exact mechanism underlying this phenomenon is not known, we suspect that pathways related to ER stress may be involved. Despite the more dramatic contraction phase associated with antigens that traffic through the ER, the memory phenotype was unchanged. Interestingly, the CD4+ T cell response was not influenced by antigen structure and displays a sharp contraction phase regardless of

whether the antigen traffics through the ER or is produced in the cytosol. We further investigated the relationship between CD4+ T cell help and CD8+ T cell immunity produced by rHuAd5. Based on the partially-exhausted phenotype of the CD8+ T cells produced by rHuAd5 (diminished TNF-α production and little IL-2 production), we suspected that inadequate CD4+ T cell help may have been responsible. However, removal of CD4+ T cells did not further impair the CD8+ T cell response produced by rHuAd5. Rather, a lack of CD4+ T cell help only impacted the magnitude of the primary CD8+ T cell response generated by rHuAd5; the functionality of the CD8+ T cell population, including the ability to proliferate following secondary stimulation, were not affected by the absence of CD4+ T cells. Thus, although CD8+ T cell expansion following immunization with rHuAd5 is dependent upon the availability of CD4+ T cell help, the memory functions of the CD8+ T cell population appears to be independent of CD4+ T cell help. Finally, we compared the magnitude of the CD8+ T cell response produced by rHuAd5 and recombinant vaccinia virus. Our results demonstrated that the functionality of the early T cell response produced by both vectors were identical. However, the primary transgene-specific CD8+ T cell responses produced by rHuAd5 were significantly larger than rVV because the vector specific responses were negligible in the case of rAd but very strong following rVV inoculation. This research has contributed to our understanding of T cell immunity following rAd immunization and will assist in the construction and implementation of future vaccines.

—ACKNOWLEDGEMENTS—

First and foremost, I would like to thank my wife, Jaime, who was definitely unaware of the road ahead of her when we started my graduate school journey. You were my counsellor during the tough times, my cheerleader during the good times and my rock throughout the entire process. Just saying "thank you" doesn't seem like enough to show my gratitude for all of your support and love. You are my best friend and my soul mate and I could not imagine my life without you.

To my mentor and supervisor, Dr. Jonathan Bramson: I would like to thank you for taking a chance on an underachieving undergraduate that you saw some promise in. I truly admire your passion for science and your ability to assimilate knowledge, identify key issues and devise an experimental approach to tackle them. Once again, I would like to thank-you for your support, guidance and giving me a kick in the butt when I needed it, but most of all for your continued belief in my abilities.

I would like to thank the members of my supervisory committee, Dr. Martin Stämpfli, and Dr. Zhou Xing, for their insight and important contributions to my scientific development.

Thank you to Carole Evelegh and Chuyan Ying for providing many of the scientific reagents and tools that I employed in my research projects.

To my first instructor in the lab, Kelley Putzu: Thanks for teaching me the ropes and for all of the laughs.

I am thankful for my friends and colleagues, Dr. Tony Yang, Robin Parsons, Dr. Natalie Grinshtein and Jennifer Basset, who were always willing to lend a helping hand with my oversized experiments. Your early morning help in the CAF and the late night companionship at the flow cytometer made it all worthwhile.

To the crew, Dave Smyth, Steve Lee, Tim Ferris, Justin Beaulieu, Pete Channan, Deven Bhatt, and Tony Gaymes, what can I say? Grad school wouldn't have been the same without you.

To my past and present colleagues in the lab, Gord Gaschler, Ramzi Fattouh, Alina Lelic, Jon Finn, Patrick Palidino, Derek Cummings, Jeff Hummel, Greg Harder, Michael Santostusso, Michael Ventresca, Korosh Kianizad, Kristy Jenkins and Leslie Latham: Thanks for the scientific talks and all the memorable times.

My acknowledgements would not be complete without thanking my family for all of their love and support over the years. My parents, Ruth and Bryce, are fantastic role models who have steered me in the right direction and provided me with all the tools that I needed to succeed. It's been a long road. Dad, I can't wait for the hand shake accompanied by "Congratulations, it's about time". You are right. It is about time. To my sister, Miriam, and my brothers, Matthew and Jonathan: Thank-you for your support throughout my graduate studies. I couldn't ask for better siblings.

—List of Abbreviations—

α	Alpha
β	Beta
βgal	Beta galactosidase
γ	Gamma
aa	Amino acid
Ad	Adenovirus
APC	Antigen-presenting cell
BCG	*Bacille Calmette-Guérin*
BCL-2	B-cell lymphoma-2
BCR	B cell receptor
BrdU	Deoxybromouridine
C2D	MHC-II-deficient
CCR	CC chemokine receptor
CD	Cluster of differentiation
CD40L	CD40-ligand
CD62L	CD62-ligand
CFSE	Carboxy-fluorescein diacetate, succinimidyl ester
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DLN	Draining lymph node
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EMP-1	Erythrocyte membrane protein 1
ER	Endoplasmic reticulum
GrB	Granzyme B
H. influenza	*Haemophilia influenza*
HBV	Hepatitis-B virus
HCV	Hepatitis-C virus
hi	High
HIV	Human immunodeficiency virus
HSV	Herpes-simplex virus
HuAd	Human adenovirus
IFN-γ	Interferon-gamma
IL	Interleukin
iTreg	Inducible T regulatory cell
KLRG-1	Killer cell lectin-like receptor G1
LCMV	Lymphocytic choriomeningitis virus
LM	Leishmania major
L. monocytogenes	*Listeria monocytogenes*
LN	Lymph node
Lo	Low

LPS	Lipopolysaccharide
LTNP	Long-term non-progressor
Luc	Luciferase
M. tb.	Mycobacterium tuberculosis
MCMV	Murine cytomegalovirus
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility complex
MHC-I	Major histocompatibility complex class-I
MHC-II	Major histocompatibility complex class-II
MPEC	Memory precursor effector cell
mRNA	Messenger RNA
NFκβ	Nuclear factor kappa beta
nTreg	Natural T regulatory cell
OVA	Ovalbumin
PC	Peritoneal cavity
PD-1	Programmed death 1
PEG	Polyethylene glycol
pfu	Plaque forming unit
pHPMA	Poly-N-2-hydroxy-propyl methacrylamide
R	Receptor
rAd	Recombinant adenovirus
rHuAd5	Human recombinant adenovirus-5
rVV	Recombinant vaccinia virus
RLU	Relative light unit
RNA	Ribonucleic acid
sem	Standard error of the means
SIV	Simian immunodeficiency virus
SLEC	Short lived effector cell
T-bet	T-box expressed in T cells
T_{cm}	Central memory T cell
TCR	T cell receptor
T_{eff}	Effector-memory T cells
T_{em}	Effector T cells
TGFβ	Transforming growth factor beta
Th1	T helper 1 cell
Th2	T helper 2 cell
Th17	T helper 17 cell
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
VLP	Virus-like-particle
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
WT	Wild type

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- Chapter 1 –

Introduction

Importance of vaccines

The development of vaccines is arguably one of the greatest public health achievements. Throughout history, infection has been a leading cause of human mortality. Severe disease outbreaks in the form of epidemics and pandemics have devastated populations and permeated all aspects of our society, influencing social policies, cultural beliefs, and scarring survivors for life. Smallpox was a deadly disease with a mortality rate as high as 30%, and survivors were often scarred with deep pockmarks or rendered blind (World Health Organization http://www.who.int/en/). However, in 1798 Edward Jenner demonstrated that inoculation of material from cowpox scabs prevented smallpox infection; he later termed this process "vaccination". Jenner was unaware of how his vaccine worked, but his cowpox formulation is credited as being the first vaccine (1). As a result of the World Health Organization's smallpox eradication program, the last naturally occurring case of smallpox was reported in 1977 in Somalia (World Health Organization http://www.who.int/en/). In 1979, following a rigorous verification process, smallpox was declared to be eradicated (World Health Organization http://www.who.int/en/). In addition to smallpox, the worldwide effort to eliminate polio has also been extremely successful; in 2008 only four countries remained polio-endemic and there are less than 2000 reported cases per year (World Health Organization http://www.who.int/en/). Health Canada currently recommends routine vaccination against 13 different pathogens which has greatly reduced the incidence of infections such as measles, mumps and *H. influenza* (Public Health Agency of Canada; http://www.phac-aspc.gc.ca). The importance of effective vaccines to public health cannot be overstated,

yet there remain many important pathogens for which no vaccine exists (2-6). Therefore, additional investigation is required to elucidate mechanisms of immune protection and design vaccines to recapitulate these effects.

1.1 Current vaccines

Vaccines are traditionally classified into three categories based on their composition and infectious potential: sub-unit, inactivated, and attenuated (1). Sub-unit vaccines are composed of one or more purified pathogen components such as toxins or structural proteins. In order for sub-unit vaccines to be effective, they must contain the primary immunological target(s) that the immune system attacks to neutralize the pathogen. Sub-unit vaccines have proven to be very effective and extremely well tolerated with few side-effects. However, toxins such as tetanus and diphtheria have to be treated to reduce or eliminate their toxic effects prior to administration (7). Furthermore, to generate and maintain effective levels of immunity, subunit vaccines often require adjuvants and/or multiple booster immunizations (8). In contrast to sub-unit vaccines, inactivated vaccines are composed of entire organisms that have been either chemically- or heat-treated rendering them non-infectious and incapable of replication. Similar to sub-unit vaccines, there are few adverse events associated with inactivated vaccine administration but several booster immunizations maybe required to maintain life-long immunity. The final category, attenuated vaccines are defined as live replicating pathogens; however, their ability to replicate effectively in humans has been greatly reduced through selective processes. The capacity of attenuated vaccines to replicate *in vivo* makes them

significantly more immunogenic compared to subunit and inactivated vaccines. However, high immunogenicity due to replication is a double edge sword, because these properties are associated with more adverse reactions in comparison to the sub-unit and inactivated vaccines (9). One of the most significant concerns with attenuated vaccines is reversion mutants. Following reversion mutations, attenuated organisms regain the functionality and virulence of the original pathogen resulting in a severe infection in vaccine recipients (10-12). In extreme cases, infection with reversions mutants can result in death. In addition to the risk of reversion mutants, attenuated vaccines are unsafe for immunocompromised people such as transplant recipients and HIV-infected individuals because their weakened immune systems are unable to cope with infections by the attenuated pathogen (13). Nevertheless, two of the most successful vaccination programs ever, polio and smallpox employed attenuated vaccines. Therefore, when attenuated vaccines are utilized with the proper precautions and in the appropriate context, they are extremely effective.

2.0 Adaptive Immunity: The basis of vaccination

The human body is a finely tuned organism and can only operate within a limited range of physical tolerances. To ensure survival, numerous homeostatic mechanisms have evolved to compensate and control for our constantly changing environment. Although there are numerous threats to homeostasis, infection is one of the greatest. The homeostatic mechanism that has evolved to combat, control and eliminate pathogenic organisms is the immune system. We are continually exposed to a variety of different pathogens such as

bacteria, viruses and parasites and our immune system has evolved an elaborate and extremely complicated system to identify and eliminate them (14, 15).

Immunity can be grossly subdivided into two categories: innate and adaptive. Innate immunity is the first line of defence against infection and is activated immediately upon pathogen detection. Although innate immunity has the potential to prevent and/or eliminate pathogenic organisms, numerous studies have demonstrated that innate immunity alone is often insufficient for survival. Nevertheless, the absence of innate immunity can also have lethal consequences because adaptive immunity cannot be generated quickly enough to prevent death. Therefore, it is commonly believed that innate immune response acts to limit pathogen infection providing an adequate window for the development of appropriate adaptive immune responses.

The adaptive immune system is composed of two complementary components, termed humoral and cellular immunity. Humoral immunity refers to immune effectors that are present within the serum and is represented by antibodies. In practice, antibody-mediated immune responses protect extracellular spaces and mucosal surfaces from infection through antibody mediated neutralization and opsonization (16, 17). Antibodies are produced by plasma cells, which are B cells that have been activated in response to external stimuli and identification of their cognate antigen (18-20). A defining feature of humoral immunity is that it can be transferred to unimmunized recipients following inoculation of sera from an immunized donor. In contrast to humoral immunity, cellular

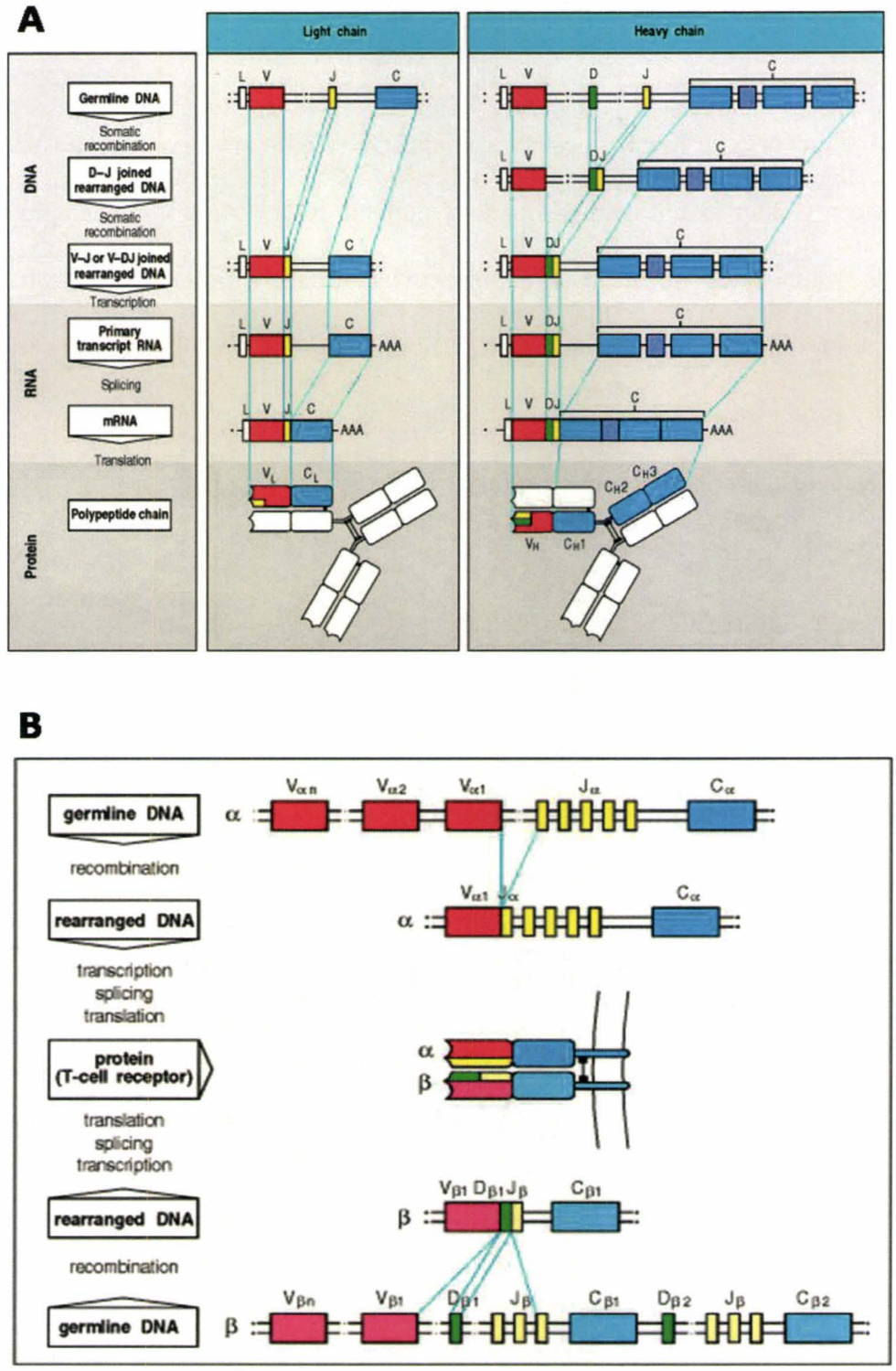
immunity is found within the cellular fraction of the blood and it cannot be transferred to unimmunized allogeneic recipients. Roughly defined, cellular immunity describes adaptive immunity mediated by antigen specific T cells. Furthermore, cellular immunity is complementary to humoral immunity because it controls and eliminates intracellular infections through the actions of T cells.

Although the two arms of adaptive immunity differ by cells types, modes of action and target locations, they share several key features, namely: diversity, specificity and long-term memory. Specificity and diversity are achieved through the expression of antigen specific receptors on T cells and B cells termed T cell receptors (TCRs) and B cell receptors (BCRs) respectably (1). TCRs and BCRs are generated during lymphocyte development a process called lymphopoiesis. Lymphopoiesis begins in the bone marrow following differentiation of the lymphoid progenitor cells from the pluripotent hematopoietic stem cell. Lymphoid progenitor cells destined to become B cells are retained within the bone marrow while those that will become T cells must first migrate to the thymus prior to receptor selection and development (21, 22). To generate a highly diverse receptor repertoire the TCR and BCR are generated through semi-random rearrangement of gene fragments (see Figure 1) (23).

As a consequence of this diverse lymphocyte population, only a small number of circulating cells are specific for any given antigen. This small number of cells is insufficient to combat or prevent an infection and requires the activation and clonal

Figure 1 TCR and BCR gene rearrangement: A) Membrane bound immunoglobulin sereves as the B cell receptor which are composed of both light and heavy chains. Unique specificities are created by somatic gene rearrangements of variable genes (V), junction genes (J), diversity genes (D) and constant genes (C). The light chain is composes of a V, J and C region and the heavy chain contains a V, D, J and C region and together they deterimine the BCR specificity. **B)** Functional TCRs are heterodimers consisting of an α-chain and a β-chain that are generated by somatic gene recombination of variable (V), diversity (D) and junctional (J) gene segments for the β-chain, and V and J gene segments for the α-chain. During T-cell development, gene segments recombine and are spliced together with the constant region (C) to form the functional αβTCR, with each T cell expressing only one type of recombined receptor complex. (Figure adapted from Immunobiology 5th edition, Garland publishing, New York, 2006.)

Figure 1


A
Light chain
Heavy chain
DNA
RNA
Protein
Germline DNA
Somatic recombination
D–J joined rearranged DNA
Somatic recombination
V–J or V–DJ joined rearranged DNA
Transcription
Primary transcript RNA
Splicing
mRNA
Translation
Polypeptide chain
B
germline DNA
recombination
rearranged DNA
transcription
splicing
translation
protein (T-cell receptor)
translation
splicing
transcription
rearranged DNA
recombination
germline DNA

expansion of T and B cells following recognition of their cognate antigens (24). Once the infection has been contained, the remaining T and B cells are termed memory cells (25). These memory cells display remarkable longevity and are physiologically distinct from their naive precursors. Memory cells respond more rapidly to pathogen exposure and can expand to a greater extent than the original population (1, 24, 25). These properties of memory lymphocytes are the basis for prophylactic vaccination. While both T and B cells play an important role in immunological memory, my thesis deals only with T cell immunity produced by vaccination. Therefore, the remainder of this document will be directed at a discussion of T cell immunity.

2.1 T Cell Immunity following vaccination

It is generally believed that the current commercially available vaccines confer protection through antibody-mediated mechanisms (26-32). However, there is now considerable evidence in both animal models and human studies, that many of these vaccines also elicit robust T cell responses (33-36). These observations have in turn prompted further consideration of the possibility that cellular immunity contributes to protective immunity.

A cross-sectional human study retrospectively examined parameters of adaptive immunity in subjects who were immunized against measles 1 to 34 years earlier. The authors detected cytokine-secreting T cells capable of proliferating in response to measles proteins and these results have been corroborated by others (37, 38). Experimental models in nonhuman primates and mice have demonstrated the essential role of cellular immunity

in controlling measles virus infection. For example, as compared to control animals, T cell-depletion in rhesus monkeys prior to measles virus inoculation resulted in 20-fold higher peak viral loads, prolonged serum viremia and skin rash (39, 40). Likewise, murine models have also demonstrated the essential role of T lymphocytes and their cytokines in controlling measles infection in the central nervous system (41, 42). Therefore, although antibody responses in humans may correlate with protection, it appears that the T cell response may be equally, if not more, important.

Inoculation of mice with Vaccinia virus (VV), a poxvirus, results in the generation of robust antibody and T cell responses (33, 43). Furthermore, depletion of T cells prior to inoculation results in increased morbidity and mortality. Evaluations of secondary immune responses to VV challenge have demonstrated that antibodies are protective in the absence of T cells and that T cell responses are protective in the absence of neutralizing antibodies (44, 45). Thus, in the case of VV, either arm of the adaptive immune system appears to be sufficient. However, complete protection against infection with highly pathogenic agents may require the coordinated efforts of both humoral and cellular immunity. While VV is not a natural mouse pathogen, Ectromelia virus (mousepox) is the only poxvirus to naturally infect mice causing skin lesions and lethality at sufficiently high doses. Resistance to lethal challenge with mousepox required the coordinated effects of both T cells and antibodies demonstrating the importance of both arms of the adaptive immune response (46). In humans, the importance of T cell immunity in the control of poxvirus infections comes from observations of disseminated

Vaccinia infection in HIV-positive patients following immunization with VV (13). Consistent with these observations, evaluation of T cell immunity in smallpox vaccinees has demonstrated that these responses are long-lived and highly-functional (33). Although these data suggest that T cell responses play a vital role in primary poxvirus infections in humans, it appears that the presence of antibody levels above a protective threshold is sufficient for secondary protection. However, as anti-smallpox immunity wanes, the combined effects of T cells and antibodies may be required to prevent symptomatic disease.

Another example of T cell-mediated protection comes from studies with influenza. Antibody levels in the serum and mucosal surfaces are considered to be accurate indicators of influenza immunity. However, a recent report by McElhaney *et al* found that in elderly patients, proliferation and cytokine production by T cells correlated with protection following vaccination while antibody levels did not correlate with protection (35). Furthermore, as influenza viruses change through antigenic drift and antigenic shift, vaccine-induced neutralizing antibodies may not be effective against heterotypic influenza. Recently, a cold-adapted, live-attenuated influenza virus has been licensed for human use and has shown greater efficacy (as defined by onset of influenza-like symptoms and confirmed by viral culture) than traditional inactivated vaccines in a randomized, double-blinded, placebo-controlled trail of children 6 to 59 months of age, even against significantly drifted influenza (47). In a murine model where the mechanism of action of cold-adapted viruses was evaluated, in particular the ability of these viruses

to elicit protection against distinct serotypes of influenza where antibodies do not cross-neutralize, it was found that the development of T cells specific for core proteins of the influenza virus played a key role in protection against challenge (48). Therefore, T cells can play a significant role in vaccine-induced protection against serologically distinct influenza.

Varicella Zoster virus is a herpes virus that causes latent infections in humans. Primary infection with Varicella causes chicken-pox, while re-emergence later in life due to waning varicella immunity results in herpes zoster or shingles. The live attenuated Varicella zoster vaccine was first administered to the public in the mid 1990's and was found to elicit both strong T cell and antibody responses (49). However, the clinical efficacy of the vaccine was found to correlate with T cell immunity and not humoral immunity (49, 50). These findings are consistent with the clinical data demonstrating that waning cellular immunity associated with aging is linked to the re-emergence of Varicella in the aged (51).

Collectively, these animal and human cross-sectional studies demonstrate an important role for T cell in vaccine-mediated protection against infectious diseases.

Figure 2

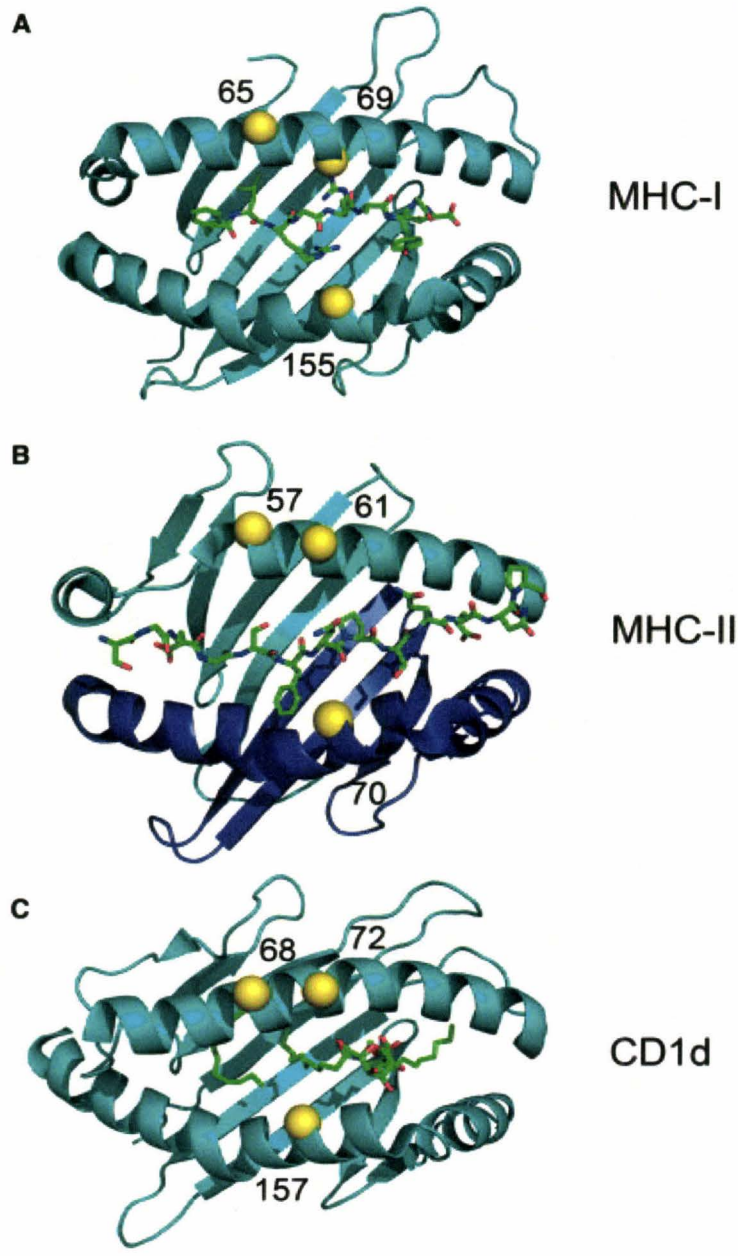


Figure 2: Antigen-Binding Clefts of MHC-I, MHC-II, and CD1d. Antigens are coloured green, and residues which contact and determine TCR restriction are coloured yellow. A) Antigen-binding cleft of MHC-I, which binds peptides of 8-11aa's in length. B) Antigen binding cleft of MHC-II, which binds peptides of 10-18aa's in length. C) Antigen binding cleft of CD1d which binds various lipid based molecules. (Figure adapted from Godfrey, DI. *Et al*. Immunity, 2008 Mar;28(3):304-14.)

3.0 T cell biology: The cast of characters

To fully appreciate the possible utility of T cell immunity for the development of vaccines for unmet pathogens, it is necessary to consider the biology of the T cell compartment. T cells can be subdivided into two main groups based on the surface expression of CD4 or CD8 molecules (52). CD4 and CD8 act as co-receptors that serve to enhance the binding of the TCR to its specific ligand (1). TCRs typically identify short amino acid sequences that are loaded into the antigen-binding pocket of the major histocompatibility complex (MHC) but can also recognize other structures (see Figure 2). Most "classic" MHC are loaded with peptides derived from antigenic proteins although other macromolecules, including lipids and carbohydrates, can be loaded on "non-classic" MHC molecules (53, 54). Display of antigen material on MHC is commonly referred to as "presentation". To be presented on MHC, antigens must first be enzymatically digested into short elements that can fit into the peptide binding grooves of MHC molecules (1). MHC molecules are divided into two categories, class I and class II. MHC class I molecules are universally expressed by almost all nucleated cells (1). MHC class I molecules are composed of 2 distinct polypeptide chains, the MHC α-chain which consists of a transmembrane motif and the peptide binding groove bound to β-2 microglobulin which acts to stabilize the peptide binding groove. The peptide binding groove located in the α-chain is closed at both ends restricting the size of the peptide fragments it can hold to 8-11aa's in length. MHC class I molecules are assembled in the endoplasmic reticulum (ER) with peptides fragments of endogenous origin. Following assembly and peptide loading they are transported to the cell surface for presentation to CD8+ T cells (55).

The distribution of MHC class II molecules is restricted to cells of the professional antigen presenting family, such as, dendritic cells (DCs), macrophages and B cells (1). MHC class II molecules are composed of two homologous peptide chains, α andβ, which both contain transmembrane motifs and portions of the peptide binding groove. The peptide binding groove is created by the dimerization of the α and β chains and is open at both ends permitting the binding of peptides that are 10-18aa's in length (1). Similar to MHC class I molecules, MHC class II molecules are also assembled in the ER; however, the peptide binding groove is bound by an invariant chain which prevents loading of endogenous peptides in the ER (56). Additionally, the invariant chain helps facilitate vesicular transport of MHC class II molecules and their fusion with late endosomes that contain peptides from endocytosed proteins that have been degraded. Following fusion with the late endosomes, the invariant chain is broken down and removed from the peptide binding groove and is replaced by a peptide fragment (56). Following peptide binding the MHC class II molecules are transported to the cell surface where they present peptides to CD4+ T cells.

T cells originate from lymphoid progenitor cells in the bone marrow and migrate to the thymus to complete their development (57). In the thymus, T cells undergo selective processes termed positive and negative selection. Positive selection selects for TCRs that are compatible with the host's complement of MHC molecules; i.e. MHC class I for CD8+ T cells and MHC class II for CD4+ T cells (1). Negative selection eliminates T

cells whose TCRs are potentially auto-reactive against MHC molecules and self peptides (1). T cells that survive both positive and negative selection exit the thymus and enter the vascular system as naive T cells. In the periphery, naive T cells travel through the vascular and lymphatic circulation and survey antigens presented by MHC (58).

CD8+ T cells are commonly referred to as cytotoxic T lymphocytes (CTLs) because their primary function is to mediate cytotoxicity. This function is not universal for all CD8+ T cells as subpopulations have been identified that have immune-regulatory functions, but these populations are rare (59). To simplify matters, we will restrict our discussion to cytotoxic CD8+ T cells. Activated CD8+ T cells identify target cells through the recognition of their cognate antigen presented in the context of MHC class I. MHC class I binding allows CD8+ T cells to maintain close contact with the target cell while it induces cell death through the process of apoptosis. The primary mechanism that CD8+ T cells employ to induce apoptosis in target cells is through the secretion of perforin and granzymes (60). T cells possess preformed vesicles that contain perforin and granzymes called lytic granules. Upon contact with a target cell, lytic granules are translocated to the synapse and are exocytosed, a process termed degranulation (61, 62). Although the exact functions of perforin are unclear, the most widely accepted hypothesis predicts that its polymerization creates pores in the target cell membrane permitting the entrance of granzymes (63). Once in the target cell, granzymes initiate apoptosis through the activation of several different pathways, such as caspases, which fragment cellular DNA (64). A secondary mechanism by which T cells can induce apoptosis is through

expression of fas ligand (fasL) (60). Interaction of fasL with its receptor fas, results in a signalling cascade that terminates with the activation of caspases and cellular death (65). In addition to their cytolytic functions, CD8+ T cells also secrete cytokines such as, IFNγ, TNFα, and IL-2, which assist in propagating an inflammatory state as well as helping to promote T cell proliferation and survival (66-68).

CD4+ T cells are an extremely diverse subset of cells with functions that range from activation of neighbouring cells (ex. B cells, CD8+ T cells, and macrophages), recruitment of cells to sites of infection, to immunoregulation. Currently CD4+ T cells can be divided into at least 5 distinct subsets based on cytokine production and functional characteristics, they are natural regulatory cells (nTreg), inducible T regulatory cells (iTreg), T helper 1 cells (Th1), T helper 2 cells (Th2) and T helper 17 cells (Th17) (69). NTreg cells are a distinct lineage of CD4+ T cells, while the other 4 subsets are derived from a common precursor lineage and their fate is determined by the signals they receive during activation (70). The initial studies separating CD4+ T cell helpers into subsets relied on the mutually exclusive expression of IFNγ and IL-4, IL-5 and IL-13 respectively to define Th1 (IFN-γ) and Th2 (IL-4, -5, -13) (71, 72). In terms of immunological importance, Th1 cells are vital for the control of intracellular pathogens while Th2 cells are required for control of helminth infections. In addition, it has been found that aberrant Th2 responses contribute to allergic disease while it was thought that deviant Th1 responses contributed to autoimmunity (73). However the recent discovery of Th17 cells has cast some doubt on the role of Th1 cells in autoimmunity (74, 75). Th17 cells

produce IL-17, IL-21 and IL-22 and are required for protection against extracellular bacterial and fungal infections (76). However, aberrant Th17 responses have been implicated in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (77, 78). NTreg cells are generated through the natural processes of the thymus and they constitutively express the surface protein CD25 (79). In contrast iTreg cells are generated in the periphery following TCR stimulation in the absence of proinflammatory mediators or to help control T cell responses to infectious agents. Furthermore iTregs can be subdivided into Th3 cells which produce TGF-β and are induced by oral tolerance and T_R1 cells which produce IL-10 (80, 81). Although nTreg and iTreg cells are generated differently they are crucial for the maintenance of peripheral tolerance, prevention of autoimmunity and control of immunological responses to infection.

Even though each CD4+ T cell subset is critical for a fully functioning immune system, Th1 cells are the primary cell type generated following immunization protocols that elicit cellular immunity. Th1 cells are critical for control of intracellular pathogens such as viruses and their effects are mediated by assisting in the activation of B cells, CD8+ T cells, macrophages and they help to generate an antiviral state through the secretion of IFNγ and TNFα (1). In addition to their "helping" functions, Th1 cells also possess the ability to kill antigen specific targets in a MHC class II restricted fashion (82, 83). However, their lytic functions are significantly slower compared to CD8+ T cells and as such it is currently unclear how much they contribute to host protection.

3.1 T CELL RESPONSES TO INFECTION

T cell responses are multifaceted and have traditionally been divided into 3 phases (84). Research employing acute infections models of Lymphocytic choriomeningitis virus Armstrong (LCMV), *L. monocytogenes* and influenza observed that the T cell response is rapidly engaged and expands to peak levels within 7-8 days, termed the expansion phase (85, 86). T cell expansion is followed by a period of significant loss, termed the contraction phase, where approximately 90% of the effector T cells are eliminated (86, 87). Finally, following T cell contraction, a stable memory population is established representing approximately 10% of the maximum response (86, 87). However, it is now known that the timing of the peak response and the rate and extent of contraction varies between organisms and that one of the greatest influences on this process is the kinetics of antigen presentation (88-90).

4.0 T CELL PRIMING: THE CRITICAL ROLE OF DENDRITIC CELLS

T cells exit the thymus as naive antigen inexperienced cells that circulate throughout secondary lymphoid tissues but remain inactive until they receive the appropriate stimuli to facilitate their activation. T cell activation is an intricate process requiring the coordinated actions of 3 distinct sets of signals, peptide stimulation, ligation of co-stimulatory molecules and cytokine stimulation (91). Effective antigen presentation is a critical step in the priming of T cell responses and is mediated by professional antigen presenting cells (APCs). APCs consist of B cells, macrophages and DCs with the latter being the most instrumental in T cell activation (88-90). DCs are a unique cell type

because they possess an innate ability to acquire process and display antigen in the context of MHC class I and II. DC progenitors originate in the bone marrow and they traffic to secondary lymphoid (lymph nodes and spleen) and non-lymphoid tissues (skin, mucosa, lungs) (92). In the periphery, DCs initially possess an immature phenotype that endows them with the ability to continually endocytose antigenic material from their environment, which is in turn processed and display on their cell surface in the context of MHC molecules (92). During an infection, DCs are stimulated by inflammatory conditions and pathogen components triggering the up-regulation of co-stimulatory molecules and trafficking to secondary lymphoid organs, a process referred to as maturation (92). Mature DCs are generally considered to be the most efficient at priming T cell responses.

To date numerous subsets of DCs have been identified in mice and the majority of them express CD11c with the notable exception of plasmacytoid DCs (93). Plasmacytoid DCs appear to play a major role in the early release of type I interferon's following infection, however; their relative contribution and importance to T cell priming is unclear (94). Therefore, in murine models CD11c is a reasonably good marker for the identification of DCs that are involved with T cell priming. CD11c+ DCs can be further subdivided based on surface marker expression. The identification of distinct DC subsets has led to the assessment of their relative contributions to CD8+ and CD4+ T cell activation.

For numerous pathogens it has been demonstrated that CD8α+ CD11c+ DCs are critical for the activation of CD8+ T cells. CD8α+ CD11c+ DCs are highly efficient at both direct and cross presentation of antigens on MHC class I. Consistent with these results, CD8α+ CD11c+ DCs have been identified as the central antigen presenting cell for CD8+ T cells following infection with herpes simplex virus (HSV), influenza, VV, LCMV and *L. monocytogenes* using 4 different routes of infection (subcutaneous, intranasal, intraperitoneal and intravenous) (95-100). With regards to CD4+ T cell immunity, it was initially thought that CD8α+ CD11c+ DCs were not important and that CD8α- CD11c+ DCs facilitated their activation (101). In support of this concept it has been demonstrated that CD8α- CD11c+ DCs have a greater capacity to present antigens in the context of MHC class II while CD8α+ CD11c+ DCs are more efficient at MHC class I presentation (102). However, recent reports have found that CD8α+ CD11c+ DCs can also prime CD4+ T cells in response to viral, bacterial and parasitic infection (103, 104). In addition, CD8α+ CD11c+ DCs have been shown to be efficient producers of IL-12, which is critical for the activation of CD8+ T cells and Th1 CD4+ T cells (105, 106). Therefore, CD11c+ DCs are critical for the priming of both CD8+ and CD4+ T cell immunity and more specifically it appears that CD8α+ CD11c+ DCs appear to play an integral role in the activation of CD8+ T cells and Th1 CD4+ T cells.

4.1 T CELL PRIMING: DENDRITIC CELL/T CELL INTERACTIONS

The process of T cell priming occurs in the T cell areas of the secondary lymphoid tissues. Naive T cells continually recirculate between the circulatory system and

secondary lymphoid tissues surveying cells for the presence of specific antigen (58). In a similar fashion, DCs are constantly acquiring antigen from the periphery to be presented to T cells in secondary lymphoid tissues. As T cells migrate through secondary lymphoid tissues, they make serial contacts with DCs using their TCR to probe the peptides presented by DCs. Upon identification of their cognate antigen, the patterns of T cell movement within T cell zones changes resulting in contacts that are longer in duration (107, 108). Research into the timing and length of DC-T cell interactions has found that they can be divided into three phases (107). The first phase is characterized by short-lived T cell-DC interactions and the up-regulation of T cell activation markers. The second phase involves long-lived T cell-DC interactions and slower movement of T cells. The third phase is defined by a reduction in contact time between DC's and T cells. Initially, it was observed that the first phase lasted approximately 8 hours, the second phase took 12 hours and the third phase began on the second day post infection (107). However, antigen density on DCs and peptide affinity can influence the timing of each phase. Presentation of higher antigen densities or peptides with higher affinity results in faster progression through each stage (109, 110). At this time, we do not know the significance of the phases of T cell-DC interactions. However, it has been hypothesized that during the first phase, T cell activation and differentiation occurs while during the second and third phases T cells receive stimuli that promote survival and continued proliferation.

4.2 T CELL PRIMING: THE AUTOPILOT MODEL

Initially, T cell responses were thought to be dependent upon the presence of antigen such that the T cell response would continue to increase in magnitude until the pathogen was cleared at which point the T cells would die off due to antigen withdrawal. However, a number of reports suggested that CD8+ T cells and CD4+ T cells required only 24 hours of antigen exposure to fully activate a pre-set program which involved a massive amplification of daughter cells (often 12 – 15 rounds of replication within a 7 to 10-day period) followed by abrupt contraction and development of a stable memory population (111, 112). These data resulted in the "auto-pilot" hypothesis which postulated that T cell expansion, contraction and memory formation was a pre-set program initiated within each naïve T cell upon their activation. The initial evidence in support of the programming model was provided by Mercado *et al*, and Corbin *et al. (113, 114)*. These groups illustrated that cessation of *L. monocytogenes* infection through antibiotic treatment 24 hours post infection had no impact on the kinetics or magnitude of the CD8+ T cell and CD4+ T cell responses compared to control mice who did not receive antibiotic treatment (113, 114). However, antibiotic treatment prior to the 24 hour time point reduced the magnitude of the peak 6 days later (113, 114). Employing transgenic T cell models, researchers have also observed that a brief TCR stimulation was sufficient for T cell activation and initiation of sustained replication. Furthermore, replication and stable memory development were not dependent on continued antigenic stimulation (112, 115-117). Groups employing more elaborate models that terminate antigen expression without influencing the inflammatory milieu or the timing of pathogen clearance

demonstrated that 7 hours of TCR stimulation was sufficient to activate CD8+ T cells (118, 119). However, if the duration of antigen exposure was extended the magnitude of the CD8+ T cell response increased but functionality was unaltered (118, 119). These studies suggested that as little as 7 hour of antigen stimulation was required to activate CD8+ T cells, while 24 hours of antigen exposure was sufficient to program both naive CD4+ T cells and CD8+ T cells to differentiate, expand, contract and form a stable memory population without further antigenic stimulation.

Although there is a substantial body of evidence in support of the programming model there is also considerable evidence to suggest that additional antigenic stimulation beyond 24 hours may be required for T cell responses to achieve their full potential. Treatment with ampicillin up to 5 days post infection with *L. monocytogenes* can produce some reduction in T cell immunity, albeit greater reductions were observed when ampicillin was administered at earlier time points (120). In addition, adoptive transfer of splenocytes from *L. monocytogenes* immune mice treated with ampicillin 24 hours post-infection into naive recipients was unable to protect them from lethal *L. monocytogenes* challenge (120). The Bevan group also found that while termination of antigen presentation 24-48 hours post infection with *L. monocytogenes* did not affect primary CD8+ T cell expansion, it did reduce the size of the memory population (121). Similar results were observed by Tseng *et al* who also observed that ampicillin treatment resulted in reduced levels of the co-stimulatory molecules CD80 and CD86 on DCs (122). Furthermore, mice treated with ampicillin 24 hours post *L. monocytogenes* inoculation

were less capable of controlling a secondary challenge with *L. monocytogenes* compared to non-ampicillin-treated controls (122). Based on these results, the authors hypothesized that reductions in co-stimulation may be responsible for the reduced memory responses in the ampicillin-treated mice. In addition premature termination of *L. monocytogenes* infection has been shown to affect CD4+ T cell immunity. Treatment with ampicillin, 24 and 48 hours following *L. Monocytogenes* inoculation resulted in 5-10 fold and 2-3 fold lower CD4+ T cell responses respectively in spleen 7 days post infection (123). Early termination of *L. monocytogenes* infection also resulted in reduced numbers of effector CD4+ T cells in peripheral tissues and reduced the magnitude of the memory response (123). In addition, a recent study observed that CD4+ T cell proliferation does not occur in a linear fashion and that there is a 2-3 day lag prior to a period of explosive proliferation (86). Furthermore, a couple of studies have found that the memory CD4+ T cell response is composed of both early and late activated cells (124-126). Early activated CD4+ T cells were found to express an effector memory phenotype ($CD62L^{lo}$), while late activated CD4+ T cells possessed a central memory phenotype ($CD62L^{hi}$) and had a greater ability to proliferate following secondary infection (124, 126).

Therefore, it would appear that a brief exposure of antigen is capable of activating T cells; however, it may not be sufficient to achieve complete activation and development of optimal memory.

5.0 Generation of T cell memory

Following pathogen clearance, T cell responses go through a period of contraction where a large portion of the responding cells are eliminated. The speed and the magnitude of the contraction process are dependent on several stimuli, such as antigen persistence which will be discussed in section 6.0 (127, 128). The net result of the contraction phase is a population of long-lived T cells that are significantly faster at mobilizing effector functions and proliferation following exposure to cognate antigen when compared to naïve T cells. Investigation into T cell memory has revealed that contrary to initial beliefs there is significant heterogeneity within memory responses and between different pathogens. These observations have inspired researchers to determine what constitutes effective and optimal T cell memory (129). Not surprisingly, there remains considerable controversy regarding the mechanisms by which T cell memory is formed and the type of memory response that is most effective. Furthermore, although much of the work regarding memory T cell differentiation and classification has been conducted on CD8+ T cells, the available research on CD4+ T cells have shown similar results suggesting that the fundamental concepts are similar between the two cell types.

5.1 Memory T cells subsets

The concept of distinct memory T cell subsets was first proposed in the late 1990s (130, 131). The original studies were conducted in humans, however; their findings have since been translated to murine models as well. Two distinct memory subsets were initially proposed termed central memory (T_{cm}) and effector memory (T_{em}) T cells (131, 132).

These cell types were distinguished by their differential expression of the cell surface markers CD44, CD62L and CCR7, T_{cm} cells were defined as $CD44^{hi}$ $CD62L^{hi}$ $CCR7^{hi}$ and T_{em} cells were defined as $CD44^{hi}$ $CD62^{lo}$ $CCR7^{lo}$. Interestingly, naive T cells express both CD62L and CCR7 as they are required for homing and trafficking of T cells into lymph nodes (130-133). Upon activation naive T cells down-regulate both CD62L and CCR7 which presumably enables them to exit lymph nodes and migrate into the periphery and sites of active infection (134, 135). Following activation naive T cells up-regulate expression of CD44 which thus permitting the distinction between $CD62L^{hi}$ $CCR7^{hi}$ naive and memory T cells. The disparity in expression of CD62L and CCR7 by T_{cm} and T_{em} cells results in different circulation patterns as well as residence in separate anatomical compartments. Expression of CD62L and CCR7 enables T_{cm} cells to gain access into the lymph nodes; whereas their T_{em} counterparts lack these molecules, and preferentially localize in the peripheral tissues (130, 131). However, the localization of the T_{cm} and T_{em} cells are not mutually-exclusive, as T_{em} may still access to the lymph nodes *via* afferent lymphatics and T_{cm} cells can be found in peripheral compartments (136). Furthermore, in murine models, CD62L expression patterns on T lymphocytes is correlated with that of CCR7; therefore, murine memory subsets are often defined based only on CD62L (133).

Since the initial distinction between T_{cm} and T_{em} memory subsets a subsequent memory population has been identified within the $CD62L^{lo}$ population (137). Stimulation by IL-7 through its receptor (IL-7Rα or CD127) is required for survival and renewal of naive

lymphocytes and in conjunction with IL-15 is required for maintenance and homeostatic proliferation of memory cells (138-140). Following activation, naive T cells transiently down-regulate CD127 expression as they differentiate into effectors, however, $CD127^{lo}$ T lymphocytes regain CD127 surface expression as effectors become memory cells (136, 141-143). The re-expression of CD127 has been found to be necessary but not sufficient for the development of memory T cells (144). In concordance with these findings evaluation of CD127 expression on memory T cells revealed that T_{cm} cells were $CD62L^{hi}$ $CD127^{hi}$ and T_{em} were $CD62L^{lo}$ $CD127^{hi}$ (137). However, in addition there was also a population of $CD62L^{lo}$ $CD127^{lo}$ cells, which were termed effector T cells (T_{eff}). Studies directly examining the functionalities of the three T cell subsets defined by CD62L and CD127 indicated that, as compared to T_{cm} and T_{em}, the $CD62L^{lo}$ $CD127^{lo}$ population or T_{eff}, were the most cytolytic but had the least *in vivo* proliferative potential after re-challenge (137, 141). Therefore, T_{eff} cells are not expected to be sustained within the memory pool since they are unable to maintain themselves through either homeostatic or antigen-driven proliferation. With regards to the T_{cm} and T_{em} subsets, many studies have demonstrated that CD62L-positive T_{cm} cells possessed a greater homeostatic turnover potential relative to T_{em} cells (137, 141, 145). Therefore, over time, one should expect a gradual enrichment of $CD62L^{hi}$ central-memory cells in the memory population as they gradually out-grow the other, less proliferative memory populations.

5.2 *Functionality Of memory CD8+ T cell subsets*

T_{cm} are typically non-cytotoxic and display limited effector function. They do display remarkable proliferative capacity and give rise to fully functional T_{eff} when activated. Thus, while the T_{em} have a higher degree of immediate effector function, the T_{cm} can give rise to a large T_{eff} population following secondary antigenic exposure. How these different properties influence protective immunity has been the subject of much investigation.

A study by Wherry *et al* analyzed extensively the relative protective ability of T_{cm} and T_{em} by adoptively transferring each subset into separate naïve hosts and subsequently infecting these chimeric mice with various pathogens *via* multiple routes (143). In all challenge studies, T_{cm} cleared the pathogens more rapidly than their T_{em} counterparts (143). Wherry *et al* attributed the superior protection of T_{cm} to their enhanced proliferative capacity. In contrast, other studies presented data demonstrating that T_{em} cells were indeed the more proliferative of the two memory subsets (146, 147). In the study by Roberts *et al*, equal numbers of memory Sendai virus-specific $CD62L^{hi}$ and $CD62L^{lo}$ T cells were adoptively transferred into naïve mice, which were subsequently infected with the same virus and the relative expansion of the T cells was monitored over time. From day 7 to day 40 after challenge, $CD62L^{lo}$ lymphocytes were the predominant population within the donor fraction across multiple tissues, suggesting that T_{em} expanded to a greater extent than T_{cm} after recall (147).

The donor cell source for the Sendai virus experiments described in the previous paragraph were derived from mice which were immunized 1 to 5 months previously ("young" memory). When a similar experiment was conducted using mice which were immunized one year earlier as donors ("aged" memory), the authors found that $CD62L^{hi}$ "aged" central-memory cells constituted 85 to 95% of the responding population; in sharp contrast to their previous observation using "young" memory T cells (148). Further adoptive transfer studies revealed that regardless of CD62L expression, "aged" memory T cells consistently outperformed their "young" counterparts by becoming the predominant population (148). These results suggest that memory stage (i.e. age of T cell population following immunization) is more reflective of the capacity to respond following secondary stimulation rather than surface expression of CD62L. Indeed, similar results were reported by Jackson *et al* who found that expression of CD62L was not related to the ability of the CD8+ T cells to respond in a secondary stimulation (149).

It is also important to note that proliferative capacity of a memory pool may not always be the best predictor of protective immunity. In a study where mice were immunized with virus-like-particles (VLPs) carrying a model antigen, the authors observed that protection from a lytic virus (vaccinia virus) required the presence of high numbers of cytolytic effector CD8+ T cells at the site of viral replication and T cell proliferation only occurred due to the failure to rapidly contain the virus (145). By contrast, control of a non-lytic virus (LCMV) could be mediated by low numbers of CD8+ T_{cm} cells which

underwent high levels of recall proliferation (145). Therefore, the degree of immune protection conferred by each T cell subset likely depends upon the invading pathogen.

Collectively, these studies argued in favour of three important concepts: 1) the relative capacity of each memory subset to confer protection is dependent on the pathogen in question; 2) although the term "memory" is often used by scientists to describe antigen-specific T cells as early as 30 days after their initial activation, "young" memory T cells may likely continue to differentiate and are not functionally identical to their "aged" memory counterparts; 3) we have not yet found surface markers that permit accurate assessment of recall potential.

5.3 Models of memory CD8+ T cell differentiation

A currently unresolved question relates to how and when memory cells differentiate following infection. Traditionally, it was believed that memory cells developed following pathogen clearance during the contractions phase; however, recent evidence suggests that this paradigm maybe too simplistic. Furthermore, memory cell development may not be identical between all infectious models suggesting that there may be more than one developmental pathway. Outlined below are a few of the proposed mechanisms of memory cell differentiation.

- *Uniform potential model* – This model predicts that effector cells and memory cells represent homogenous populations and that each cell possesses the same potential to

develop into a memory cell. The paradigm proposes that competition and/or withdrawal of survival factors such as cytokines and antigen limit the number of effector cells that can survive contraction and develop into memory cells (150). Experimental systems employing the adoptive transfer of large numbers of transgenic T cells provided initial support for this model. In these models, most of the memory population ultimately acquires a T_{cm} phenotype and T_{em} cells could convert to T_{cm} upon transfer into naïve hosts (141, 143). However, these results were shown to be an artefact of artificially increasing precursor frequencies through the use of TCR transgenic T cells. When limiting numbers of TCR transgenic T cells were adoptively transferred, which better reflect normal physiology, the resultant memory population was heterogeneous and cells with a T_{em} phenotype were unable to convert to a T_{cm} phenotype (151).

- *Decreasing potential model* – Similar to the previous model, this model predicts that all effector T cells possess equal potential to develop into memory cells, but that the degree of TCR stimulation is inversely related to their capacity to become memory cells (152, 153). In addition, increased or prolonged stimulation by other sources than TCR stimulation may also result in decreased memory potential. Using this model, the development of memory T cell subsets can be explained by their relative exposure to activation stimuli, the order of most stimulated to least stimulated would be $T_{eff} > T_{em} > T_{cm}$ (153). This model is supported by experiments showing that T cells engaged early in the response acquire an effector phenotype while those engaged later following acute

infection acquire a memory-like phenotype (124, 125). In addition to data generated by acute infections, this model can explain the heterogeneity of memory responses to persistent infections and T cell exhaustion during chronic infection (89, 154, 155). This model is challenged by the findings of Chang *et al* who demonstrated that T cells can divide asymmetrically producing one daughter cell that is destined to become an effector cell while the other cell was destined to become a memory cell which suggest that memory may have nothing to do with the degree of antigen exposure (156).

- *Fixed lineage model* – The fixed lineage model predicts that following T cell activation cells are committed to become either memory cells or effector cells. This model suggests that memory cells are present at all points of the response and they are the survivors of contraction (157, 158). This model is supported by the observation of asymmetric separation of daughter cells following the first division by Chang *et al*, and the findings of Stemberger *et al* that activation of 1 T cell can give rise to effector, and memory cells (156, 159). In addition, in a recent report by Teixeiro *et al*, they found that differences in TCR stimulation influence the ability of T cells to differentiate into memory cells. The authors adoptively transferred T cells containing a point mutation in their TCRβ chain into mice and immunized them with recombinant *L. monocytogenes* expression OVA (160). The point mutation resulted in reduced TCR polarization and improper NFκβ organization at the immunological synapse (160). T cell containing the point mutation efficiently developed into effectors but were unable to differentiate into memory cells, while wild type cells showed normal effector and memory development

(160). Although their findings do not definitively prove that the lineage model is correct, they do suggest that differences in signalling between T cells can impact their fate.

- *Fate commitment and progressive differentiation model* – This model largely combines the concepts of the previous models. In this model it is hypothesized that there exists both memory precursor effector cells (MPECs) destined to become long-lived memory cells and terminally differentiated short-lived effector cells (SLECs) that will die during contraction (161). It is postulated that cell fate is determined by the strength of stimulation and the degree of differentiation is correlated to the duration of stimulation (162, 163). Furthermore, MPECs are created as memory precursor cells that require additional stimulation to differentiate into long lived memory cells and exhibit effector functions such as granzyme B-mediated cytolysis and limited replicative capacity (164). Importantly, the MPEC fate is not fixed and following adequate stimulation they can convert into terminally differentiated SLECs.

Although the various models are supported by available literature, this field remains highly controversial. This is likely a result of the complexity of the process and the likelihood that the mechanism of memory development may be highly dependent upon the experimental model.

6.0 Impact of Prolonged antigen presentation on T cell immunity

Many of the models employed to study T cell immunity involve agents that cause acute infections. In these models, pathogen clearance typically correlates with the peak of the T cell response 7-12 days post infection and is followed by a period of T cell contraction. Following acute infection, the memory population that develops does not require subsequent exposure to antigen and is maintained through homeostatic cytokines such as IL-7 and IL-15. However, recent reports have identified numerous instances in which the peak response does not correlate with complete antigen clearance and, in some cases, antigen presentation and microbial replication can persist indefinitely.

Currently, models of prolonged antigen presentation can be categorized based on criteria such as the amount and duration of antigen presentation, persistence of microbial replication and effect on T cell function.

- *Chronic infections*: T cell responses are unable to eliminate the pathogen following infection. The microbe continues to replicate resulting in presentation of large amounts of antigen in an inflammatory setting. This environment results in T cell dysfunction which manifests as a gradual loss of cytokine production and finally complete loss of T cell function, termed exhaustion (154, 155, 165-169).

- *Persistent infections*: Persistent infections are initially controlled by the development of strong adaptive immune responses (170). However, sterilizing immunity is not

achieved and the pathogen is maintained either as a latent infection or through infection of immune privileged tissues. T cell immunity does not become dysfunctional but persistent reactivation of latent pathogens or constant low-level replication results in continual T cell stimulation (89, 171, 172). In comparison to chronic infections the magnitude of antigen presentation and inflammation is much lower and T cells do not become exhausted but the memory population is typically composed of a high frequency of effector-like T cells.

- *Acute infections with prolonged antigen presentation*: These infectious agents elicit potent T cell responses that effectively inhibit their replication by 7-12 days post infection. However, low level antigen presentation in the absence of obvious microbial replication and inflammation can persist for days to weeks following the peak T cell response (90, 126, 173-175). Similar to persistent infections, T cells are constantly stimulated with low-levels of antigen producing a heterogeneous memory population that is not dysfunctional.

7.0 CD4+ T CELL HELP FOR CD8+ T CELL IMMUNITY

As previously stated the development of efficacious, long-lived CD8+ T cell memory requires the co-ordinated effects of antigen, co-stimulatory molecules and cytokine stimulation (91, 128). In addition, it has been widely reported that input from CD4+ T cells is a prerequisite (176). Priming CD8+ T cell responses in the absence of CD4+ T cell help yields "unhelped" CD8+ T cells, which display numerous defects in expansion,

memory cell maintenance and functionality (177-186). There is discord among researchers regarding the timing of CD4+ T cell help and the actual functional attributes of CD8+ T cells that are influenced by CD4+ T cell help. The controversy notwithstanding, CD4+ T cell help appears to be vital for CD8+ T cell function because in most models, "unhelped" CD8+ T cells show some evidence of dysfunction when compared to their helped counterparts.

7.1 CD4+ T CELL HELP INFLUENCES CD8+ T CELL PROGRAMMING; THE DC LICENSING HYPOTHESIS

Initial studies evaluating the role of CD4+ T cells in CD8+ T cell immunity employed vaccines that were non-inflammatory, such as peptide pulsed DCs, free peptide and antigen-loaded allogenic splenocytes (185, 187-190). In those studies, it was reported that CD8+ T cell immunity was not elicited following immunization of mice that lacked CD4+ T cells. Furthermore, it was also established that help from CD4+ T cells was mediated through CD40L/CD40 signalling (185, 188, 191). Interestingly, in the absence of CD4+ T cells, the addition of agonist CD40 antibodies was capable of restoring the primary CD8+ T cell response to wild-type levels (188, 191). Taken together, these studies demonstrated that CD4+ T cells provide essential help to CD8+ T cells through the CD40-CD40L signalling pathway. Given the important role for DCs in priming T cell responses, it was suggested that DCs provide a temporal bridge between activated CD4+ T cells and antigen-specific naïve CD8+ T cells. The "DC licensing" hypothesis postulates that cognate interactions between CD40L-positive CD4+ T cells and antigen-

loaded CD40-positive dendritic cells, are required to provide DCs with a "license" to activate CD8+ T cells.

Further support for the DC licensing model came from studies which examined whether the MHC class I and MHC class II molecules needed to be presented by the same APC. To address this issue, bone marrow chimeric mice harbouring DCs defective in either MHC I or II peptide presentation were immunized with protein-loaded splenocytes and antigen-specific CD8+ T cells were monitored (187, 189). Compared to wild-type controls, chimeric mice did not exhibit an antigen-specific CD8+ T cell response, thus demonstrating that both MHC I and II peptide need to be present on the same DC. Taken together these reports suggested that CD4+ T cell help was required to fully activated DCs, endowing them or "licensing" them, to activate CD8+ T cell immunity.

7.2 CD40-CD40L Interaction: Help May Not Involve DC Licensing

The DC licensing model hypothesizes that CD40/CD40L interactions between CD4+ T cells and DCs is required to initiate CD8+ T cell immunity. However in a report by Bourgeois *et al* suggested that CD40/CD40L interactions may not be between CD4+ T cells and DCs but rather, between CD4+ T cells and CD8+ T cells. Bourgeois *et al* employed an immunization model where H-Y-specific transgenic CD8+ T cells were adoptively transferred into T cell-deficient female mice and subsequently immunized with male bone marrow (192). In this model antigen-specific proliferation of the H-Y specific transgenic CD8+ T cells was observed regardless of whether H-Y-specific CD4+ T cells

were co-injected at the start of the experiment. However, "unhelped" CD8+ T cells as early as 5 days after immunization were unable to produce cytokines such as IFN-γ and IL-2, and did not undergo antigen-driven proliferation two months after initial immunization (192). Therefore, although in the H-Y model a primary "unhelped" CD8+ T cell response is observed, the functionality of "unhelped" CD8+ T cells was not maintained long-term. Interestingly, compared to the DC licensing model, the presence or absence of CD40 on the male bone marrow immunization cells which, presumably contain dendritic cells presenting both MHC-I and –II peptides, did not affect the transgenic CD8+ T cell functionality (192). Instead, functionality of the CD40-deficient transgenic CD8+ T cells correlated with the "unhelped" phenotype, suggesting that CD4+ T cell help is provided directly to the responding CD8+ T cells (192). This study argues that in addition to indirectly providing help to CD8+ T cells by first licensing DCs, CD4+ T cells may also offer help directly to CD8+ T cells *via* CD40-CD40L interaction.

However, the concepts that help through CD40 expressed on CD8+ T cells has been refuted by several studies. CD8+ T cell immunity to influenza was unaffected by the presence or absence of CD40 expression on CD8+ T cells. However, when CD40 expression was deleted from non-T cells but maintained on CD8+ T cells, the resultant CD8+ T cell population was dysfunctional (193). In concordance, following *L. monocytogenes* infection, Sun *et al*, did not observe a defect in CD8+ T cell immunity when CD40-/- CD8+ T cells were compared to CD40+/+ CD8+ T cells (179). Therefore,

the latter models confirm the DC licensing hypothesis and fail to support the direct interaction hypothesis.

7.3 Infectious agents: CD8+ T cell immunity in the absence of CD4+ T cell help

While most non-infectious vaccinating agents display an absolute dependence on CD4+ T cell help to generate a primary CD8+ T cell response, the same is not true for infectious agents. Following immunization with influenza (186, 194), *L. monocytogenes* (195-197), LCMV-Armstrong (197, 198), and VV (178), the generation of CD8+ T cell immunity is independent of CD4+ T cell help. Consistent with the DC licensing hypothesis, it has been suggested that the inflammatory nature of these infectious agents may promote DC licensing without the need for CD40-CD40L signalling. Indeed, ligation of toll like receptors (TLRs) has been shown to trigger an immature DC to express co-stimulatory molecules, endowing them with the capabilities to activate naive CD8+ T cells (199-201). With that said it has also been shown that CD4+ T cell help can be bypassed by artificially increasing the precursor frequency of naïve CD8+ T cells prior to vaccination (202). Mintern *et al* found that at high enough frequencies, CD8+ T cells provide help for themselves through the interaction of CD40 and CD40L. However, although infectious agents are capable of eliciting CD8+ T cell immunity in the absence of CD4+ T cell help, in the majority of cases there are still observable defects in CD8+ T cell immunity when compared to helped controls.

7.4 Defects in "unhelped" CD8+ T cell immunity

Although it is known that CD4+ T cell help contributes to CD8+ T cell immunity, the mechanisms of "help" are not entirely known. As previously stated, unhelped CD8+ T cells have shown defects in expansion, memory maintenance, recall and functionality (177, 179-184, 186, 195, 203-206). In light of these widely varying reports it is quite likely that depending on the immunization model employed, the requirements for CD4+ T cell help may vary in both its nature and timing.

A considerable amount of evidence has been produced to suggest that defects in "unhelped" CD8+ T cells are generated during priming. The Armstrong stain of LCMV elicits an acute infection that results in memory CD8+ T cells that producers both IFNγ, TNFα and, to a lesser extent, IL-2 (155, 207). In addition, the memory CD8+ T cell population is predominantly $CD127^{hi}/CD62L^{hi}$; a phenotype consistent with T_{cm} (143). However infection with LCMV-Armstrong in the absence of CD4+ T cells elicits memory CD8+ T cells that are diminished in their ability to produce TNFα and IL-2 and they produce less IFNγ compared to helped CD8+ T cells (208). In addition "unhelped" CD8+ T cells are predominantly $CD62L^{lo}$ and $CD127^{lo}$, consistent with a T_{eff} phenotype (208). Defects in cytokine production have been attributed to epigenetic changes of CD8+ T cells primed in the absence of CD4+ T cell help. Indeed, the inability of ""unhelped"" CD8+ T cells to secret cytokines such as IFN-γ and IL-2 was shown to be a result of epigenetic remodelling of their corresponding loci (209). More specifically, increased histone acetylation at the IFN-γ promoter and enhancer as well as the failure of

the cells to demethylate the IL-2 promoter region correlated with the inability of the ""unhelped"" CD8+ T cells to produce these cytokines, presumably due to the relative inaccessibility of these gene regions for transcription compared with wild-type controls (209).

Furthermore, the development of CD8+ T cell memory in the absence of CD4+ T cell help may be skewed. As previously stated, memory CD8+ T cell responses generated following LCMV infection without CD4+ T cell help are dominated by effector memory cells. A recent report by Intlekofer *et al* found that exposure to LCMV in the absence of CD4+ T cells yielded "unhelped" CD8+ T cells that expressed higher levels of the transcription factor T-bet compared to their helped counterparts (205). Over expression of T-bet was shown to repress expression of the IL-7α receptor which is required for T_{cm} differentiation (205). In addition, deletion of T-bet was shown to correct the observed defects in "unhelped" CD8+ T cell memory (205). Taken together these studies suggested that the defect in "unhelped" CD8+ T cell responses is possibly due to epigenetic changes that prevented appropriate differentiation and the development of functional memory cells.

Another hallmark of "unhelped" CD8+ T cell immunity is defective secondary proliferative responses. Janssen *et al* found that defective secondary expansion by "unhelped" CD8+ T cells was not due to lack of proliferation but rather an increased rate of cell death (206). They found that "unhelped" CD8+ T cells up-regulated the pro-

apoptotic molecule TNF-related apoptosis-inducing ligand (TRAIL), a soluble pro-apoptotic molecule that promotes programmed cell death in many different cell types (206). While the receptors for TRAIL were equally expressed by both helped and "unhelped" CD8+ T cells, TRAIL mRNA was selectively up-regulated in the "unhelped" CD8+ T cells, suggesting that proliferating helpless CD8+ T cells were inducing their own death (206). Furthermore, the authors demonstrated that TRAIL mediated apoptosis of "unhelped" CD8+ T cells could be overcome by blocking TRAIL signalling.

However, up-regulation of TRAIL may not be the complete reason for defective recall responses of helpless CD8+ T cells. To further our understanding of the role of TRAIL in CD8+ T cell biology, Badovinac *et al* evaluated the effect of TRAIL deficiency in CD8+ T cells primed in the presence or absence of CD4+ T cells (203). In the presence of CD4+ T cell help, the phenotype, kinetics and numbers of TRAIL-deficient CD8+ T cells were similar to TRAIL sufficient CD8+ T cells following LCMV infection. In line with previous results, "unhelped" CD8+ T cells maintained higher levels in the absence of TRAIL (203). However, the authors found that TRAIL deficiency only delayed, but did not prevent, the erosion of "unhelped" CD8+ T cell memory (203). Two months after infection, the "unhelped" TRAIL-deficient memory cells lost their ability to produce cytokines and were unable to proliferate in response to challenge (203). Similarly, TRAIL-deficiency did not seem to influence the memory CD8+ T cell population produced by L. monocytogenes or VV (210). Therefore, these reports suggests that CD4+ T cell help may influence the CD8+ T cell response both at the time of priming and

during the memory phase, but TRAIL-mediated death of "unhelped" CD8+ T cell death was only a factor during the early expansion phase.

An alternative reason for defective recall responses of "unhelped" CD8+ T cells was recently reported by Fuse *et al.* (204). Following VV infection they found that "unhelped" CD8+ T cells were defective in their ability to mediate recall responses. Interestingly, both as a percentage of cells and on a per cell basis, helpless VV specific memory CD8+ T cells were found to express higher levels of the inhibitory surface receptor programmed death 1 (PD-1) (204). PD-1 has been shown to repress CD8+ T cell proliferation and production of IL-2 which is required for T cell replication (154, 211). Furthermore, blocking PD-1 resulted in the restoration of recall responses by "unhelped" CD8+ T cells to levels comparable to helped CD8+ T cells (204). In addition, *in vivo* administration of IL-2 to helpless CD8+ T cells down-regulated PD-1 expression and restored their proliferative capabilities. However, the authors also found that if CD40 stimulating monoclonal antibodies were added during the priming of "unhelped" responses, defective CD8+ T cells that expressed PD-1 were not elicited (204). Their results suggest that following Vaccinia virus infection CD4+ T cell help may prevent defective CD8+ T cell responses through both CD40- and IL-2 mediated mechanisms that prevent PD-1 up-regulation.

In contrast to the "programming" hypothesis, others have argued that CD4+ T cells are, in fact, dispensable during priming, and are required only later on in an antigen-nonspecific

fashion to sustain the and longevity and functionality of the CD8+ memory pool. In these experiments employing LCMV-Armstrong and *L. monocytogenes*, Sun *et al* adoptively transferred CD8+ T cells primed in wild-type and MHC class II-deficient mice into naïve hosts and monitored the donor T cells over time (179). Increased loss and functional erosion of the transferred T cells was observed in the MHC-II-deficient recipients, regardless of whether the CD8+ T cells were primed in a CD4+ T cell-deficient or sufficient environment (179). Thusly, CD4+ T cells were only essential in the maintenance of the memory CD8+ T cell population. According to Sun *et al*, CD8+ T cells primed in a CD4-deficient environment will differentiate into functional memory cells as long as subsequent CD4+ "help" is provided.

Finally, CD4+ T cell help may not directly influence CD8+ T cells. Instead, the apparent failure of the "unhelped" CD8+ T cells to re-expand upon challenge may be a reflection of inadequate help given to DCs. In a study by Marzo *et al*, the helped and "unhelped" CD8+ T cells proliferated to the same extent when placed in a CD4+ T cell-sufficient setting (184). In addition, adoptive transfer studies demonstrated that helped and "unhelped" memory CD8+ T cells were indistinguishable in terms of their cytokine-secreting and target cell lysis capability on a per-cell basis. However, the magnitude of the secondary peak CD8+ T cell response was reduced by 5-fold when mice were treated with a CD4-depleting antibody at the time of recall challenge. Concurrent administration of anti-CD40L and CD4-depleting antibodies during challenge diminished the peak magnitude by a further 13-fold, indicating that CD40-CD40L signalling was essential for

CD8+ T cell recall proliferation (184). The same group has demonstrated that DCs were essential for maximizing a secondary proliferative CD8+ T cell response to many pathogens including *L. monocytogenes*, influenza, and VSV (99). Other groups have also demonstrated the absolute importance of DCs for secondary responses to HSV (212) and LCMV (213). Therefore, it is reasonable speculate, at least in some models, that CD40-CD40L signalling during the recall response serves to activate the antigen-presenting DCs, although direct stimulation of the responding CD8+ T cells themselves cannot be excluded.

Together, these studies demonstrate that CD4+ T cells may provide help to CD8+ T cells during the priming, maintenance, as well as the challenge phase of the response, although the precise timing of help that will lead to maximal primary and secondary CD8+ T cell immunity may vary amongst individual pathogens

8.0 Protective T cell responses: The importance of polyfunctional T Cell immunity

The primary functions of CD8+ T cells appear to be the lysis of infected cells and the generation of an anti-microbial state to limit the spread of infection (214). CD4+ T cells also contribute to the creation of an inflammatory environment. Furthermore, CD4+ T cell play a key role in B cell (1), CD8+ T cell (176) and macrophage activation (1). CD4+ T cells also seem to express lytic functions; however, the contribution of cytolytic CD4+ T cells to the elimination of infected target cells is currently unknown (215-218).

Traditionally, T cell immunity was measured based on the magnitude of the response. However, T cell express multiple effector molecules, such as IFNγ, TNFα, IL-2, perforin, granzymes, fasL (CD95) that all contribute to their anti-microbial activity. The development of multi-parametric flow cytometry in combination with advances in methods to identify individual antigen specific T cells has permitted the simultaneous evaluation of multiple effector molecules a per cell basis. Therefore, each antigen-specific T cell can be interrogated for multiple effector functions simultaneously. The term "polyfunctional" has been coined to describe T cells that can elaborate two or more effector molecules. Interestingly, investigations using polychromatic flow cytometry to evaluate T cell functions have revealed that T cell responses are extremely heterogeneous and can change during the course of a response (219). Furthermore, these findings suggest that there can be significant differences in the quality of antigen-specific T cell responses elicited by different vaccination platforms which in turn, can influence their protective ability (216, 220-225).

One of the primary effector mechanisms of CD8+ T cells, and some CD4+ T cells, is the cytolysis of pathogen-infected cells. Target cells are identified through TCR/MHC interactions and cell death is mediated through the release of cytotoxic granules or ligation of the fas receptor on target cells via fasL on the T cell (226). The key effector molecules in cytotoxic granules are perforin and granzymes which serve to permeabilize the target cell and instigate apoptosis. Similarly, ligation of fas on the target cell also initiates apoptotic pathways. The importance of these lytic function is highlighted by the

fact that they are critical in the clearance of numerous intracellular pathogens (226). Further proof of the importance of these pathways comes from studies demonstrating that numerous pathogens express molecules design to subvert the action of the cytolytic mediators (227).

T cells also produce cytokines which are critical for clearance and control of infectious agents. Two hallmark cytokines, IFN-γ and TNFα, have been shown *in vivo* to control the growth of several pathogens including vaccinia virus, *L. monocytogenes*, mycobacterium tuberculosis (*M. tb.)*, hepatitis B virus (HBV), influenza virus and murine cytomegalovirus (MCMV) (66, 67, 228-230). Although IFNγ or TNFα on their own are sufficient to control numerous pathogens, there is considerable evidence that their simultaneous production results in synergistic actions that yield faster and more efficient microbial clearance (231-233). Another important cytokine produced by T cells is IL-2, which is essential for T cell expansion and survival (68, 234, 235), although the source of IL-2 remains controversial.

Evaluation of cytokine production by each T cell subset has revealed that there are significant differences between CD8+ T cells and CD4+ T cells as well as variations within each subset depending on the infectious agent. Researchers have found that all activated CD8+ T cells initially express IFNγ and that a significant number will also acquire the ability to produce TNFα, however; the fraction of antigen-specific cells producing both cytokines can vary depending on the stage of the response and the specific

pathogen (155, 207, 228, 236). A smaller proportion of antigen-specific CD8+ T cells express IL-2 and the expression of this cytokine is typically delayed relative to IFNγ and TNFα (155, 236). However, similar to TNFα the percentage of CD8+ T cells that express IL-2 varies at different stages of the response and between different infectious agents (155, 236). For CD4+ T cells, TNFα appears to be ubiquitous expressed by all activated cells with a large percentage also expressing IFNγ (86, 228, 237, 238). In contrast to CD8+ T cells, a larger proportion of CD4+ T cells express IL-2, however IL-2 expression is almost always associated with either TNFα or IFNγ production (237, 238). Similar to CD8+ T cells, the proportion of cells with the capacity to produce two or more cytokines can vary between pathogens.

To date, significant differences have been observed in the polyfunctionality of antigen-specific T cells evoked by different infectious agents. Therefore, it may be fair to assume that T cells will possess different abilities to protect against pathogenic challenges. Furthermore, considerable evidence has accumulated suggesting that the protective capacity of T cells correlates with the number of effector functions they possess. These findings have important implications in the design and evaluation of potential vaccines.

8.1 CD8+ T CELL POLYFUNCTIONALITY

Perhaps the best example of the importance of T cell polyfunctionality is in the case of human immunodeficiency virus (HIV) infection. Researchers have found that the magnitude of the HIV specific CD8+ T cell responses does not correlate with disease

progression (239-241). It is not unusual to find high frequencies of HIV-specific CD8+ T cells in patients with progressive disease. Initially, it was suggested that the T cells present in progressors may not be targeting the correct antigens. More recently, investigators have found that CD8+ T cells isolated from long-term non-progressors (LTNPs) have increased lytic and proliferative capacities compared to CD8+ T cell isolated from progressors (242). In addition, a higher frequency of CD8+ T cells from LTNPs produced multiple cytokines (IFNγ, TNFα, IL-2) compared to progressors (220). Furthermore, a higher proportion of CD8+ T cells from progressors only produced IFNγ compared to LTNP, suggesting that the HIV-specific CD8+ T cells may be functionally exhausted as described earlier in section 6.0 (220). Indeed, this evidence of functional exhaustion correlates with increased expression of PD-1 on CD8+ T cells within the progressor population and blockade of PD-1 signalling can improve T cell functionality (167, 243).

Hepatitis C virus (HCV) infection evokes a CD8+ T cell response with a high-degree of functionality as assessed by measurements of proliferative capacity and cytokine production (244). Patients co-infected with HCV and HIV display a less functional HCV-specific CD8+ T cell response characterized by a lack of CD8+ T cells producing both IFNγ and IL-2 and diminished proliferative capacity (244). The diminished polyfunctionality of the CD8+ T cell response in co-infected patients correlated with higher loads of HCV and more severe liver fibrosis (244).

Vaccinia virus is considered to be a prototypic "effective" vaccine because it was used to eradicated smallpox. CD8+ T cells elicited by VV immunization display a high-degree of polyfunctionality (222). VV-specific CD8+ T cells produce IFNγ, TNFα, IL-2, display cytotoxic activity and high proliferative capacity (222). VV has also been engineered to be used as a recombinant vector for vaccination. Interestingly, the authors observed that CD8+ T cell immunity directed at HIV transgenes encoded by recombinant VV vectors displayed comparable polyfunctionality to CD8+ T cells reactive against VV itself (222).

8.2 CD4+ T CELL POLYFUNCTIONALITY

HIV has also revealed that polyfunctionality is an important aspect of CD4+ T cell immunity. Comparison of CD4+ T cell responses between LTNPs and progressors has demonstrated differences in their ability to produce cytokines. LTNPs have a higher frequency of CD4+ T cells that produce two or more cytokines based on the analysis of IFNγ, TNFα and IL-2 than progressors (224, 225, 245-247). In contrast, up to 50% of CD4+ T cells in progressors were found to only produce IFNγ (224, 225). These results suggest that control of HIV requires the development of multifunctional CD4+ T cell responses. Interestingly, the CD4+ T cell populations in patients on antiviral therapies also demonstrated increased polyfunctionality relative to the progressor population (245). These results beg the question of whether polyfunctionality is the cause or effect of reduced virus loads.

CD4+ T cell polyfunctionality has been found to correlate with protection against Leishmania major (LM). Darrah *et al*, found that in mice, the efficacy of LM vaccines could be predicted by the cytokine secretion profile of the CD4+ T cells produced by the vaccine (221). More specifically, the degree of protection against Leishmania challenge correlated with the frequency of CD4+ T cells that could produce IFNγ, TNFα and IL-2 (221).

Taken together, these studies suggest that polyfunctionality is a better measure of the T cell response than the magnitude of the antigen-specific T cell population. Therefore, investigations of novel vaccines need to consider all parameters.

9.0 A need for vaccine platforms that elicit robust cellular immunity

Although successful vaccines have been developed for an array of pathogens, we still lack effective vaccines for several serious diseases such as, HIV (3), malaria (2) and tuberculosis (248). To this point, traditional vaccine strategies designed to elicit neutralizing antibody responses for these agents have been largely unsuccessful (249-251). In the case of HIV, the envelope protein is the primary target for neutralizing antibodies. However, the conserved antibody epitopes are surrounded by glycosylation sites that mask them from neutralizing antibodies (252). Although, antibodies can be generated against these carbohydrate moieties, the glycosylation sites map to highly variable regions of the *env* protein, resulting in continual alterations in glycosylation patterns and the development of mutant viruses that can evade neutralizing antibodies

(253). In line with these observations, promising vaccination results were initially observed against the erythrocyte membrane protein 1 (EMP-1) from the most pathogenic strain of malaria, plasmodium falciparum. However, EMP-1 is highly diversified and extremely variable due to antigenic switching, limiting the number of strains that can be neutralized by antibody produced by a given variant of EMP-1 (254-256). Similarly, each year novel killed influenza virus vaccines must be created due to antigenic drift and antigenic shift of the hemagglutinin protein which can evade antibodies produced by vaccines from previous years (257). In summary, although neutralizing antibodies can be highly effective, due to constant changes within the target proteins, it would be necessary to regularly revise the vaccine formulation to match the existence strains. It is unlikely that such a strategy would work for pathogens such as HIV which exist as a complex collection of different strains within a given individual. While epitopes within structural proteins may be highly mutable, allowing pathogens to evade antibody responses, it has been suggested that epitopes within non-structural proteins that the viruses uses for replication may be less mutable and, thus, represent more stable targets for vaccine development. Recognition of non-structural proteins, which are largely intracellular, requires a T cell-based vaccine as antibodies cannot effectively identify proteins inside infected cells.

In the context of current vaccination platforms, live attenuated vaccines are the most effective at eliciting long-lived cellular immune responses (33, 258). However, the basic strategy of developing attenuated vaccines is not always an option for highly-pathogenic

agents due to the potential for reversion mutants within the vaccine inoculum. This conundrum has prompted the investigation of alternative vaccination platforms that fulfill the requirement of eliciting both humoral and cellular immunity while eliminating the risk of reversion mutants. One promising solution to this dilemma is the development of recombinant vectors, termed genetic vaccines. Viral vectors have shown the most promise because they readily infect recipient cells and are efficient at eliciting both cellular and humoral immune responses. Application of this approach involves introduction of pathogen specific genes or epitopes into the genome of a non-pathogenic vector (259). To increase the safety of these vector platforms, their ability to replicate *in vivo* and evade adaptive immunity can be reduced or terminated through the removal of genes critical for *in vivo* replication and immune evasion (259).

10.0 ADENOVIRUS AND THEIR USE AS RECOMBINANT VIRAL VECTORS

Recombinant adenoviruses have emerged as a highly-robust vaccination platform. Adenovirus (Ad) was first discovered in the adenoids of children and army personnel suffering from acute respiratory infection (260, 261). In immune-competent individuals, Ad infection results in a mild self-limiting infection, but can occasionally cause conjunctivitis and gastroenteritis in infants. In immunocompromised individuals, Ad infection can be fatal due to fulminant hepatitis, pneumonia or encephalitis. To date, 51 different human Ad serotypes have been identified which can be further classified into 6 different subgroups based on hemagglutination properties, genomic organization and

tumorigenic potential in rodents (262). In addition, Ads can infect numerous different vertebrates such as, humans, monkeys, pigs, cows, horses, sheep, dogs, and birds (262).

Ads are non-enveloped icosahedral shaped viruses, 90nm in diameter and contain a double stranded DNA genome of approximately 36kb (262). The faces of the icosahedral shaped shell (termed "the capsid") are primarily composed of 240 hexon capsomeres which form 12 facets of 20 hexons each and 12 penton capsomeres forming each of the vertices (263). The vertices are composed of a penton base attached to a protruding fibre. The fibre is composed of three domains, an N-terminal domain that binds to the penton base, a central shaft and a globular C-terminal knob that binds the primary receptor on host cells (263). Following initial cellular binding, the penton base binds to integrins in the cellular membrane, the fibre is removed and the Ad virion is endocytosed (262). Upon entry into the cell, the virion associates with microtubules, gets transported to the nucleus where it docks to a nuclear pore complex and the viral genome is imported into the nucleus and episomal viral transcription is initiated (262). The Ad genome encodes at least 11 genes and even more proteins due to extensive splicing of the RNA transcripts. Ad genes are grouped into two categories termed early and late as defined by the transcription of these genes before or after viral genome replication, respectively. The 6 early genes (E1-E4) encode proteins that are mainly involved with viral replication; whereas the 5 late genes (L1 – L5) encode structural proteins (262).

Advances in molecular biology enabled the engineering of recombinant Ad (rAd) vectors that express foreign gene(s) under the control of heterologous promoters. Most work with rAd vectors has focused on human Ad type 5 (HuAd5) as this virus has been extensively characterized and the expression system associated with recombinant HuAd5 is well-established (264). The HuAd5 capsid is extremely rigid and will only accept genetic material of $\leq$ 105% of the wild type genome, permitting insertion of approximately 2kb of exogenous DNA (265). To facilitate the incorporation of larger gene inserts, additional regions of the virus have been removed. The vectors that we, and many laboratories, employ have the E1 and E3 regions removed thereby allowing the insertion of up to 8kb of exogenous DNA (266). Deletion of the E1 region renders the virus replication-defective due to the critical role the E1 protein plays in initiating viral DNA replication (267). The E3 protein mainly acts to down-regulate host immune response, so the deletion of the E3 region does not affect the viral replication *in vitro* and perhaps might enhance its immunogenicity *in vivo (267)*. Consequently, propagation of the E1, E3-deleted rAd only requires E1 function which can be provided *in trans* by cell lines that constitutively express the E1 protein (268, 269). Much of the development of rAd vectors spawned from an interest in employing these viruses for gene replacement therapy. However, the rapid appearance of cellular immunity against the transgenes carried by E1, E3-deleted rAd vectors severely limits the durability of gene expression *in vivo*. While this is a hurdle for gene replacement therapy, it is an opportunity for vaccination (270, 271).

10.1 Advantages & Disadvantages Of rAd As A Vaccine Platform

Replication-deficient recombinant adenoviral vectors offer several advantages as a vaccination platform. For example, rAd can be grown to high titres in appropriate cell lines. rAd infects a variety of mammalian cell types including actively dividing and post-mitotic, quiescent cells (262). In addition, the rAd genome replicates episomally and does not integrate into the DNA of the host cell, thus minimizing the risk of disrupting crucial host genes(262). As well, the magnitude and the duration of the transgene expression can be manipulated by using various heterologous promoters. Finally, many preclinical and clinical trials using recombinant adenovirus-based vaccines have shown excellent efficacy and safety records (272-276).

Despite the aforementioned advantages, the efficacy of rAd-based vaccines can be severely limited due to the presence of pre-existing anti-Ad immunity (263, 264, 277). HuAd5 is the most widely studied and commonly used Ad vector, but is also highly prevalent in most human population. Neutralizing antibodies can be directed against fibre and hexon components; however, while antibodies directed against the fibre are responsible for *in vitro* neutralization, hexon-specific antibodies appear to be responsible for *in vivo* neutralization (278). The hexon protein is composed of 7 hypervariable regions which have been identified as the primary target of neutralizing antibodies. Recent reports found that swapping the hexon hypervariable regions from HuAd5 with a rare serotype HuAd48 overcame neutralizing immunity to HuAd5 (279). Another approach to overcome the inhibitory effects of neutralizing antibodies is coating rAd

virions with polymers to block antibody binding. To date, two polymers have been employed, polyethylene glycol (PEG) and poly-N-2-hydroxy-propyl methacrylamide (pHPMA) (280, 281). PEG coating does not inhibit the biological activity of the virus, while pHPMA inhibits infection. However, this problem has been overcome through the addition of cellular ligands such as fibroblast growth factor and vascular endothelial growth factor onto the coated vectors. One final method to circumvent pre-existing anti-rAd immunity is the use of serologically-distinct human and nonhuman adenoviruses. Rare human adenovirus serotypes 2, 6, 7, 11, 24, 34, 35 have been engineered for the expression of heterologous genes (282-287). As well, nonhuman adenoviruses such as those isolated from chimpanzees, pigs, cows, sheep, dogs and birds are being investigated as possible vaccine candidates (288-294). Although the use of serologically-distinct Ad vectors circumvents the problem of neutralizing antibodies, the immunogenicity of these rAd vectors may still be muted by cross reactive anti-Ad specific T cells. However, the effects Ad-specific T cells does not appear to be as significant as the humoral response (295).

10.2 Protective Cellular Immunity elicited by replication defective recombinant adenoviral vaccines

Recombinant Adenoviral vectors have received considerable attention as a recombinant vaccination platform because of their innate ability to elicit robust humoral and cellular immunity. This has fostered development of rAd vaccines against pathogens for which no vaccine exists and in an attempt to improve on vaccines with poor efficacy.

Furthermore, numerous rAd vaccines have been evaluated in murine models, primate models and human clinical trials.

The current vaccine for tuberculosis is Bacillus Calmette-Guerin (BCG). In children, BCG is effective at preventing *M.tb.* infection; however, for reasons that we don't completely understand, BCG immunization is ineffective in adults. Therefore extensive vaccine research is currently being conducted to improve *M .tb.* vaccine efficacy. Wang *et al* created a rHuAd5 vaccine that encodes the *M. tb* Ag85A gene (rAdAg85A) and have demonstrated that intranasal inoculation of rAdAg85A in mice elicits antigen specific CD8+ and CD4+ T cell responses against Ag85A (273). Furthermore, immunity generated by rAdAg85A immunization cleared *M. tb* infection more rapidly and significantly prevented *M.tb* dissemination more effectively than following BCG immunization. The superior protection elicited by rAd85A inoculation was found to result from the accumulation of cytolytic CD8+ and CD4+ T cells in the airway lumen (296). Similar experiments in macaques have demonstrated that prime boost immunizations with recombinant BCG and rHuAd35 expressing several *M.tb* epitopes generated CD4+ and CD8+ T cell responses that were larger in magnitude and displayed greater polyfunctionality compared to BCG immunization (297).

Recombinant adenoviral vectors have also been generated as vaccines against influenza. Experimental murine vaccine models have demonstrated that rAd encoding various influenza antigens elicit potent humoral and cellular immunity that is protective against

lethal influenza challenge. A study by Lo *et al*, demonstrated that an rHuAd5 vaccine was more protective compared to the killed influenza vaccine (298). Hoelscher *et al*, demonstrated that immunization with a rHuAd5 encoding the hemagglutinin of a H5N1 strain of influenza provided complete protection against a lethal challenge with a heterologous H5N1 strain; protection was correlated with the development of strong CD8+ T cell responses and there was no significant antibody cross-reactivity with the vaccine strain measured (272). These results further reinforce the importance of T cell-mediated immunity in vaccine-mediated control of influenza infection.

Although Ebola and Marburg viruses do not pose an immediate threat to the western world, they are among the most deadly diseases known to man with mortality rates as high as 90%. Sullivan *et al* demonstrated that immunization of macaques with rHuAd5 expressing the glycoprotein and nucleoprotein from Ebola alone or in combination with a plasmid DNA prime provided robust protection from lethal challenge (275, 276). In this case, protection correlated with the development of potent CD8+ T cell and antibody responses. These results have been recapitulated by other groups in both murine and primate models (299, 300). RAd vaccines against Marburg virus have displayed similarly promising results. Wang *et al* demonstrated that immunization of guinea pigs with a rHuAd5 expressing the glycoprotein from two strains of Marburg virus protected them from lethal virus challenge (301). Most notably, immunization of non-human primates with a rAd vaccine expressing numerous Ebola and Marburg virus antigens generated

protective immunity against 2 strains of Ebola virus and 3 strains of Marburg virus when at ministered at 1000 times the lethal dose (302).

Recombinant adenovirus vaccines have also shown some promise as a vaccine candidate against HIV. In primate SIV models, rAd vaccine given 3 times over the course of 24 weeks induced an antigen-specific CD8+ T cell level that was far greater than vaccinia virus-based vaccine given in the same manner (303). When plasmid DNA or vaccinia-virus primed mice were boosted with rHuAd5, the levels of CD8+ T cells as well as antibody response were greatly enhanced (303-305). Importantly, rAd vaccine induced CD8+ T cells were cytotoxic and produced IFN-γ upon short-term stimulation (303-305). In subsequent studies, it was found that potent CD8+ T cell response observed in vaccinated macaques correlated with protection from subsequent viral challenge and with preservation of the CD4+ T cell compartment post-challenge (306, 307). Based on these findings, rHuAd5 vectors expressing the gag pol and nef HIV genes were tested in humans. Initial reports indicated the vaccine generated HIV-specific CD4+ and CD8+ T cell responses that were detected in the majority of vaccinees at 4 weeks (308). Anti-HIV antibodies also developed in response to the vaccine but these antibodies were non-neutralizing (308). However, during phase II clinical testing the vaccine trials were halted because vaccinated subjects were not protected from HIV infection, and HIV viral loads were not reduced in comparison to non-vaccinated control subjects (309, 310). The reasons for the vaccine failure are not completely known. Although most of the vaccinees developed HIV-specific T cell responses, on average, these responses were

only 10-20% of the magnitude observed during natural HIV infection (309, 310). HuAd5 was the vector used for these studies and a large percentage of humans have pre-existing immunity which might have muted the immune response. Furthermore, only 3 T cell responses were observed against unique epitopes in pol, gag and nef, which may be insufficient to mediate protection (309, 310). Based on these findings, new proposals and suggestions have been put forward to enhance the immunity and efficacy of future HIV vaccines (311).

As a means of augmenting the immune response produced by rAd, replication-competent vectors, which maintain the E1 functions, have been evaluated as vaccines. The transgene expression cassettes in these vectors were introduced into the E3 region. Such vectors have been evaluated as HIV vaccine candidates in preclinical primate studies and have successfully induced HIV-specific CD8+ T cell response (312, 313). Lui *et al*, employed a heterologous rHuAd26 prime/rHuAd5 boost immunization protocol expressing SIV gag to generate cellular immunity in macaques (314). Compared to Ad5 immunization alone, the heterologous prime/boost regimen elicited T cell responses that were larger, more diverse and had a higher degree of polyfunctionality (314). In addition, following pathogenic SIV challenge prime/boost immunized macaques had lower levels of viremia and AIDS associated pathologies.

11.0 SCOPE AND IMPACT OF MY THESIS RESEARCH

To further our understanding of T cell immunity following immunization with rAd, my project has evaluated numerous aspects of CD8+ T cell and CD4+ T cell immunity. Contained within the body of the thesis are the following 4 manuscripts:

- *Manuscript #1.* The magnitude of the CD8+ T cell response produced by recombinant virus vectors is a function of both the antigen and the vector (Cell Immunol. 250: 55-67, 2007): To optimize CD8+ T cell immunity following rAd immunization we must understand how vector specific responses as well as the transgene influence CD8+ T cell immunity. This body of work analyzes the impact of both the vector and the transgene on CD8+ T cell immunity following recombinant viral immunization by directly comparing rAd and rVV vectors.

- *Manuscript #2.* Persistence of transgene expression influences CD8+ T cell expansion and maintenance following immunization with recombinant adenovirus (In revision at Journal of Virology): Our own data as well as others have identified persistent antigen presentation past the peak response following rAd immunization. This body of work investigated the impact of prolonged antigen presentation with regards to T cell priming, memory development, phenotype and functionality.

- *Manuscript #3.* CD8+ and CD4+ T cell responses produced by adenovirus vaccines are differentially influenced by antigen selection (to be submitted to the Journal of

Immunology): Compared to CD8+ T cell immunity significantly less is known regarding CD4+ T cell immunity following rAd immunization. In addition, it was unknown what impact prolonged antigen presentation has on rAd elicited CD4+ T cell immunity. This paper characterizes CD4+ T cell immunity following rAd immunization, and evaluates how transgene configuration impacts both CD4+ T cell and CD8+ T cell immunity.

- *Manuscript #4.* On the role of CD4+ T cells in the CD8+ T-cell response elicited by recombinant adenovirus vaccines (Mol Ther. 15: 997-1006, 2007): It is well established that CD4+ T cells have a critical role in CD8+ T cell immunity, however; the timing of CD4+ T cell help and observed defects in CD8+ T cell immunity vary between different models. This manuscript evaluates the timing and mechanism of CD4+ T cell help for CD8+ T cells following rAd immunization.

Furthering our understanding of T cell immunity produced by rAd vaccine is necessary to maximize their clinical utility. To this end, the results of my thesis have demonstrated that transgene configuration can influence the magnitude and kinetics of the CD8+ T cell response but has little impact on phenotype or functionality. With regards to CD4+ T cell immunity, transgene configuration can also influence response magnitude but has little impact on response kinetics or functionality. Moreover, it appears that longevity of transgene expression following infection has a definite impact upon the maintenance of CD8+ T cell immunity but play less of a role in CD4+ T cell immunity. The requirement

for transgene expression in sustaining CD8+ T cell memory is limited and the population appears to progress to a stage where it is independent of transgene expression. With regard to CD4+ T cell – CD8+ T cell interactions, CD4+ T cell help is required for maximal expansion of the CD8+ T cell population, but does not seem to influence the functionality of the CD8+ T cells elicited by recombinant adenovirus. These findings have contributed to our understanding of T cell immunity following rAd immunization as well as to our general comprehension of T cell biology.

- Chapter 2 –

The magnitude of the CD8+ T cell response produced by recombinant virus vectors is a function of both the antigen and the vector

James Millar, Dilan Dissanayake, Teng Chih Yang, Natalie Grinshtein, Carole Evelegh, Yonghong Wan and Jonathan Bramson

Prologue

RAd and rVV are highly effective at eliciting CD8+ T cell immunity and have shown great promise as vaccination platforms. However, little is known regarding their influence on the transgene specific CD8+ T cell population. Our comparison of these genetic vaccine platforms has revealed the following:

- RHuAd5 primarily elicits transgene specific CD8+ T cell immunity while CD8+ T cell responses elicited by rVV are primarily directed against viral targets.
- Transgene selection influenced the magnitude of the CD8+ T cell response by both vectors
- The functionality of the primary and secondary CD8+ T cell responses were similar for each vector
- Plasmid priming influenced the magnitude but not the functionality of CD8+ T cell responses elicited by both vectors.

The works contained within this study, where planned, executed and assembled by myself, with technical assistance from Dilan Dissanayake, Teng Chih Yang, Natalie Grinshtein and Carole Evelegh. Dr. Jonathan Bramson provided general supervision and along with Dr Yonghong Wan assisted with experimental design and interpretation of the results. The described in this chapter was published in:

The magnitude of the CD8+ T cell response produced by recombinant virus vectors is a function of both the antigen and the vector.

Millar J, Dissanayake D, Yang TC, Grinshtein N, Evelegh C, Wan Y, Bramson J.

Cell Immunol. 2007 Nov-Dec;250(1-2):55-67

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Cellular Immunology 250 (2007) 55–67

Cellular Immunology

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The magnitude of the CD8+ T cell response produced by recombinant virus vectors is a function of both the antigen and the vector

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Received 11 November 2007; accepted 17 January 2008
Available online 3 March 2008

Abstract

Virus-based recombinant vaccines have proven highly effective at generating protective CD8+ T cell responses. Multiple vector platforms are available, however, little is known about the relative influence of the different vectors on the transgene-specific CD8+ T cell population. To address this question, we compared several characteristics of the CD8+ T cell response elicited by recombinant adenovirus (rAd) and vaccinia virus (rVV). We found that following rAd immunization the transgene-specific CD8+ T cell response peaked around day 12 and was larger and more sustained than the response produced by rVV. In addition, the CD8+ T cell response generated by rAd was directed primarily against the transgene, whereas the CD8+ T cell response produced by rVV principally targeted the vector backbone. In addition, we also observed that transgene selection also impacted on the magnitude of the CD8+ T cell response elicited by both vectors. Despite differences in the magnitude of the anti-transgene CD8+ T cell response, both vectors elicited CD8+ T cell populations with similar cytokine production, functional avidity and cytolytic activity. In addition, plasmid priming prior to immunization with either rAd or rVV only impacted the magnitude of the transgene gene specific CD8+ T cell response. Our study demonstrates that both vector and transgene selection can influence the magnitude of the CD8+ T cell response, but they do not influence functionality.

Keywords: Adenovirus; Vaccinia virus; CD8+ T cell

1. Introduction

Cellular immunity mediated by CD8+ T cells is essential for the resolution of many diseases and infections [1–4]. CD8+ T cell responses play a central role in the elimination of intracellular pathogens, which is accomplished by both direct and indirect mechanisms [5]. Recombinant viruses, have proven to be useful tools for eliciting specific CD8+ T cell immunity, and are presently being investigated clinically as vaccination agents [6]. A complete understanding of the immune response elicited by recombinant virus vaccines is necessary to effectively apply these strategies clinically. While it is clear that the various vector platforms elicit quantitatively different CD8+ T cell responses, it still remains to be resolved how much is due to the transgene and how much is vector dependent.

The capacity of a CD8+ T cell to clear infected cells is related to a number of functional qualities, including: responsiveness to limiting amounts of MHC/peptide complex (i.e. functional avidity), cytotoxic activity and cytokine production [7–10]. Since the efficacy of the CD8+ T cell response is a reflection of multiple effector pathways, CD8+ T cell immunity must be evaluated using multiple parameters to provide a proper measure of quality. Our previous studies have focused on recombinant Adenovirus vectors (rAd). Adenovirus is a non-enveloped virus with a linear double-stranded DNA genome of approximately 35 Kb that encodes 35–40 genes [11]. The rAd employed in our study is replication-defective and possesses deletions

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doi:10.1016/j.cellimm.2008.01.005

of the E1 and E3 regions [12]. Genetic vaccines based on rAd have been used to immunize against Ebola [13], SARS [14], Herpes simplex virus 2 [15] and HIV [16–18], and the latter vaccines are currently being evaluated in clinical trials. We have recently reported that intramuscular immunization with rAd yields a CD8+ T cell population that peaks on day 12 post-immunization followed by a period of protracted contraction. Phenotypic evaluation of the memory CD8+ T cell population has demonstrated that it is primarily composed of effector memory cells ($CD44^{hi}CD62L^{lo}$) that either produce IFN-γ and TNF-α or only IFN-γ [19]. Furthermore, the CD8+ T cell population was able to control virus infection to undetectable levels as late as 90 days post-immunization.

To determine whether this CD8+ T cell phenotype and kinetics was unique to rAd, we have compared the anti-transgene CD8+ T cell population elicited by rAd to the CD8+ T cell populations elicited by recombinant Vaccinia Virus (rVV), a biologically distinct DNA virus. Vaccinia virus (VV) is an enveloped double-stranded DNA virus with a genome of approximately 200 kb that encodes approximately 200 genes [20]. VV was first employed as a vaccine against Smallpox, but has since been used as a vector to express heterologous genes [21]. The rVV used in our studies is replication-competent and the transgenes are inserted into the TK region. Similar to rAd, rVV vaccines have been shown to effectively generate protective CD8+ T cell responses against numerous agents and a number of rVV vaccines have been tested in clinical trial [22,23].

Interestingly, while we observed differences with regard to the kinetics and magnitude of the CD8+ T cell response elicited by the two vector systems, we only observed small functional differences among the CD8+ T cell populations. Thus, the functionality of the CD8+ T cell response elicited by these two vector systems does not appear to be influenced by the vector backbone, however, the kinetics of the transgene-specific CD8+ T cell response is influenced by both vector selection and transgene configuration.

2. Materials and methods

2.1. Plasmids and viruses

A number of antigens were used for these studies: (1) chicken egg ovalbumin (OVA), (2) ER-SIINFEKL, which is the SIINFEKL peptide linked to an ER-targeting element, and (3) SIINFEKL-Luc, which is a modified version of luciferase bearing the SIINFEKL epitope at the N-terminus. A plasmid vector (pSIINFEKL-Luc-003) and a rAd vector (AdSIINFEKL-Luc-004) expressing SIINFEKL-Luc have been described previously [24,25]. An rAd expressing ER-SIINFEKL (AdssOVA) was graciously provided by M. Bevan (U. Washington) [26]. The adenovirus *dl*70-4 is replication-competent and lacks the E3 region. The rVVs expressing OVA (rVV-OVA) and ER-SIINFEKL (rVV-ESOVA) were graciously provided by Jonathan Yewdell (NIAID, Bethesda, MD) [27]. rVV-β-gal expressing the *Escherichia coli* β-galactosidase was a kind gift from N. Restifo (NCI, Bethesda, MD). VV-WR is wild type vaccinia virus. To generate rVVs expressing either SIINFEKL-Luc or Alan (a cDNA similar to SIINFEKL-Luc where KAVYNFATM is linked to the N-terminus of luciferase), the cDNAs were cloned into pSC11 and rescued according to the method of Moss et al. [28]. Since some of these vectors were produced in our laboratory while others were obtained from external sources, the nomenclature is not consistent among the recombinant viruses. For the sake of simplicity, all the vectors will be referred to hereafter by the prefixes "rAd" or "rVV" to denote the vector and the suffixes "OVA", "ER-SIINFEKL", "SIINFEKL-Luc", "β-gal" or "Alan" in reference to the antigen they express (ex. rAd-OVA, rVV-ER-SIINFEKL, rVV-SIINFEKL-Luc).

2.2. Animals and immunizations

All experiments were conducted using female C57BL/6 mice purchased from Charles River Breeding laboratories (Wilmington, MA). Mice were immunized with 10^5–10^8 pfu of rAd or rVV diluted in sterile PBS and injected intramuscularly. For plasmid immunization, 100 μg pSIINFEKL-Luc was injected intramuscularly followed by electroporation as described previously [29].

2.3. Cell culture

All cells were cultured using Falcon plasticware (BD Biosciences, Franklin Lakes, NJ). C57SV cells and lymphocytes were cultured in cRPMI [RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (Life Technologies), 1 mM HEPES, 50 μM β-mercaptoethanol (Life Technologies)].

2.4. Flow cytometry reagents

The following flow cytometry antibodies were purchased from BD Pharmingen (Franklin Lakes, NJ). Anti-CD8α (clones 53-6.7) labelled with FITC or PE-Cy5, anti-IFN-γ (clone XMG1.2) labelled with PE or APC, anti-TNF-α (clone MP6-XT22) labelled with FITC, anti IL-2 (clone JES6-5H4) labelled with PE, anti-CD62L (clone MEL-14) labelled with FITC and PE, anti-CD43 (clone 1B11) labelled with PE and anti-CD44 (clone 1M7) labelled with PE-Cy5. APC-labelled granzyme B (clone Gb12) was purchased from CALTAG (Burlingame, CA). FITC-labelled CD127 (clone A7R34) was purchased from eBiosciences (San Diego, CA). PE and APC-labelled K^b/SIINFEKL tetramers were obtained from the Molecular Biology core at the Trudeau Institute (Saranac Lake, NY). The 25.DI.16 hybridoma [30] which produces an antibody specific for K^b/SIINFEKL was kindly provided by J. Yewdell (NIAID, Bethesda,MD). Antibodies produced by

25.DI.16 were purified from spinner culture supernatants and purified on Protein G-sepharose columns (Amersham Pharmacia). Samples were analyzed using either a FACScan, LSRII or Facs Canto flow cytometer.

2.5. Preparation of tissues for flow cytometry

Spleens, lymph nodes (popliteal, inguinal and ileac), femur, tibia, blood, and lung cells were harvested from mice sacrificed at various time points following immunization. Splenic cell suspensions were prepared by disrupting the spleen between the frosted tips of two glass slides. Lymph node cell suspensions were generated by crushing them against an etched Petri dish with a plunger. Lung cell suspensions were generated by dicing whole lungs and incubating them in HBSS containing 150 U/ml of collagenase type 1 from Invitrogen (Carlsbad, CA) for 1 h at 37 °C. Digested lung tissues were then pressed through a 70 μm cell strainer (BD) to obtain single cell suspensions. Bone marrow cell suspensions were generated by flushing the femur and the tibia with a syringe containing PBS. Red blood cells were lysed from all preparations by treatment with 0.15 M NH_4Cl lysis buffer for 5 min (peripheral blood samples were treated twice with lysis buffer). For antibody and tetramer staining, all cell suspensions were aliquoted into 96-well round-bottomed plates at 2×10^6 cells/well (BD Pharmingen). Prior to all antibody and tetramer staining, samples were incubated at 4 °C for 15 min with Fc block (Clone 2.4G2, BD Pharmingen) diluted in FACS buffer (0.5% BSA in PBS).

2.6. Tetramer staining

This method has been described by our group previously [25].

2.7. Intracellular cytokine staining

Intracellular cytokine was visualized using a protocol we described previously [25].

2.8. Preparation of virus-infected target cells

To prepare virus-infected targets, C57SV cells were plated at 2.5×10^6 cells per 10-cm tissue culture dish and infected with *dl*70-4 (MOI = 50) or VV-WR (MOI = 45) for 30 min. Target cells were harvested 18 h after Ad infection and 4 h following VV infection.

2.9. Functional avidity

Splenocyte samples were restimulated using serial 10-fold dilutions of SIINFEKL, ranging from 1 μM to 1 pM and stained for intracellular IFN-γ as described above. Functional avidity was determined as the peptide concentration that resulted in stimulation of 50% of the maximum number of IFN-γ^+ $CD8^+$ T cells [31].

2.10. Measurement of K^b/SIINFEKL complexes following virus infection

C57SV cells were seeded into six-well plates and infected at 90% confluence with rAd-SIINFEKL-Luc, rAd-ER-SIINFEKL, rVV-SIINFEKL-Luc or rVV-ER-SIINFEKL at MOIs ranging from 1 to 50. Cells were harvested 24 h after infection and K^b/SIINFEKL complex and luciferase levels were determined. To measure K^b/SIINFEKL complexes, cells were washed in FACS buffer and stained with 25.DI.16 [30]. Bound 25.DI.16 antibody was visualized by addition of biotinylated Goat anti-mouse anti-IgG1 (Southern Biotechnology Associates, Birmingham, AL), and followed by streptavidin-linked PE-Cy5 (BD Pharmingen). To measure luciferase levels, $1.2–1.5 \times 10^6$ cells were lysed in 500 μl of 1× Reporter Lysis Buffer (Promega) and assayed as described previously [32]. The amount of luciferase in each lysate was determined using a standard curve using recombinant luciferase (Sigma). To determine the percentage of virus-infected cells, a parallel set of wells were infected with rAd and rVV expressing β-gal, loaded with the fluorescent β-gal substrate by hypo-osmotic shock, FDG (Molecular Probes), and analyzed by flow cytometry.

2.11. In vivo cytotoxicity assay

Single cell, red blood cell depleted splenic suspensions were obtained from naïve B6.PL-Thy1.1 mice, re-suspended at a concentration of 20×10^6 cells/ml in PBS 10%FBS and loaded with peptide (1 μg/ml) for 1 h at 37 °C. Two peptide-loaded populations were prepared: one with SIINFEKL and the other with KAVYNFATM to provide Ag-specific and Ag-non-specific targets, respectively. Cells were then washed with PBS/10% FBS and re-suspended in PBS/10% FBS at 10×10^6 cells/ml. Ag-specific targets were labelled with a final concentration of 5 μM CFSE while antigen non-specific targets were left unlabelled. The two target cell populations were mixed at a 1:1 ratio and adoptively transferred into mice. Four hours later, lymphocytes were isolated from lymph nodes and spleens. Cells were prepared for flow cytometry as previously described and stained with anti-Thy1.1-PE (clone OX-7, BD Pharmingen). Cells were analyzed by flow cytometry for Thy1.1 and CFSE. Generally 500,000 events were collected per sample. Specific lysis was determined using the calculation described by Coles et al [33].

2.12. Statistical analysis

Statistical analysis was conducted using Microsoft excel on log transformed data to normalize variations. Differences were considered significant at $p < 0.05$. Data are presented as means ± SEM.

3. Results

3.1. Measuring the transgene-specific CD8+ T cell response elicited by rAd and rVV vectors

To compare the anti-transgene CD8+ T cell populations generated by rAd and rVV vectors, we monitored the CD8+ T cell response to SIINFEKL, a well-defined K^b-binding epitope. To provide insight into the impact of antigen structure on immunization, three different antigens containing SIINFEKL (Table 1 and described in Materials and methods) were used. Mice were immunized intramuscularly with rAd and rVV expressing each antigen over a range of doses and transgene-specific CD8+ T cell responses were monitored using either intracellular cytokine staining or tetramer analysis since we have found that they generate comparable results. Initially, we monitored the CD8+ T cell response in the blood using tetramers (Fig. 1). The most obvious differences between the vaccinations were the kinetics and magnitudes of the SIINFEKL-specific CD8+ T cell response. Whereas the response to the rVV vectors peaked at day 7, similar to previous reports [34], the CD8+ T cell response peaked at day 14, consistent with previous data from our lab [25]. At the peak of the response, the CD8+ T cell population elicited by rAd-ER-SIINFEKL and rAd-OVA represented approximately 5% and 25% of total CD8+ T cells, respectively (Fig. 1A and C), while the SIINFEKL-specific populations elicited by the corresponding rVV vectors ranged between 0.5%

Table 1
Transgene formulations expressed in both rAd and rVV vectors

Name	Schematic	Description
SIINFEKL-Luc	$OVA_{257-264}$ Luciferase	CD8+ T cell epitope of chicken egg ovalbumin fused to N-terminus of firefly luciferase
ER-SIINFEKL	ER Targeting Sequence $OVA_{257-264}$	CD8+ T cell epitope of ovalbumin linked to an endoplasmic reticulum targeting sequence.
OVA	OVA	The full length ovalbumin protein

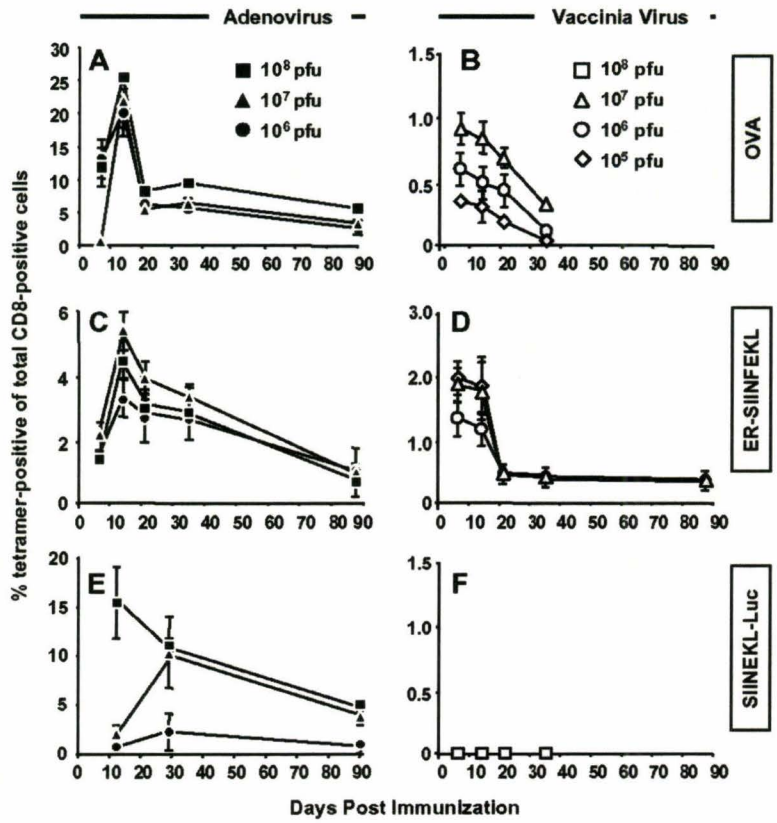

Fig. 1. Tetramer analysis of SIINFEKL specific CD8+ T cells in the blood following IM immunization with different pfu's of rAd or rVV. C57BL/6 mice were immunized with either rAd or rVV. Mice were bleed at various time points post-immunization and the frequency of antigen-specific CD8+ T cells was assessed by staining with K^b/SIINFEKL tetramer. Closed symbols represent mice immunized with rAd and open symbols represent mice immunized with rVV. (A) rAd-OVA; (B) rVV-OVA; (C) rAd-ER-SIINFEKL; (D) rVV-ER-SIINFEKL; (E) rAd-SIINFEKL-Luc; (F) rVV-SIINFEKL-Luc. Squares, 10^8 pfu; triangles, 10^7 pfu; circles, 10^6 pfu; diamonds, 10^5 pfu. Each point represents means ± SEM for 5 10 mice.

and 2.0% of the total peripheral CD8+ T cell population (Fig. 1B and D). Strikingly, even though the SIINFEKL-Luc antigen provoked a robust CD8+ T cell response following rAd immunization (upwards of 15% of total CD8+ T cells; Fig. 1E), we were unable to measure any SIINFEKL-specific CD8+ T cells following rVV-SIINFEKL-Luc immunization, even when doses of 10^8 pfu were used (Fig. 1F). Over the range of doses employed in this study (10^6–10^8 pfu), we observed minimal differences in the frequencies of blood–borne tetramer-positive CD8+ T cells following vaccination with the vectors expressing ER-SIINFEKL and OVA while the response to rAd-SIINFEKL-Luc showed a marked dose-dependence, particularly at the early time points (Fig. 2E).

To determine whether the minimal dose-dependence observed in Fig. 1 was a unique property of the blood–borne CD8+ T cells, we also enumerated the CD8+ T cells in peripheral tissues [draining lymph nodes (DLN), spleen, lungs and bone marrow (Fig. 1). Consistent with the results from peripheral blood, rVV-SIINFEKL-luc immunization did not elicit measurable levels of SIINFEKL-specific CD8+ T cells in the spleen or lymph nodes (Fig. 6B and data not shown), so we have only presented comparative data of the primary response following immunization with viruses expressing ER-SIINFEKL and OVA (Fig. 2). Tissues were harvested at the peak of the CD8+ T cell response (day 7 for rVV and day 12 for rAd) and SIINFEKL-specific CD8+ T cells were enumerated based on intracellular staining of IFN-γ following stimulation with specific peptide. The CD8+ T cell populations elicited by both rVV and rAd exhibited comparable distribution among the analyzed tissues. Unlike the analysis of peripheral blood lymphocytes, enumeration of CD8+ T cells in the tissues revealed a dose-dependent relationship that was evident for all the viruses but to different extents. Differences in the CD8+ T cell responses elicited by rAd-OVA ranged by 3- to 5-fold, depending upon the tissue. Increasing the dose of rVV-OVA resulted in a 3- to 10-fold increase in SIINFEKL-specific CD8+ T cells the lungs and spleen (Fig. 2). The CD8+ T cell response generated by rVV-ER-SIINFEKL was found to be less affected by virus dose, as increasing the dose from 10^6 to 10^7 pfu only

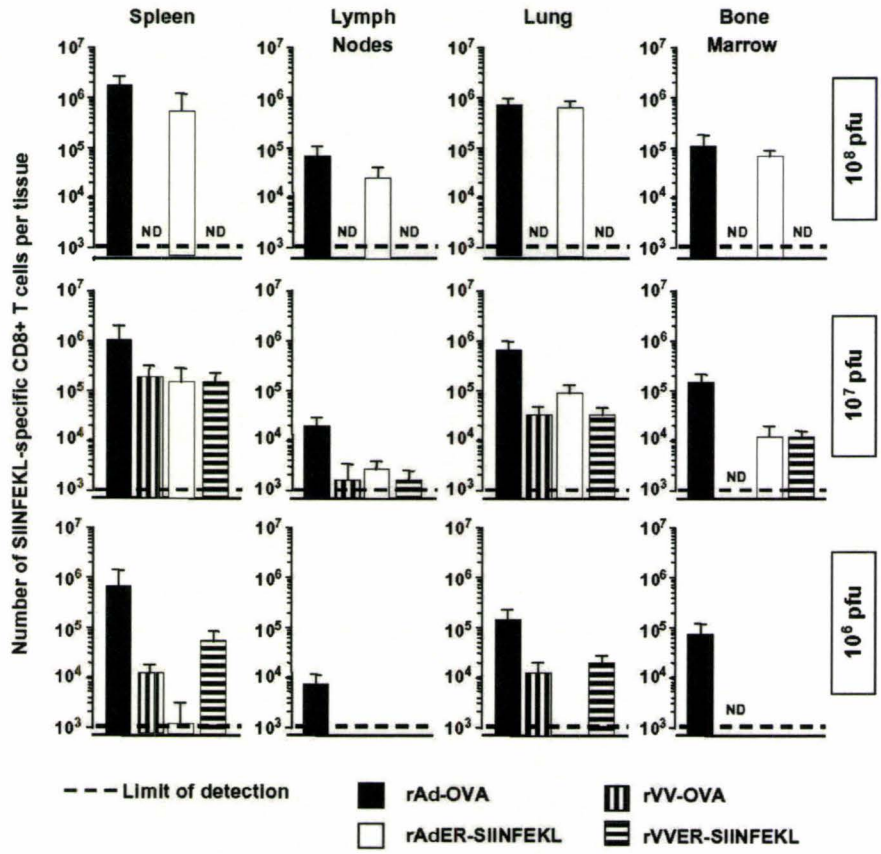

Fig. 2. Enumeration and distribution of SIINFEKL-specific CD8+ T cell responses following rAd and rVV immunization. C57BL/6 mice were immunized IM with various doses of rAd-OVA (closed bars), rAdER-SIINFEKL (open bars), rVV-OVA (vertical hatches), or rVVER-SIINFEKL (horizontal hatches). RAd-immunized mice were sacrificed 12 days post-immunization and rVV-immunized mice were sacrificed 7 days post-immunization. SIINFEKL-specific CD8+ T cells were enumerated using IFN-γ ICS. Each bar represents means ± SEM for 4 5 mice per group. ND, not determined.

resulted in a 2-fold elevation in the spleen and lungs. The most striking differences were observed following rAd-ER-SIINFEKL immunization, minimal numbers of antigen-specific CD8+ T cells were observed at the low dose (10^6 pfu) while cell numbers at the high dose (10^8 pfu) were comparable to the mice immunized with rAd-OVA. Thus, each vector/transgene combination seems to have unique properties that will influence the magnitude of the CD8+ T cell response. Also, the frequency of antigen-specific CD8+ T cells detected by tetramer in the blood does not appear to be an accurate reflection of the peptide-responsive CD8+ T cells identified by ICS in the tissues.

3.2. K^b/SIINFEKL presentation following rAd and rVV infection

To be certain that the absence of a SIINFEKL-specific CD8+ T cell response following rVV-SIINFEKL-Luc immunization was not due to a defect in processing the SIINFEKL peptide from this antigen when expressed by rVV, we infected C57SV cells, a C57Bl/6 murine fibroblast cell line *in vitro* and monitored the surface expression of SIINFEKL using an antibody specific for the SIINFEKL/K^b complex (Fig. 3). C57SV cells were infected with either rAd (rAd-SIINFEKL-luc or rAd-ER-SIINFEKL) or rVV (rVV-SIINFEKL-luc or rVV-ER-SIINFEKL) using MOIs of 1, 5 or 50. Cells were harvested at various times post-infection and K^b/SIINFEKL levels were measured using a specific antibody (Fig. 4 and data not shown). Following infection with either rVV or rAd, the highest levels of K^b/SIINFEKL were observed at 24 h post-infection with no further increase at 36 h (data not shown). At an MOI of 50 with either virus, 100% of the C57SV cells were infected based on parallel experiments using infection of viruses expressing β-galactosidase followed by staining with FDG (data not shown), so we have only shown the results at this dose since they reflect populations where all cells express the SIINFEKL transgenes. A number of interesting points can be made from this data. First, the ER-SIINFEKL transgenes load K^b with more peptide that the SIINFEKL-Luc transgenes. Second, rVV-SIINFEKL-Luc yields more K^b/SIINFEKL complexes than rAd-SIINFEKL-Luc which was consistent with the luciferase levels in these cells (61.2 μg Luc/10^6 cells following rVV infection and 3.2 μg Luc/10^6 cells following rAd infection). Thus, the failure to observe SIINFEKL-specific CD8+ T cells following rVV-SIINFEKL-Luc immunization is not due to inappropriate processing of the SIINFEKL epitope but may be due to insufficient gene expression levels in vivo. These results also demonstrate that the magnitude of the CD8+ T cell response *in vivo* is not directly related to the efficiency of K^b loading by the vaccine.

3.3. Virus-specific CD8+ T cell responses

Another possible explanation for the reduced levels of SIINFEKL-specific CD8+ T cells elicited by rVV may be

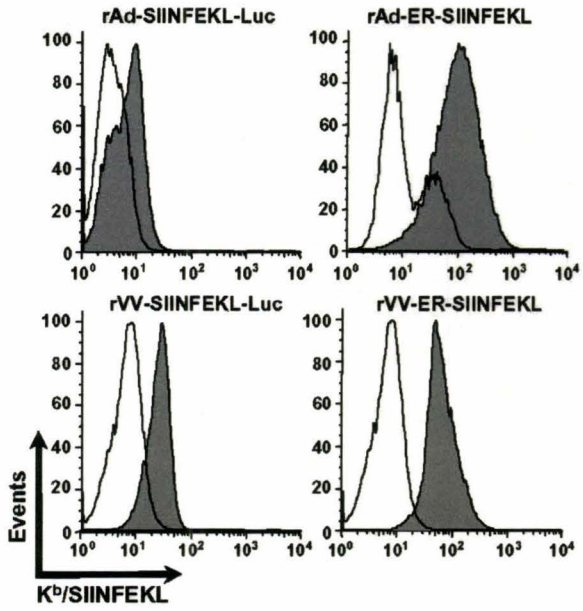

Fig. 3. K^b/SIINFEKL presentation following rAd and rVV infection. C57SV cells were infected with rAd-SIINFEKL-Luc, rAd-ER-SIINFEKL, rVV-SIINFEKL-Luc and rVV-ER-SIINFEKL at an MOI of 50. Cells were harvested 24 h later and assayed for SIINFEKL/K^b complexes using biotinylated 25-D1.16 antibody and streptavadin-PE-Cy5 (shown in the figure as the shaded gray histograms). The black line represents control samples stained with only streptavidin-PE.

that the intramuscular route is a poor route for immunization with rVV. Since the CD8+ T cell response against SIINFEKL is only a portion of the overall CD8+ T cell response generated by infection with the recombinant viruses, we also measured the CD8+ T cell response against the vector backbone. To this end, C57Bl/6 mice were immunized with either rVV-SIINFEKL-luc or rVV-ER-SIINFEKL and sacrificed 7, 14 or 21 days later. Virus-specific CD8+ T cells were evaluated in the spleen, lungs and DLNs by ICS. The peak VV-specific CD8+ T cell response following immunization with 10^7 pfu rVV-SIINFEKL-luc was observed at day 7 ($3.6 \times 10^6 \pm 0.5 \times 10^6$ cells/spleen; Fig. 4A) similar to the anti-transgene response. Similarly, following 10^7 pfu rVV-ER-SIINFEKL immunization, sizeable VV-specific CD8+ T cells responses were observed in the spleen ($1.02 \pm 0.2 \times 10^6$ cells/spleen; Fig. 4B). VV-specific CD8+ T cell responses of a similar magnitude were also observed following immunization with rVVs expressing different transgenes (Alan and β-gal; Fig. 4C). The VV-specific CD8+ T cell response did not vary much across the doses employed in our study indicating that the strong anti-vector response was not a dose-dependent phenomenon. These results demonstrate that intramuscular delivery is an efficient route for rVV immunization. Additionally, these data suggest that the low transgene-specific CD8+ T cell responses observed following rVV immunization are likely a result of dominance by epitopes from the vector backbone.

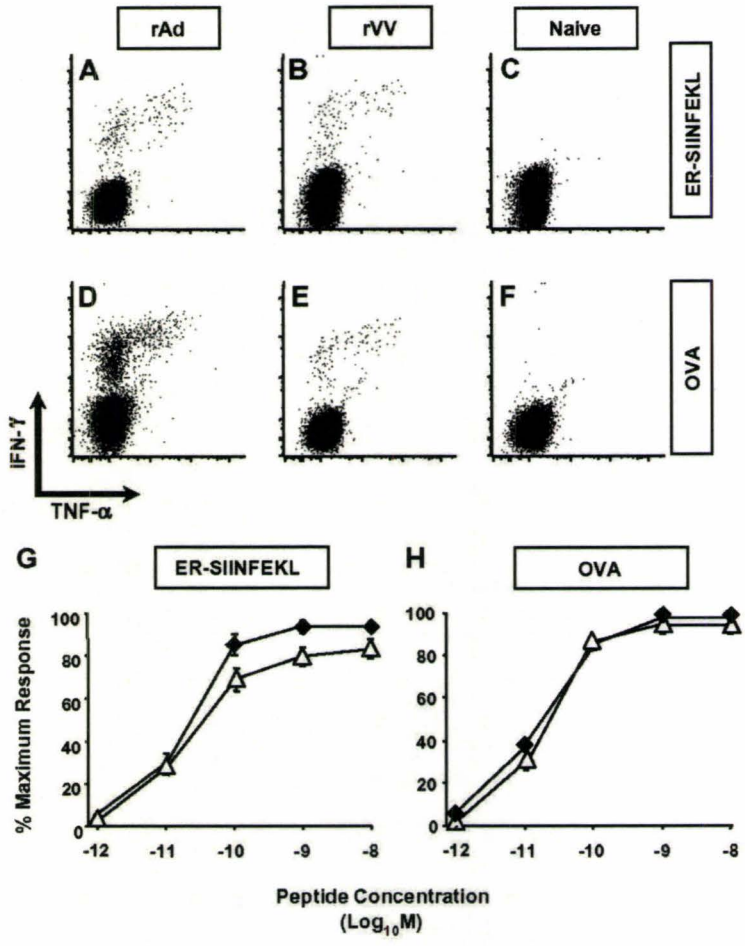

Fig. 5. Functional characterization of the SIINFEKL-specific CD8+ T cells following rAd and rVV immunization. (A F) IFN-γ and TNF-α production in the splenocyte population following SIINFEKL stimulation was measured by flow cytometry at the peak of the response following immunization with (A) rAd-ER-SIINFEKL, (B) rVV-SIINFEKL, (D) rAd-OVA, or (E) rVV-OVA. As negative controls, we have included naïve mice (Panels C and F). The data shown was gated first on CD8+ cells. (G H) C57BL/6 mice were immunized IM with rAd (closed diamonds) or rVV (open triangles) expressing either the ER-SIINFEKL (G) or the FL-OVA (H) transgene. At the peak of the CD8+ T cell response, splenocytes were stimulated with serial dilutions of SIINFEKL peptide and IFN-γ positive cells were determined by ICS. Results are displayed as the percent of the maximum response elicited by the highest concentration of peptide. Each point represent means ± SEM for 4 5 mice.

Table 2
Ratio of IFN-γ/TNF-α double positive cells to IFN-γ positive cells

Virus	Dose (PFU)	Frequency of IFN-γ/TNF-α positive cells relative to total IFN-γ positive cells (%)		
		Spleen	Lymph nodes	Lung
Ad-OVA[a]	10^8	29 ± 4	28 ± 3	32 ± 1
	10^7	15 ± 2	17 ± 6	23 ± 4
	10^6	21 ± 14	13 ± 2	19 ± 7
AdER-SIINFEKL[a]	10^8	23 ± 2	39 ± 6	28 ± 2
	10^7	39 ± 4	10 ± 9	37 ± 6
rVV-OVA[b]	10^7	60 ± 5	43 ± 7	50 ± 3
	10^6	49 ± 16	ND	52 ± 7
rVVER-SIINFEKL[b]	10^7	57 ± 3	36 ± 11	50 ± 5
	10^6	52 ± 4	28 ± 8	48 ± 4

[a] Mice were sacrificed 12 days post-immunization.
[b] Mice were sacrificed 7 days post-immunization.

at the peak of their respective transgene-specific CD8+ T cell response. The populations looked similar after immunization with any of the viruses (mainly $CD44^{hi}$, $CD43^{hi}$ and $CD62L^{lo}$) and the phenotype was not influenced by dose (data not shown).

As a final measure of CD8+ T cell function, we conducted an *in vivo* CTL assay using C57BL/6 mice immunized with 10^7 pfu rAd-ER-SIINFEKL or rVV-ER-SIINFEKL since similar frequencies of SIINFEKL-specific CD8+ T cells were measured at the peak of the response (Fig. 2). Comparable cytolytic activity was observed in the lymph nodes following immunization with rAd and rVV (Fig. 7A–C), however, significantly greater activity was measured in the spleen in the rVV group compared to the rAd immunized mice ($p < 0.05$) (Fig. 7D–E).

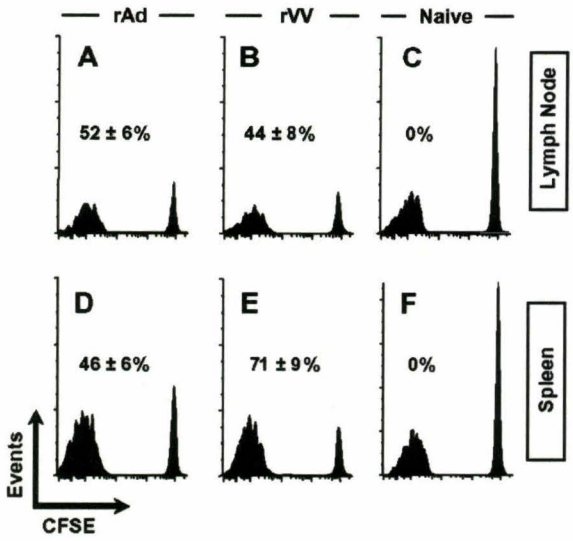

Fig. 6. *In vivo* lytic activity of SIINFEKL-specific CD8+ T cells elicited by rAd and rVV. C57BL/6 mice were immunized IM with 10^7 pfu of rAdER-SIINFEKL (A and D) and rVV-ER-SIINFEKL (B and E). At the peak of the SIINFKEL-specific CD8+ T cell response, 5×10^6 CFSE-labeled, SIINFEKL-pulsed and 5×10^6 CFSE-negative, KAVYNFATM-pulsed Thy1.1 positive splenocytes were adoptively transferred IV into immunized mice. Mice were sacrificed 4 h post transfer, Draining lymph nodes (A C) and spleens (D F) were harvested. The data shown was gated on Thy1.1-positive cells. The average percent killing for each group (means ± SEM; $n = 3$) is displayed in each panel.

3.5. Impact of plasmid priming on SIINFEKL specific CD8+ T cell responses following rVV and rAd immunizations

Since virus vectors have also been explored extensively as agents to boost vaccine responses, we examined the CD8+ T cell population elicited by rVV and rAd in mice previously immunized with plasmid DNA. Although immunization with rVV-SIINFEKL-Luc alone did not generate detectable levels of SIINFEKL-specific CD8+ T cells, when mice were immunized with pSIINFEKL-Luc followed by rVV-SIINFEKL-Luc, antigen-specific CD8+ T cells levels greater than 10^6 cells/mouse were observed at the peak response and high levels of CD8+ T cells were sustained for at least 3 weeks (Fig. 7B). Similar observations were made following immunization with rVV-ER-SIINFEKL (data not shown). Plasmid priming with pSIINFEKL-Luc followed by rAd-SIINFEKL-luc immunization dramatically increased the SIINFEKL specific CD8+ T cell response at day 7 relative to non-primed mice (Fig. 7A). However, at later time points following rAd-SIINFEKL-luc immunization, the number of SIINFEKL specific CD8+ T cells in the plasmid-primed mice was comparable to non-primed mice (Fig. 8A). Interestingly, the anti-vector CD8+ T cell response was not influenced by the presence of pre-existing SIINFEKL-specific CD8+ T cells generated by the plasmid priming (Fig. 7C and D).

Priming with pSIINFEKL-Luc did not appear to influence cytokine production by the CD8+ T cells (Fig. 5I–K) and MFI analysis demonstrated that activated SIINFEKL specific CD8+ T cells using either immunization protocol produced similar levels of IFN-γ and TNF-α as the CD8+ T cells elicited in the primary response to rAd and rVV (compare Fig. 7 to Fig. 4). Finally, we observed no difference in the functional avidity of SIINFEKL specific CD8+ T cells elicited by either rAdSIINFEKL-Luc or rVVSIINFEKL-Luc (Fig. 7E).

4. Discussion

Recombinant viral vectors are being increasingly evaluated as a vaccination platform for intracellular pathogens [1,36,37]. These investigations have led to the assessment of many different vector systems that induce protective CD8+ T cell responses. Although the principles of each strategy are similar, vectors vary in characteristics such as transgene expression, tissue tropism, and immunogenicity, all of which directly influence vaccination efficacy [6]. The present study has examined differences in CD8+ T cell responses generated by immunization with rAd and rVV. We found that rAd was capable of eliciting higher levels of transgene-specific CD8+ T cells than rVV, consistent with previous studies [16,25,37,38] and these differences did not appear to be due to differential lymphocyte circulation. Our results suggest that rAd and rVV vectors may actually saturate the anti-transgene CD8+ T cell response at different levels. Interestingly, the number of SIINFEKL-specific CD8+ T cells in the spleen at the peak of the response following immunization with any of the rVV used in this study did not get much higher than 10^5 cells/spleen, while immunization with rAd vectors readily achieved levels of $\geqslant 10^6$ cells/spleen. These results were not unique to SIINFEKL-specific CD8+ T cells because we observed similar differences using vectors that elicit CD8+ T cells against KAVYNFATC, an immunodominant epitope of LCMV GP (J. Millar and J. Bramson, unpublished results). Further evidence that the viruses may "saturate" the transgene-specific CD8+ T cell response comes from the studies comparing Ad-OVA to Ad-SIINFEKL-Luc. While at low doses of vector (10^6 pfu/mouse), the SIINFEKL-specific response elicited by rAd-SIINFEKL-Luc was several orders of magnitude lower than the response generated by rAd-OVA, at high doses of vector (10^8 pfu/mouse) both vectors elicited comparable responses (Fig. 1). These results were due to a greater than 2 log increase in CD8+ T cells elicited by rAd-SIINFEKL-Luc across this dose range, whereas the response generated by rAd-OVA varied by only 2- to 3-fold. In contrast to both rAd-OVA and rAd-SIINFEKL-Luc, rAd-ER-SIINFEKL generated a maximum response that was 2- and 4-fold lower respectably. In contrast, rVV-ER-SIINFEKL produced more antigen-specific CD8+ T cells at 10^6 pfu compared to rVV-OVA, both vectors elicited comparable responses at 10^7 pfu (Fig. 1). Thus,

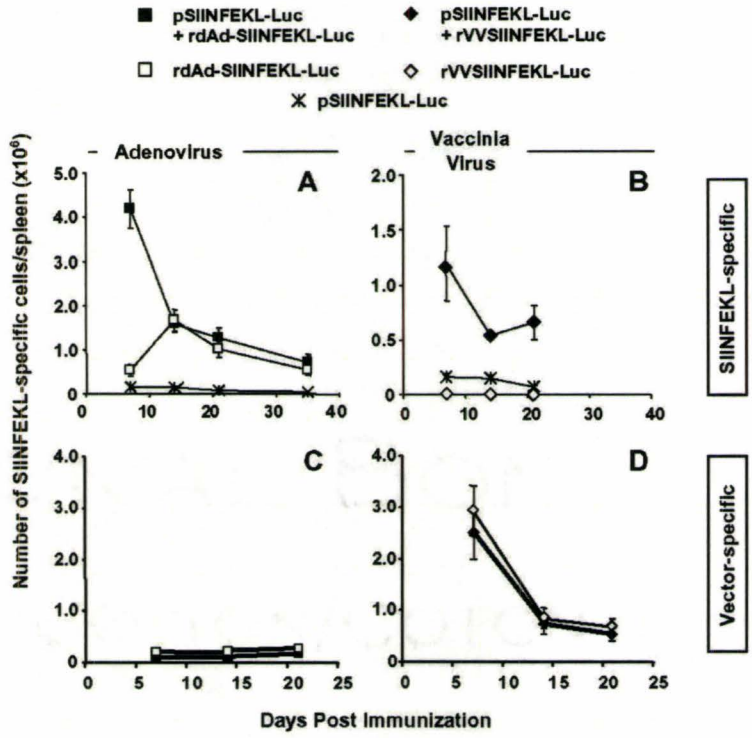

Fig. 7. SIINFEKL-specific and vector-specific CD8+ T cells following recombinant virus immunization with or without pSIINFEKL-Luc priming. C57BL/6 mice were immunized IM with 100 μg of plasmid pSIINFEKL-Luc, 21 day post-plasmid immunization, mice were immunized IM with either 10^8 pfu rAd-SIINFEKL-Luc or 10^7 pfu rVV-SIINFEKL-Luc. Mice were sacrificed 7, 14, 21 or 35 days post virus immunization and antigen-specific CD8+ T cells were enumerated using IFN-γ ICS. Panels A B represent the SIINFEKL-specific responses and Panels C D represent the vector-specific responses. (A and C) Mice were immunized with pSIINFEKL-luc (stars), AdSIINFEKL-Luc (open squares) or pSIINFEKL-Luc/AdSIINFEKL-Luc (closed squares). (B and D) Mice were immunized with pSIINFEKL-Luc (stars), rVV-SIINFEKL-Luc (open diamonds) or pSIINFEKL-Luc/rVV-SIINFEKL-Luc (closed diamonds). Each point represent means ± SEM for 5 10 mice.

the magnitude of the anti-transgene CD8+ T cell response is a result of a combination of factors including antigen structure, vector and dose. However, these results indicate that unknown factors may limit the absolute level of transgene-specific CD8+ T cells such that quantitative differences must also be considered in the context of an immunization strategy.

In addition, differences in transgene-specific CD8+ T cell response magnitude appear to be due to altered immunodominance of epitopes derived from the transgene relative to the vector backbone. This is not altogether surprising when one considers the differences between these vectors with regard to viral gene expression and lifecycle. The rVV encodes approximately 200 viral genes and is replication-competent, therefore, the viral proteins are being expressed at high levels following infection. In contrast, rAd expresses fewer viral genes (~30) and is replication-deficient. Thus, there are likely many more CD8+ T cell targets provided by the rVV backbone relative to the rAd. The large number of VV-specific epitopes increases the likelihood that the anti-transgene response will be reduced following rVV immunization due to immunodominance of the vector-specific response. Furthermore, Fischer et al., recently demonstrated that inhibition of endogenous VV gene expression resulted in amplification of the transgene-specific CD8+ T cell responses [39]. Therefore, it appears that a threshold may exist for presentation of peptide epitopes to elicit a transgene-specific CD8+ T cell response. Failure to exceed this threshold results in an inability to generate a measurable transgene-specific CD8+ T cell response following rVV immunization.

The lack of primary SIINFEKL-specific CD8+ T cell response following immunization with rVV-SIINFEKL-Luc was unexpected. Our inability to measure a SIINFEKL specific CD8+ T cell response was not due to a defect in the processing and presentation of the SIINFEKL eptiope. Furthermore, boosting with rVV-SIINFEKL-Luc evoke a robust SIINFEKL-specific population confirming that this vector provides the SIINFEKL epitope in vivo. Comparison of K^b/SIINFEKL presentation *in vitro* following rVV-SIINFEKL-luc and rVV-ER-SIINFEKL infection (Fig. 3), revealed rVV-SIINKFEL-luc generated lower numbers of SIINFEKL/K^b complexes compared to rVV-ER-SIINFEKL. Therefore, in consideration of the observation that the CD8+ T cell response following rVV infection is dominated by VV-specific CD8+ T cells, the

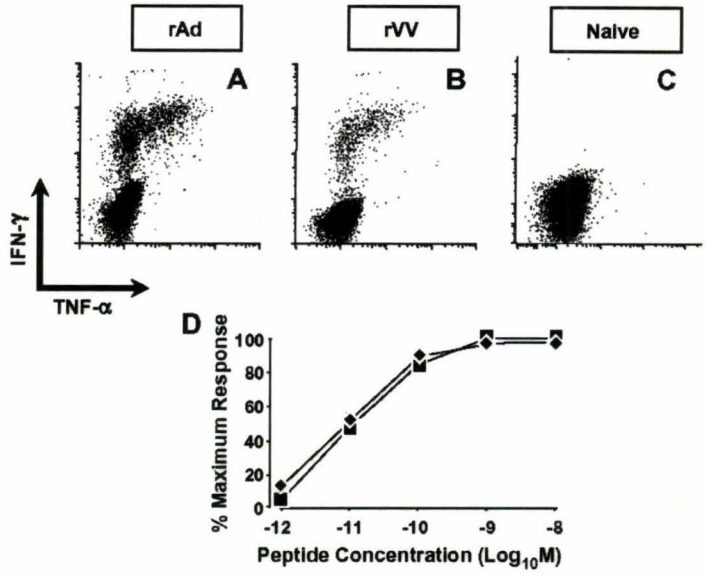

Fig. 8. Functional characterization of the SIINFEKL-specific CD8+ T cells generated by plasmid priming with recombinant virus boosting. C57BL/6 mice were immunized IM with 100 μg of plasmid pSIINFEKL-Luc and 21 days later mice received either 10^8 pfu rAd-SIINFEKL-Luc or 10^7 pfu rVV-SIINFEKL-Luc. Mice were sacrificed 7 days later. IFN-γ and TNF-α production in the splenocyte population following SIINFEKL stimulation was measured by flow cytometry. The data shown was gated first on CD8+ cells. Panel A represents a mouse immunized with pSIINFEKL-Luc/rAd-SIINFEKL-Luc. Panel B represents a mouse immunized with pSIINFEKL-Luc/rVV-SIINFEKL-Luc. Panel C represents a non-immunized mouse. (D) Splenocytes from mice pSIINFEKL-Luc/AdSIINFEKL-Luc (closed squares) or pSIINFEKL-Luc/rVV-ESOVA (closed diamonds) were stimulated with serial dilutions of SIINFEKL peptide. Results are displayed as the percent of the maximum response elicited by the highest concentration of peptide. Each point represent means ± SEM for 5 10 mice.

SIINFEKL-Luc gene construct may not provide sufficient SIINFEKL epitopes to compete with the VV-derived epitopes. The SIINFEKL-Luc antigen also gave rise to surprising results in the rAd system. Whereas the kinetics and magnitude of the SIINFEKL-specific response in the blood stream was not affected by dose following immunization with rAd-OVA and rAd-ER-SIINFEKL, the kinetics and magnitude were markedly dose-dependent following immunization with rAd-SIINFEKL-Luc. So, it is also possible that the SIINFEKL-Luc protein may have some unexpected immunological properties that distinguish it from the other antigens and these unexpected properties may be the reason we did not observe a SIINFEKL-specific CD8+ T cell response following priming with rVV-SIINFEKL-Luc.

A secondary purpose of our study was to evaluate the functional characteristics of the transgene-specific CD8+ T cells elicited by the two vector platforms. Despite the marked difference in the size of the SIINFEKL-specific CD8+ T cell response generated by the two vector systems, the populations were functionally similar. Functionality did not seem to be influenced by antigen structure because we employed three different forms antigen containing the SIINFEKL peptide. Finally, *in vivo* CTL results demonstrated that rVV-ER-SIINFEKL and rAd-ER-SIINFEKL immunized mice had comparable levels of target cell killing, although the population elicited by rVV may have enhanced lytic activity. Taken as a whole, these data lead us to conclude that there is no substantial difference in the functional quality of transgene-specific CD8+ T cells elicited by either rAd or rVV. Furthermore, we would also speculate that differences in protection following rAd and rVV immunization in previous studies was probably a direct result of inadequate levels of protective CD8+ T cells elicited by rVV, not functional inferiority [37].

Our study also examined the influence of plasmid priming on the CD8+ T cell responses generated by rAd and rVV immunization. Quantitatively, our results are consistent with previous reports. We observed that plasmid priming markedly increased the expansion of transgene-specific CD8+ T cells ($\geq$10-fold) responding to either rAd or rVV immunization. While this effect was not sustained for rAd in our model, another report demonstrated that the elevation of CD8+ T cell counts following rAd immunization of plasmid-primed mice was maintained for several weeks [40], however, that study monitored the presence of tetramer-positive CD8+ T cells in the peripheral blood and our data suggests that the peripheral blood may not be an accurate reflection of the overall CD8+ T cell response. Nevertheless, studies employing other viruses have also observed reduced levels of contraction following secondary and tertiary immunizations in both lymphoid and non-lymphoid tissues [41–43]. Previously, we have reported that the CD8+ T cell response following rAd immunization does not exhibit a dramatic contraction phase and the population is maintained at high levels (>40% of peak levels) for

several weeks [19,25]. Therefore, it may not be accurate to compare the secondary response in mice primed with rAd to secondary responses in mice primed with agent that evoke a sharp contraction phase (e.g. VSV, LCMV, *L. monocytogenes*). Plasmid priming also had minimal effect on the functionality of SIINFEKL specific CD8+ T cells. Our results differed from those of Estcourt et al. [44] who examined the impact of plasmid priming on rVV immunization. In their study, the CD8+ T cell population in mice immunized with plasmid followed by rVV exhibited a 1000-fold increase in avidity relative to the CD8+ T cells in mice receiving rVV alone. A key difference between the two studies is that Estcourt et al. expanded the CD8+ T cells *in vitro* for 6 days prior to functional analysis, whereas our assays were conducted on freshly isolated splenocytes. Differential expansion of high avidity effectors during the *in vitro* expansion may have introduced an experimental bias.

The pursuit of vaccines that elicit potent cellular mediated immunity has lead researchers to utilize many different immunization protocols. The intracellular lifecycle and immunogenicity of viruses makes them the ideal vector to generate CD8+ T cell responses. Our study has provided information that advances our understanding of how different viral vectors impact on the generation of transgene-specific CD8+ T cell responses and how best to use them.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research (MOP-42433 to J.L.B.). J.L.B. is supported by an Rx & D—Health Research Foundation/CIHR Career Award in Health Research. Y.H.W. is a CIHR New Investigator. J.M. was supported through a PREA award given to J.L.B. from the Ontario Ministry of Enterprise, Opportunity, and Innovation.

References

[1] S.J. McConkey, W.H. Reece, V.S. Moorthy, D. Webster, S. Dunachie, G. Butcher, J.M. Vuola, T.J. Blanchard, P. Gothard, K. Watkins, C.M. Hannan, S. Everaere, K. Brown, K.E. Kester, J. Cummings, J. Williams, D.G. Heppner, A. Pathan, K. Flanagan, N. Arulanantham, M.T. Roberts, M. Roy, G.L. Smith, J. Schneider, T. Peto, R.E. Sinden, S.C. Gilbert, A.V. Hill, Nat. Med. 9 (2003) 729 735.

[2] L.G. Guidotti, T. Ishikawa, M.V. Hobbs, B. Matzke, R. Schreiber, F.V. Chisari, Immunity 4 (1996) 25 36.

[3] F. Villinger, G.T. Brice, A.E. Mayne, P. Bostik, K. Mori, C.H. June, A.A. Ansari, Blood 99 (2002) 590 599.

[4] H. Winter, H.M. Hu, W.J. Urba, B.A. Fox, J. Immunol. 163 (1999) 4462 4472.

[5] J.T. Harty, A.R. Tvinnereim, D.W. White, Annu. Rev. Immunol. 18 (2000) 275 308.

[6] J.L. Bramson, Y.H. Wan, Expert Opin. Biol. Ther. 2 (2002) 75 85.

[7] P. Wong, E.G. Pamer, Annu. Rev. Immunol. 21 (2003) 29 70.

[8] O.O. Yang, P.T. Sarkis, A. Trocha, S.A. Kalams, R.P. Johnson, B.D. Walker, J. Immunol. 171 (2003) 3718 3724.

[9] M.C. Villacres, S.F. Lacey, C. Auge, J. Longmate, J.M. Leedom, D.J. Diamond, J. Infect. Dis. 188 (2003) 908 918.

[10] M. Derby, M. Alexander-Miller, R. Tse, J. Berzofsky, J. Immunol. 166 (2001) 1690 1697.

[11] N. Tatsis, H.C. Ertl, Mol. Ther. 10 (2004) 616 629.

[12] P. Ng, R.J. Parks, D.T. Cummings, C.M. Evelegh, U. Sankar, F.L. Graham, Hum. Gene Ther. 10 (1999) 2667 2672.

[13] N.J. Sullivan, T.W. Geisbert, J.B. Geisbert, L. Xu, Z.Y. Yang, M. Roederer, R.A. Koup, P.B. Jahrling, G.J. Nabel, Nature 424 (2003) 681 684.

[14] W. Gao, A. Tamin, A. Soloff, L. D'Aiuto, E. Nwanegbo, P.D. Robbins, W.J. Bellini, S. Barratt-Boyes, A. Gambotto, Lancet 362 (2003) 1895 1896.

[15] W.S. Gallichan, K.L. Rosenthal, J. Infect. Dis. 177 (1998) 1155 1161.

[16] J.W. Shiver, T.M. Fu, L. Chen, D.R. Casimiro, M.E. Davies, R.K. Evans, Z.Q. Zhang, A.J. Simon, W.L. Trigona, S.A. Dubey, L. Huang, V.A. Harris, R.S. Long, X. Liang, L. Handt, W.A. Schleif, L. Zhu, D.C. Freed, N.V. Persaud, L. Guan, K.S. Punt, A. Tang, M. Chen, K.A. Wilson, K.B. Collins, G.J. Heidecker, V.R. Fernandez, H.C. Perry, J.G. Joyce, K.M. Grimm, J.C. Cook, P.M. Keller, D.S. Kresock, H. Mach, R.D. Troutman, L.A. Isopi, D.M. Williams, Z. Xu, K.E. Bohannon, D.B. Volkin, D.C. Montefiori, A. Miura, G.R. Krivulka, M.A. Lifton, M.J. Kuroda, J.E. Schmitz, N.L. Letvin, M.J. Caulfield, A.J. Bett, R. Youil, D.C. Kaslow, E.A. Emini, Nature 415 (2002) 331 335.

[17] S.M. Sumida, D.M. Truitt, M.G. Kishko, J.C. Arthur, S.S. Jackson, D.A. Gorgone, M.A. Lifton, W. Koudstaal, M.G. Pau, S. Kostense, M.J. Havenga, J. Goudsmit, N.L. Letvin, D.H. Barouch, J. Virol. 78 (2004) 2666 2673.

[18] S. Santra, M.S. Seaman, L. Xu, D.H. Barouch, C.I. Lord, M.A. Lifton, D.A. Gorgone, K.R. Beaudry, K. Svehla, B. Welcher, B.K. Chakrabarti, Y. Huang, Z.Y. Yang, J.R. Mascola, G.J. Nabel, N.L. Letvin, J. Virol. 79 (2005) 6516 6522.

[19] T.C. Yang, J. Millar, T. Groves, N. Grinshtein, R. Parsons, S. Takenaka, Y. Wan, J.L. Bramson, J. Immunol. 176 (2006) 200 210.

[20] M.W. Carroll, B. Moss, Curr. Opin. Biotechnol. 8 (1997) 573 577.

[21] B.N.K. Fields, David M. Howley, M. Peter, Fields Virology, third ed., Raven Press, 1996.

[22] H. Kwak, W. Mustafa, K. Speirs, A.J. Abdool, Y. Paterson, S.N. Isaacs, Virology 322 (2004) 337 348.

[23] S.A. Thomson, S.L. Elliott, M.A. Sherritt, K.W. Sproat, B.E. Coupar, A.A. Scalzo, C.A. Forbes, A.M. Ladhams, X.Y. Mo, R.A. Tripp, P.C. Doherty, D.J. Moss, A. Suhrbier, J. Immunol. 157 (1996) 822 826.

[24] M. Miller, G. Rekas, K. Dayball, Y.H. Wan, J. Bramson, Vaccine 22 (2004) 2517 2523.

[25] T.C. Yang, K. Dayball, Y.H. Wan, J. Bramson, J. Virol. 77 (2003) 13407 13411.

[26] P. Brossart, A.W. Goldrath, E.A. Butz, S. Martin, M.J. Bevan, J. Immunol. 158 (1997) 3270 3276.

[27] N.P. Restifo, I. Bacik, K.R. Irvine, J.W. Yewdell, B.J. McCabe, R.W. Anderson, L.C. Eisenlohr, S.A. Rosenberg, J.R. Bennink, J. Immunol. 154 (1995) 4414 4422.

[28] B. Moss, P. Earl, N. Cooper, in: Expression of Proteins in Mammalian Cells Using Vaccinia Viral Vectors, Wiley Interscience, Cambridge, 1991.

[29] K. Dayball, J. Millar, M. Miller, Y.H. Wan, J. Bramson, J. Immunol. 171 (2003) 3379 3384.

[30] A. Porgador, J.W. Yewdell, Y. Deng, J.R. Bennink, R.N. Germain, Immunity 6 (1997) 715 726.

[31] M.K. Slifka, J.L. Whitton, Nat. Immunol. 2 (2001) 711 717.

[32] J. Bramson, K. Dayball, C. Evelegh, Y.H. Wan, D. Page, A. Smith, Gene Ther. 10 (2003) 251 260.

[33] R.M. Coles, S.N. Mueller, W.R. Heath, F.R. Carbone, A.G. Brooks, J. Immunol. 168 (2002) 834 838.

[34] L.E. Harrington, Rv.R. Most, J.L. Whitton, R. Ahmed, J. Virol. 76 (2002) 3329 3337.

[35] T.C. Yang, J. Milllar, T. Groves, N. Grinshtein, R. Parsons, S. Takenaka, Y. Wan, J. Bramson, J. Immunol. 176 (2006) 200 210.

[36] S.C. Gilbert, J. Schneider, C.M. Hannan, J.T. Hu, M. Plebanski, R. Sinden, A.V. Hill, Vaccine 20 (2002) 1039 1045.

[37] D.R. Casimiro, L. Chen, T.M. Fu, R.K. Evans, M.J. Caulfield, M.E. Davies, A. Tang, M. Chen, L. Huang, V. Harris, D.C. Freed, K.A. Wilson, S. Dubey, D.M. Zhu, D. Nawrocki, H. Mach, R. Troutman, L. Isopi, D. Williams, W. Hurni, Z. Xu, J.G. Smith, S. Wang, X. Liu, L. Guan, R. Long, W. Trigona, G.J. Heidecker, H.C. Perry, N. Persaud, T.J. Toner, Q. Su, X. Liang, R. Youil, M. Chastain, A.J. Bett, D.B. Volkin, E.A. Emini, J.W. Shiver, J. Virol. 77 (2003) 6305 6313.

[38] Y. Miyahira, Y. Takashima, S. Kobayashi, Y. Matsumoto, T. Takeuchi, M. Ohyanagi-Hara, A. Yoshida, A. Ohwada, H. Akiba, H. Yagita, K. Okumura, H. Ogawa, Infect. Immun. 73 (2005) 7356 7365.

[39] M.A. Fischer, D.C. Tscharke, K.B. Donohue, M.E. Truckenmiller, C.C. Norbury, J. Gen. Virol. 88 (2007) 2378 2386.

[40] D.H. Barouch, P.F. McKay, S.M. Sumida, S. Santra, S.S. Jackson, D.A. Gorgone, M.A. Lifton, B.K. Chakrabarti, L. Xu, G.J. Nabel, N.L. Letvin, J. Virol. 77 (2003) 8729 8735.

[41] D. Masopust, S.J. Ha, V. Vezys, R. Ahmed, J. Immunol. 177 (2006) 831 839.

[42] D. Masopust, J. Jiang, H. Shen, L. Lefrancois, J. Immunol. 166 (2001) 2348 2356.

[43] J.M. Grayson, L.E. Harrington, J.G. Lanier, E.J. Wherry, R. Ahmed, J. Immunol. 169 (2002) 3760 3770.

[44] M.J. Estcourt, A.J. Ramsay, A. Brooks, S.A. Thomson, C.J. Medveckzy, I.A. Ramshaw, Int. Immunol. 14 (2002) 31 37.

- Chapter 3 –

Persistent Transgene Expression is Required for Maximal Expansion and Maintenance of the CD8+ T Cell Population Generated by Recombinant Adenovirus

Jonathan D. Finn, James B. Millar, Jennifer Bassett, Natalie Grinshtein, Teng Chih Yang, Robin Parsons, Carole Evelegh, Yonghong Wan, Robin J. Parks, and Jonathan L. Bramson

Prologue

Following rHuAd5 immunization, previous research from our lab has observed protracted CD8+ T cell contraction and effector memory generation, which we attributed to prolonged CD8+ T cell antigen presentation. To further evaluate the effects of prolonged antigen presentation on CD8+ T cell priming, memory development, phenotype and functionality, a doxycycline-regulated adenovirus vector was constructed to enable controlled extinction of transgene expression *in vivo*. The highlights of our research are as follows:

- Transgene expression is required for at least 13 days to achieve the peak primary response and for 60 days to attain the maximum memory response.
- 60 days post immunization, the memory response may convert to being maintained through antigen independent mechanisms
- Termination of transgene expression had modest yet significant effects on both functional and phenotypic characteristics

The research contained within this chapter is the result of a partnership between myself and Dr Jonathan Finn. In this study I was involved in experimental development, implementation and execution. In particular, I contributed the data displayed in figures 1, 2, 5, 6, 7 and 10. This chapter is currently in revision at the Journal of Virology.

Title: Persistence of transgene expression influences CD8+ T cell expansion and maintenance following immunization with recombinant adenovirus.

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Running title: Prolonged antigen expression sustains CD8+ T cells

Keywords: Adenovirus, Vaccine, CD8+ T cell

[1] Both authors contributed equally to this work

[2] This work was supported by grants to J.LB. and R.J.P. from the Canadian Institutes for Health Research (MOP-42433, MOP-47819).

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Abstract

Several reports have demonstrated that the CD8+ T cell response elicited by recombinant adenovirus vaccines exhibited a sustained effector memory phenotype that was associated with long-term availability of antigen. We show in this manuscript that the phenotype of the memory population was not the result of continual priming of naïve T cells. An alternate explanation for the memory phenotype is that persistent antigen expression continually restimulates antigen-experienced CD8+ T cells. To address this possibility, a doxycycline-regulated adenovirus vector was constructed to enable controlled extinction of transgene expression *in vivo*. We investigated the impact of premature termination of transgene expression at various time points (day 3 – day 60) following immunization. When transgene expression was terminated before the maximum response had been attained, overall expansion was attenuated and the memory population was not sustained. When transgene expression was terminated between day 13 and day 30, the memory population was significantly reduced at later time points demonstrating that the early memory population was antigen-dependent. Extinction of transgene expression at day 60 had no impact on memory maintenance indicating that the memory population may ultimately become antigen-independent. Premature termination of antigen expression had significant, but modest, effects on the phenotype and cytokine profile of the memory population. These results offer new insights into the mechanisms of memory CD8+ T cell maintenance following recombinant adenovirus immunization.

Introduction

Recombinant adenovirus (rAd) vaccines have garnered considerable attention as platforms for eliciting CD8+ T cell immunity due to their strong immunogenicity in numerous studies, including simian models and preliminary human trials (1-3). While the commonly used recombinant human Ad5 vectors (rHuAd5) may not represent the optimal serotype for use in humans, due to high prevalence of pre-existing immunity, vectors of different serotypes and species have been developed which should overcome these concerns (4-6). In preparation for the use of these vectors in human trials, we have been investigating their immunobiology in preclinical rodent models as a means of optimizing the vectors and identifying potential limitations.

CD8+ T cells play an important role in host defence against tumors and viral infections. During the primary phase of the CD8+ T cell response, the activated precursors undergo a rapid and dramatic expansion phase followed by a period of contraction where 80 – 90% of the antigen-specific population dies off leaving the remaining cells to constitute the memory population . CD8+ T cells mature over the course of the primary response and acquire the ability to produce IFN-γ, TNF-α and, to a lesser degree, IL-2. Memory T cells can be divided into central-memory and effector-memory T cells based on phenotype and anatomical location. These phenotypic differences have also been linked to functional differences; however, these relationships remain controversial (8-14).

Our studies have revealed some unexpected qualities of the CD8+ T cell response generated by intramuscular immunization with rHuAd5. The CD8+ T cell response exhibited a protracted contraction phase where 40-60% of the peak number of CD8+ T cells persisted 3 weeks after the peak response in multiple compartments. The rHuAd5-induced memory CD8+ T cell population was composed primarily of effector and effector-memory cells (CD62L-, CD127-/+), only a fraction (50 – 70% depending upon the antigen) of the population produced TNF-α and very few cells produced IL-2 (15, 16). The phenotype of the rHuAd5-elicited CD8+ T cell population was more consistent with the CD8+ T cell population observed in persistent infections, such as polyoma virus (17), murine herpesvirus-68 and murine cytomegalovirus (19, 20) than acute virus infection models such as LCMV , vaccinia (21) , VSV (22), or influenza . Further investigation demonstrated that, similar to a persistent infection, antigen presentation persisted for >30 days following rHuAd5 immunization in our model . Additional unpublished results from our group have demonstrated evidence of antigen presentation 60 days after rHuAd5 immunization and similar results have recently been described by another group (24). These data combined suggest that the sustained effector phenotype may arise from prolonged, low-level transgene expression by the rHuAd5 vector, although this connection remains to be formally proven. It is difficult to fully appreciate the implications of these observations at this time since chronic exposure to antigen is often associated with CD8+ T cell dysfunction, yet rHuAd5 vectors have been used successfully to elicit protective immunity in many models of pathogen infection and tumor challenge (1, 2) including models from our lab (15, 25). Nevertheless, other

reports have provided evidence that rHuAd5 vectors can, indeed, lead to dysfunctional CD8+ T cell immunity (26, 27). Therefore, further investigation is required to properly assess the implications of the prolonged antigen expression following rHuAd5 immunization in terms of sustaining a functional memory CD8+ T cell response.

We, and others, have determined that antigen presentation can be detected for weeks following exposure to agents that produce "acute" infections such as rHuAd5, VSV or influenza (15, 24, 28-30). However, the importance of antigen presentation beyond the acute phase of the immune response is unknown. Previous reports have demonstrated that a CD8+ T cell only requires 20 - 24 hours of stimulation to become fully activated, proliferate and progress to a memory phenotype (31-33) although optimal stimulation may require a longer period (40 – 60 hours) (34-36). However, antigen presentation *in vivo* is unlikely to result in synchronous activation of all available CD8+ T cell precursors; therefore it is critical to define the relationship between duration of antigen presentation and optimal development of CD8+ T cell immunity. We have previously observed that the duration of antigen expression influenced the magnitude of the CD8+ T cell response produced by plasmid DNA vaccines and a similar observation has recently been made by another group . In a model of cutaneous HSV infection where antigen presentation persisted for 7-8 days following infection , reducing the duration of antigen presentation to 4 days markedly reduced the magnitude of the CD8+ T cell response. Likewise, in a non-infectious vaccination model, CD8+ T cell expansion and acquisition of effector function of the CD8+ T cell population was markedly improved when the

vaccine was delivered repeatedly over the period of a week compared to the situation where the vaccine was only given once (36). In contrast, maximal CD8+ T cell expansion and differentiation only required 2 days of exposure to peptide-pulsed dendritic cells *in vivo* . Furthermore, antigen presentation *in vivo* only persists 2-3 days following infection with *P. falciparum* and *L. monocytogenes* yet both infections produce robust CD8+ T cell immunity (41, 42). Thus, while a brief exposure to antigen may be sufficient to drive the T cell response; maximal expansion *in vivo* may require prolonged antigen presentation although the exact timing remains to be determined and will likely be a function of the vaccination agent.

In the current report, we sought to determine the relationship between transgene expression and CD8+ T cell maintenance and memory. To this end, we constructed an Ad vector with a regulatable cassette that would permit attenuation of gene expression at various times post-infection. Using this reagent we addressed two key questions: 1) How does duration of antigen expression impact the magnitude of primary CD8+ T cell expansion? 2) Is antigen expression required beyond the peak expansion to maintain the memory CD8+ T cell population?

Materials and methods:

Construction of plasmids and replication-deficient adenovirus (rHuAd5)

All of the plasmids used in these studies were constructed using standard cloning methods (Mantiatis et al). The pTet-OFF plasmid was obtained from Clontech and was the source of the tetracycline transactivator (tTA) gene. pTREminCMV-Luc contains a modified version of luciferase bearing the immunodominant class-I epitope from chicken egg ovalbumin (SIINFEKL) tagged to the N-terminus under control of the Tet Response Element (TRE) fused with the minimal CMV promoter (from pUHD 10-3) and terminated by the bovine growth hormone polyadenylation sequence. pTREminIL2-Luc contains the SIINFEKL-Luciferase transgene under control of the TRE fused with the minimal IL-2 promoter (obtained from pZ_{12}I-PL-2 graciously provided by Ariad Pharmaceuticals) and terminated by the SV40 polyadenylation sequence. pGL3-Basic (Promega) consists of the luciferase transgene lacking any eukaryotic promoter. The plasmid, ptTA-TRE-SIINFEKL-Luc consists of an expression cassette where tTA is transcribed under the control of the MCMV IE promoter (43) and the reporter cassette from pTREminIL2-Luc on a single plasmid in a tail to tail configuration. The plasmid, ptTA-HS4-TRE-SIINFEKL-Luc is a variant of ptTA-TRE-SIINFEKL-Luc where the two cassettes are separated by an HS4 chicken β-globin locus core insulator fragment (from pNI-CD,a gift of Dr. Adam West, University of Glasgow).

A recombinant adenovirus (rHuAd5) vector carrying the expression cassette from ptTA-HS4-TRE-SIINFEKL-Luc was rescued using the E1,E3-deleted backbone described by

Ng et al. and named Ad-tTA-SIINFEKL-Luc. We also constructed a helper-dependent version of this doxycycline-regulated rHuAd5 which lacks all viral genes using the system described by Palmer and Ng . The helper-dependent virus was named **hd**Ad-tTA-SIINFEKL-Luc. A control virus, AdSIINFEKL-Luc, expressing the SIINFEKL-Luc transgene under the control of the constitutive MCMV promoter has been described previously . All rHuAd5 were propagated using 293 cells and purified using CsCl gradient centrifugation as previously described .

Immunizations and doxycycline treatment

Female C57BL/6 mice purchased form Charles River Breeding Laboratories (Wilmington, MA). For immunizations, 10^7 - 10^8 pfu rHuAd5 was diluted to 100µl in sterile PBS and subsequently injected intramuscularly (IM) in both rear thighs. A rHuAd5 expressing the SIINFEKL-Luc antigen under the control of the constitutive MCMV promoter (AdSIINFEKL-Luc-004) has been described by our group previously . To measure secondary responses, mice were challenged with 5 x 10^6 pfu of a recombinant vaccinia virus (rVV) expressing the SIINFEKL epitope linked to an ER-targeting signal (rVV-ESOVA) (graciously provided by Jonathan Yewdell, NIAID, Bethesda, MD) . Doxycycline was administered initially as an intraperitoneal injection of 500µg doxycycline and maintained by the addition of doxycycline in the drinking water. Mice were initially given a high-dose of doxycyline in their drinking water (2mg/ml) for 48 hours and subsequently maintained on 200µg/ml doxycycline. The drinking water was also supplemented with 5% sucrose. This dosing of doxycyline was found to completely

ablate the CD8+ T cell response to Ad-tTA-SIINFEKL-Luc but have no effect on the CD8+ T cell response produced by Ad-SIINFEKL-Luc, which expresses the antigen from a constitutive promoter .

Flow cytometry reagents

All flow cytometry antibodies were purchased from BD Pharmingen except anti-CD127 (clone A7R34) and anti-KLRG1 (clone MAFA), which was purchased eBiosciences (San Diego, CA), respectively. The following antibodies and fluorescent reagents were purchased from BD Pharmingen: anti-CD8α (clone 53-6.7), anti-CD62L (clone MEL-14), anti-IFN-γ (clone XMG1.2), anti-TNF-α (clone 53-2.1), anti-IL-2 (clone JES6-5H4), anti-Thy1.2 (clone 30H12). APC-labelled K^b/SIINFEKL tetramers were prepared at McMaster University or Baylor College of Medicine. Most staining conditions involved 5 fluorochromes (FITC, PE, PE-Cy5, PE-Cy7 and APC) and data was acquired using either an LSRII or a FACSCanto equipped with a 488nm and 633nm laser.

Analysis of T cell responses

Sample preparation and staining methodologies have been extensively described in our previous publications (15, 16)

Generation of partial hematopoietic chimerism

At various time points before (2 days) or after (days 12 to day 70) AdSIINFEKL-Luc-004 immunization, mice were treated with 600 μg busulfan as described by Vezys et al (49).

The next day, mice received an intravenous injection of 25 x 10^6 bone marrow cells from a Thy1.1$^+$ mouse (B6.PL-Thy1^a/CyJ; Jackson Laboratory, Bar Harbor, ME). The busulfan-treated mice were allowed to reconstitute for 70 days at which point spleens and peripheral blood were harvested for analysis of SIINFEKL-specific cells. Endogenous CD8+ T cells were identified as Thy1.2+ and donor cells were identified as Thy1.2-.

Recruitment of naïve T cells

Lymph nodes were removed from OT-I/Thy1.1+ mice and processed into single-cell suspensions. OT-I/Thy1.1+ CD8+ T cells were isolated from the lymph node preparation using negative selection magnetic separation (Stem Cell Technologies, Vancouver, BC). To characterize the recruitment of naïve CD8+ T cells into the experienced CD8+ T cell pool at different times post-immunization, limiting numbers of OT-I cells (70 cells/mouse) were transferred into C57Bl/6 mice intravenously at various time points following immunization with AdSIINFEKL-Luc-004 in accordance with the results of Badovinac et al . OT-I cells were monitored in the peripheral blood at various time points (day 7, day 12, day 19, day 35) following transfer and identified as K^b/SIINFEKL+ Thy1.2-

Measurement of vaccinia virus in mouse ovaries

Ovaries were homogenized in 2 ml of 1 mM Tris, pH 9.0. The homogenates were further disrupted by three consecutive "freeze/thaw" cycles. To determine virus titer in the homogenates, confluent CV-1 cells in 12-well plates were infected with serial dilutions of

the homogenate and, 2 days later, plaques were visualized by staining with 0.1% crystal violet in 20% ethanol.

Results

The sustained effector memory CD8+ T cell population produced by rHuAd5 is not due to continual priming of naïve CD8+ T cells.

As stated in the introduction, the phenotype of the memory population produced by rHuAd5 is more akin to a persistent infection than an acute infection. Vezys et al. demonstrated that the heterogeneity of the memory population produced by polyoma virus and LCMV clone 13 was due to continual recruitment of naïve T cells (49). Our previous report argues against this model as thymectomy prior to immunization had no impact on the maintenance or phenotype of the antigen-specific CD8+ T cell population following rHuAd5 immunization. To further examine the possibility that the memory population produced by intramuscular immunization with rHuAd5 is a mixture of long-lived CD8+ T cells and newly recruited effector CD8+ T cells, we induced partial hematopoietic chimerism using the same technique employed by Vezys et al. (49) to demonstrate the priming of naïve CD8+ T cells at late times after infection. This method involves treatment with busulfan, which is toxic to bone marrow progenitors but minimally toxic to peripheral lymphocytes, so the existing host-derived memory CD8+ T cells are minimally affected by this treatment. Following busulfan treatment, the mice are reconstituted with congenic (Thy1.1+) bone marrow to facilitate the identification of recently primed CD8+ T cells. For this experiment, mice immunized with AdSIINFEKL-Luc-004 were treated with busulfan either 2 days prior to immunization or 12, 30, and 70 days after immunization (schematic provided in Figure 1A). We chose to investigate multiple time points because our previous work suggested that the availability of antigen

wanes over time , so the effect would be expected to be most pronounced at early times following immunization. Mice were allowed to rest for 70 days following busulfan treatment at which time more than 50% of the CD8+ T cells were donor-derived (Figure 1B, upper left-hand panel). Despite the marked reconstitution by congenic CD8+ T cells, very few of these donor-derived cells were engaged into the circulating SIINFEKL-specific CD8+ T cell pool even when busulfan treatment preceded the immunization (Figure 1C). The SIINFEKL-specific CD8+ T cell population in the spleen, lungs and blood was composed almost entirely (>95%) of host-derived CD8+ T cells, regardless of the time of busulfan treatment (Figure 1C and data not shown). Since reconstitution with donor-derived cells in this model require approximately 1 month (49), it can be argued that the group where partial chimerism was induced 2 days prior to immunization demonstrates that the memory population produced by rHuAd5 is composed primarily of CD8+ T cells that were engaged within the first 30 days following immunization.

As another approach to address this question, we administered naïve OT-I T cells at various time points following immunization to define the period of time that naïve CD8+ T cells can be primed following rHuAd5 immunization. Our previous results have demonstrated that it is possible to engage OT-I cells to proliferate *in vivo* during a period of at least 30 days following AdSIINFEKL-Luc-004 immunization (15, 16). However, those experiments involved adoptive transfer of 5×10^5 OT-I and several reports have demonstrated that excessive numbers of OT-I cells (> 100/mouse) can result in non-physiological T cell priming (50, 51). Therefore, for these experiments, we employed a

Figure 1

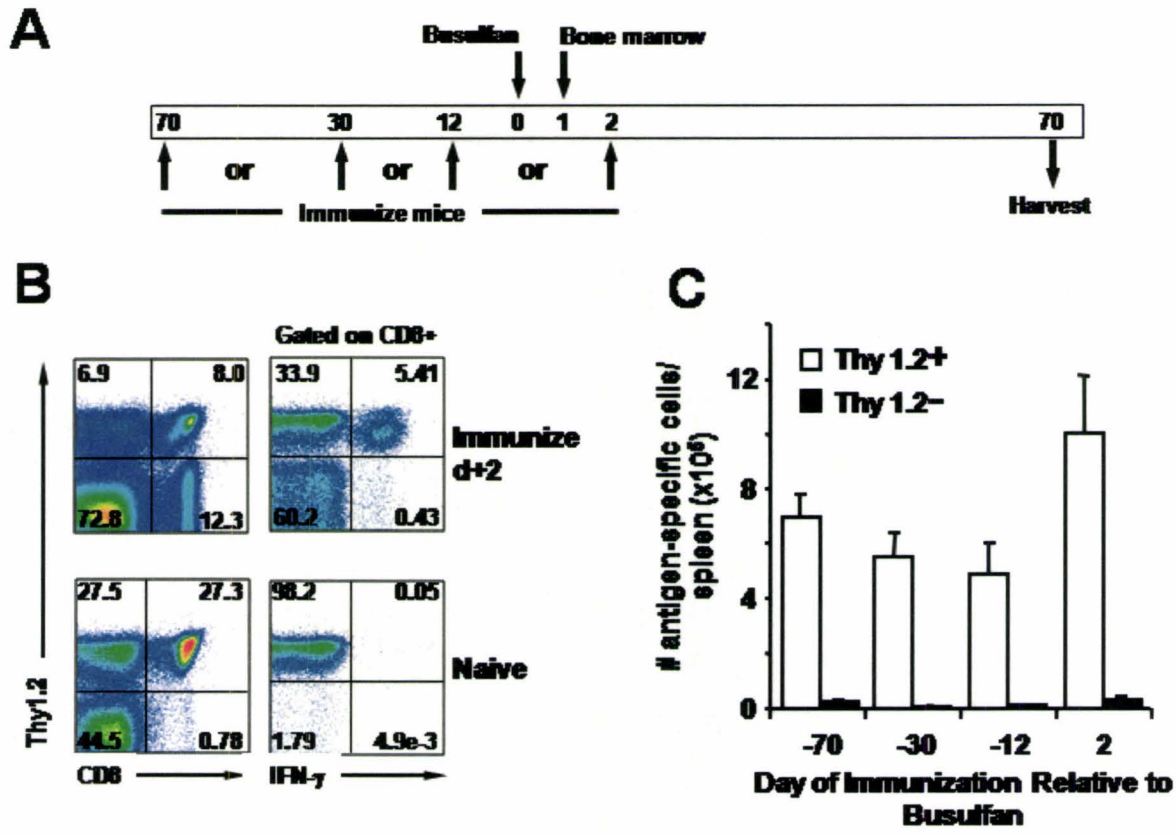


Figure 1. The induction of partial hematopoietic chimerism fails to support a role for continual priming of naïve CD8+ T cells following rHuAd5 immunization. ***A.*** Schematic representation of the experimental design. ***B.*** Examples of flow cytometry data. The upper plots are representative of a mouse treated with busulfan before immunization with AdSIINFEKL-Luc-004. The lower plots are representative of a naïve mouse. The left-hand plots are gated on live lymphocytes. The right-hand plots are gated on CD8+ T cells and represent samples that were stimulated with SIINFEKL peptide. Endogenous CD8+ T cells are Thy1.2+ and donor bone marrow-derived CD8+ T cells were identified as Thy1.2-. ***C.*** The numbers of antigen-specific CD8+ T cells in the spleen were quantified and identified as Thy1.2+ (host-derived; open bars) and Thy1.2- (donor-derived; closed bars). Each bar is representative of 5 mice +/- SEM.

dose of 70 naïve OT-I cells per mouse as this dose has been shown to faithfully recapitulate the endogenous CD8+ T cell response . To determine the time period following immunization where naïve T cells are primed, OT-I T cells were adoptively transferred into immunized mice on the day of infection (day 0), 2 days later, 4 days later and 7 days later (a schematic is provided in Figure 2A). The presence of circulating OT-I cells in the periphery was monitored in the peripheral blood 7, 12, 19 and 35 days after transfer (Figure 2B and data not shown). Only OT-I CD8+ T cells transferred on day 0 or day 2 following immunization were engaged to enter the circulating pool of SIINFEKL-specific CD8+ T cells (Figure 2B). OT-I T cells transferred at later times were not observed in the circulating SIINFEKL-specific CD8+ T cell pool at any of the time points we examined. Thus, it appears that the sustained effector phenotype associated with the memory population produced by rHuAd5 is composed of CD8+ T cells that were engaged during the first few days following infection rather than the result of continual recruitment of naïve CD8+ T cells.

Development and characterization of a repressible rHuAd5 expression system in vitro and in vivo.

Since our data failed to support the likelihood that continual priming of naïve CD8+ T cells underlies the sustained effector phenotype observed following rHuAd5 infection, we hypothesized that persistent low-level transgene expression may result in continual stimulation of circulating antigen-experienced CD8+ T cells yielding a population akin to that found in models of persistent infection. To determine the importance of the duration

Figure 2

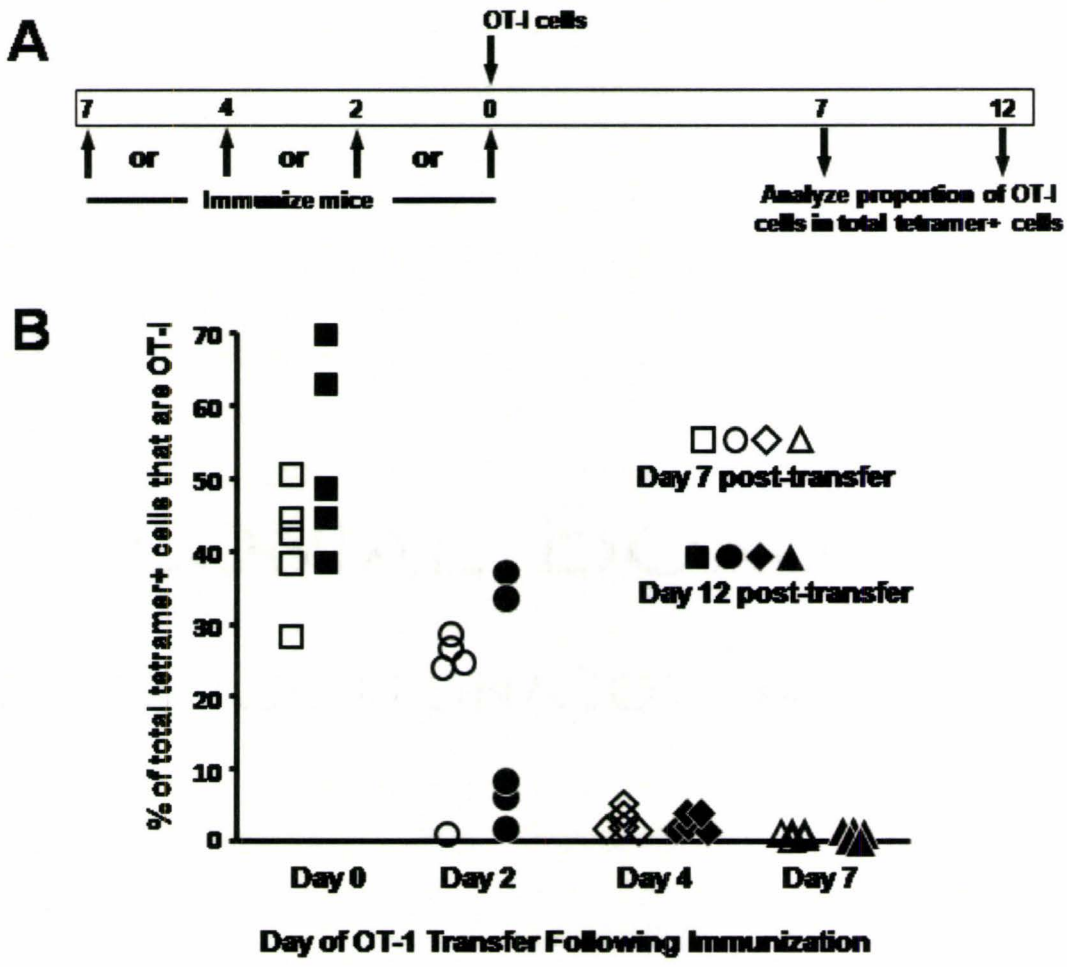


Figure 2. Adoptive transfer of limiting numbers of naïve OT-I cells suggests a limited period for naïve CD8+ T cell priming following rHuAd5 immunization. ***A.*** Schematic representation of the experimental design. ***B.*** The frequency of OT-I cells relative to the total tetramer-positive population in the peripheral blood was determined. Each symbol represents a single mouse. Open symbols represent samples taken 7 days following adoptive transfer. Closed symbols represent samples taken 12 days following adoptive transfer.

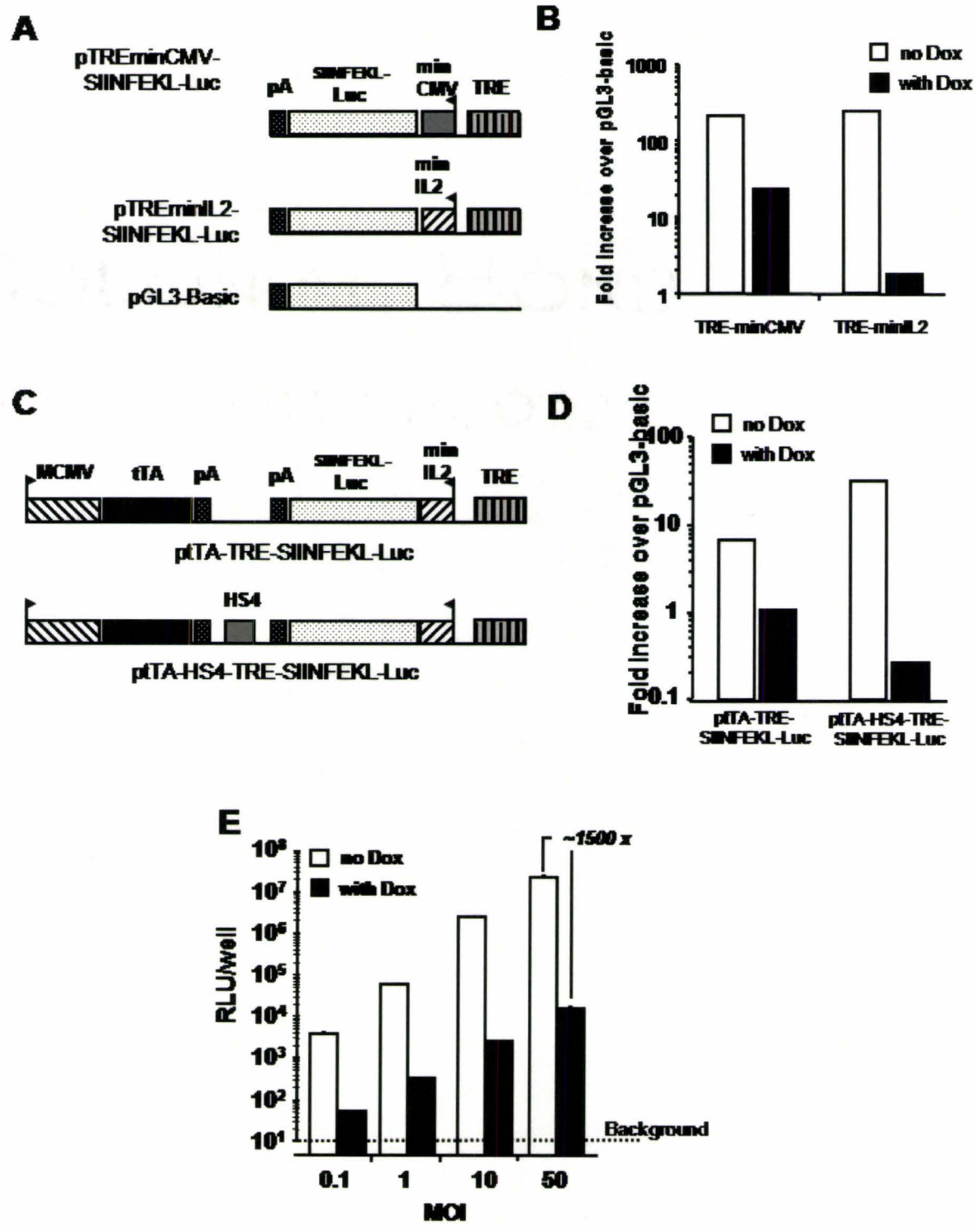
of antigen expression to the CD8+ T cell response following rHuAd5 immunization, we decided to use the "Tet-OFF" expression system where the presence of doxycycline (DOX) will terminate gene expression (52). We initially tested the minimal CMV promoter fused to the Tetracycline Response Element (TRE) as an inducible promoter to regulate SIINFEKL-Luc expression (pTREminCMV-SIINFEKL-Luc; Figure 3A), however we found that this configuration resulted in poor repression of gene expression when co-transfected with a plasmid expressing the tetracycline transactivator (tTA) under the control of constitutive promoter (pTet-OFF) in the presence of DOX (Figure 3B, TRE-minCMV). The minimal CMV promoter was replaced with the minimal IL-2 promoter to generate pTREminIL2-SIINFEKL-Luc (Figure 3A) and repression was significantly enhanced following co-transfection with pTet-OFF in the presence of DOX (Figure 3B). To produce a single vector which expressed the tetracycline transactivator (tTA) and the inducible transgene, we inserted that TRE-minIL2-SIINFEKL-Luc cassette into a plasmid that contained a cassette where tTA was expressed under the control of the MCMV immediate early promoter. The two cassettes were oriented in a tail-to-tail fashion (Figure 3C). While this configuration only provided modest suppression of transgene expression in the presence of DOX (Figure 3D), we found that suppression could be greatly increased by the inclusion of an HS4 insulator between the two cassettes (Figure 3C and 3D). We therefore rescued the HS4-containing expression system into a rHuAd5 vector (named Ad-tTA-SIINFEKL-Luc) that was used subsequently for all *in vivo* studies. We observed high repression levels (~1500 fold) in the presence of DOX across a range of MOIs when BHK cells were transduced *in vitro* (Figure 3E).

To make best use of this model, it was necessary to identify a dose of Ad-tTA-SIINFEKL-Luc that produced sufficiently high levels of SIINFEKL-specific CD8+ T cells for accurate assessment by flow cytometry. Mice were immunized with 10^8, $3x10^8$ or 10^9 pfu of Ad-tTA-SIINFEKL-Luc and SIINFEKL-specific CD8+ T cells were measured in the blood 14 days later. It was found that 10^9 pfu of Ad-tTA-SIINFEKL-Luc gave rise to a consistent and measurable response (Figure 4A), so this dose was used for all further studies. To verify that the kinetics of CD8+ T cell expansion and contraction following Ad-tTA-SINFEKL-Luc were consistent with our previously observed data, we examined the numbers of CD8+ T cells present in the spleen at various time points following immunization with Ad-tTA-SIINFEKL-Luc (Figure 4B). Similar to our previous results, we observed that the SIINFEKL-specific CD8+ T cell population expands dramatically between day 6 and day 12 following immunization and subsequently exhibits a slow decline in CD8+ T cell numbers (15, 46).

To confirm that DOX treatment would result in sufficient attenuation of gene expression to suppress the induction of SIINFEKL-specific CD8+ T cells, mice were administered various doses of DOX in their drinking water 5 days before immunization with Ad-tTA-SIINFEKL-Luc and SIINFEKL-specific CD8+ T cell responses were measured in the spleen 22 days after immunization. While DOX concentrations as low as 50 μg/mL and 100μg/ml resulted in substantial attenuation of the SIINFEKL-specific CD8+ T cell response, a dose of 200 μg/mL was necessary to completely abrogate the SIINFEKL-specific CD8+ response in all mice (Figure 4C). Similar results were observed in the

Figure 3. A Modified doxycycline-regulated expression cassettes provides robust control of gene expression. ***Panel A.*** Schematic of expression cassettes containing the minimal CMV (minCMV) and minimal IL2 (minIL2) promoters linked to the Tetracycline Responsive Element (TRE). ***Panel B.*** Plasmids containing the cassettes shown in panel A were co-transfected with pTet-OFF into BHK cells. Cells were cultured in the presence or absence of doxycycline (DOX; 10μg/mL) and assayed for luciferase activity 24 h post-transfection. Data is represented as the fold-increase in luciferase activity over a promoter-less luciferase expression plasmid (pGL3-Basic). ***Panel C.*** Schematic representation of the expression elements in ptTA-TRE-SIINFEKL-Luc, where a cassette expressing tTA under the control of the constitutive MCMV promoter was fused tail-to-tail with the TREminIL2-SIINFEKL-Luc expression cassette shown in panel A, and ptTA-HS4-TRE-SIINFEKL-Luc which is a derivative of ptTA-TRE-SIINFEKL-Luc in which an HS4 core insulator fragment was inserted between the two cassettes. ***Panel D.*** Plasmids containing the expression elements described in Panel C were individually transfected into BHK cells and cultured in the presence or absence of DOX (10μg/ml) and luciferase activity was assayed a described above. ***Panel E.*** An rHuAd5 vector (Ad-tTA-SIINFEKL-Luc) encoding the bi-functional, HS4 separated expression system shown in ***B*** was purified and tested at a range of MOI's on BHK cells in the presence or absence of DOX (10μg/mL). D)

Figure 3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pTREminCMV-SIINFEKL-Luc
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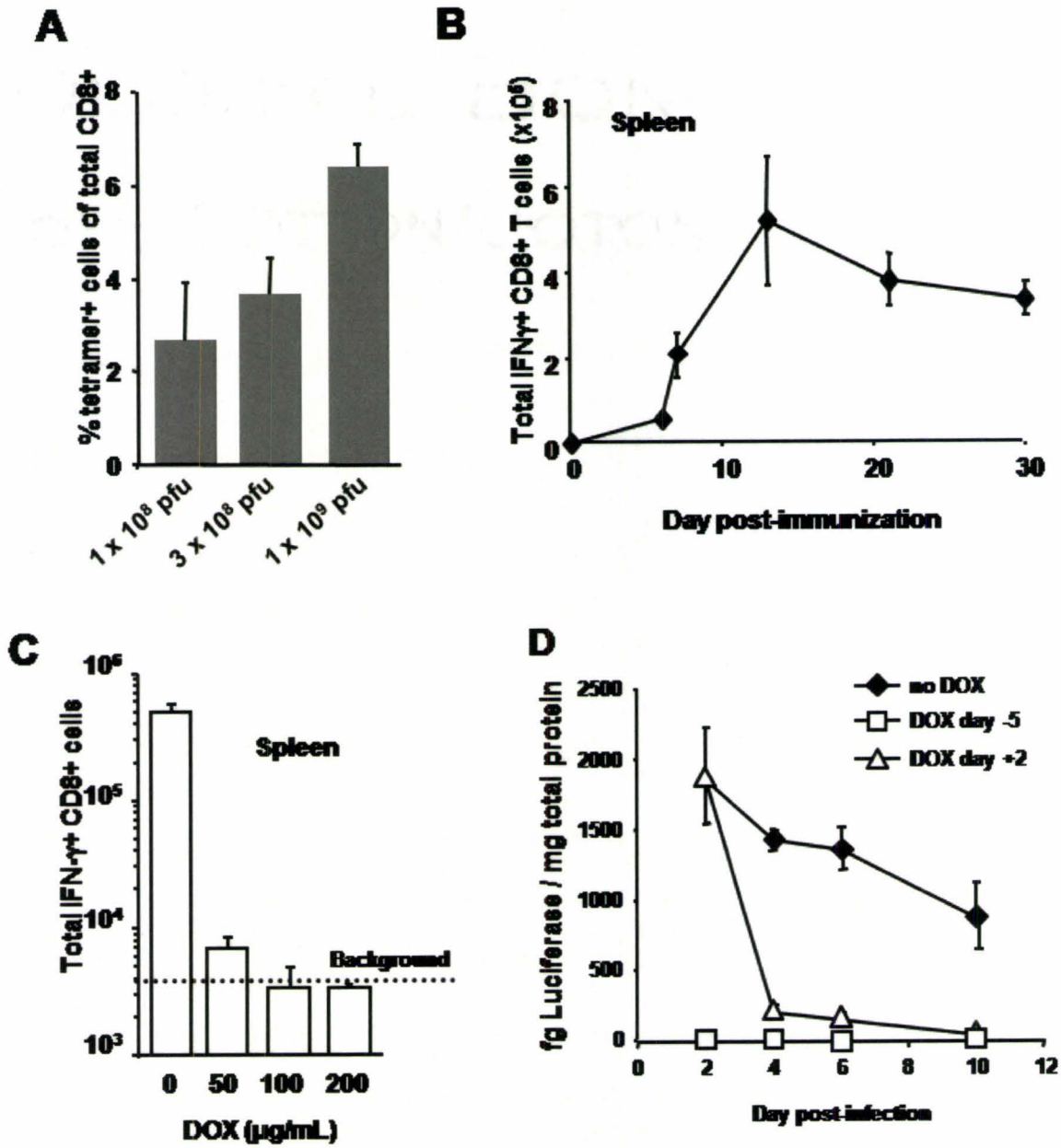
min IL2
pGL3-Basic
B
Fold Increase over pGL3-basic
1000
100
10
1
no Dox
with Dox
TRE-minCMV
TRE-minIL2
C
MCMV
tTA
pA
pA
SIINFEKL-Luc
min IL2
TRE
ptTA-TRE-SIINFEKL-Luc
HS4
ptTA-HS4-TRE-SIINFEKL-Luc
D
Fold Increase over pGL3-basic
100
10
1
0.1
no Dox
with Dox
ptTA-TRE-SIINFEKL-Luc
ptTA-HS4-TRE-SIINFEKL-Luc
E
RLU/well
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
no Dox
with Dox
~1500 x
Background
0.1
1
10
50
MOI

Figure 4. Ad-tTA-SIINFEKL-Luc elicits an immune response comparable to other rHuAd5 vectors which can be fully attenuated by treatment with DOX. ***Panel A.*** C57Bl/6 mice were immunized with increasing doses of Ad-tTA-SIINFEKL-Luc over a range of doses (10^8 – 10^9 pfu/mouse). Fourteen days later, the frequency of SIINFEKL-specific CD8+ T cells was assessed in the peripheral blood. ***Panel B.*** C57Bl/6 mice were immunized with 10^9 pfu of Ad-tTA-SIINFEKL-Luc and the number of SIINFEKL-specific CD8+ T cells was assessed in the spleens of mice sacrificed at various times post-immunization. Each point represents the mean +/- SEM for at least 3 mice per group. ***Panel C.*** C57Bl/6 mice received a single bolus intraperitoneal injection of 500μg of DOX 5 days prior to immunization and were subsequently given water containing between 50 and 200 μg/ml DOX. Five days following the initiation of DOX treatment, mice were immunized with 10^9 pfu of Ad-tTa-SIINFEKLuc. Mice were sacrificed 22 days post immunization and the total numbers of SIINFEKL-specific CD8+ T cells were assessed in each spleen. Each data point represents the mean of 3 mice +/- SEM. ***Panel D.*** C57Bl/6 mice received 10^9 pfu Ad-tTA-SIINFEKL-Luc intramuscularly in the presence or absence of DOX. One group of mice (DOX day-5, open squares) received single bolus intraperitoneal injection of DOX 5 days prior to immunization and were subsequently given water containing 200 μg/ml DOX. The second group (DOX day +2) received single bolus intraperitoneal injection of DOX 2 days after immunization and, at the same time, the mice were given water containing a high dose of DOX (2mg/mL). The third group of mice (no DOX) did not receive any DOX during the period of experiment. Thigh muscles were harvested at time points indicated and assayed for luciferase activity. The results represent the mean +/- SEM for 6 muscle samples at each time point.

Figure 4


A
% tetramer+ cells of total CD8+
0
2
4
6
8
1 x 10⁸ pfu
3 x 10⁸ pfu
1 x 10⁹ pfu
B
Spleen
Total IFNγ+ CD8+ T cells (x10⁵)
0
2
4
6
8
0
10
20
30
Day post-immunization
C
Spleen
Total IFN-γ+ CD8+ cells
10³
10⁴
10⁵
10⁶
Background
0
50
100
200
DOX (µg/mL)
D
no DOX
DOX day -5
DOX day +2
fg Luciferase / mg total protein
0
500
1000
1500
2000
2500
0
2
4
6
8
10
12
Day post-infection

peripheral blood (data not shown). For suppression of gene expression following immunization, we developed a treatment regimen where mice received a bolus injection of DOX (500μg delivered via intraperitoneal injection) which was supplemented with a high dose of DOX in their drinking water (2mg/mL) for 2 days followed by a maintenance regimen where mice were given a lower dose of DOX in their drinking water for the duration of the experiment (200μg/mL). This dosing regimen was found to result in rapid repression of gene expression (Figure 4D; diamonds). To confirm that DOX treatment was not having a non-specific effect on the CD8+ T cell response, mice were treated with DOX and immunized with AdSIINFEKL-Luc-004. The frequencies of SIINFEKL-specific CD8+ T cells in mice treated with DOX were equivalent to those mice immunized in the absence of DOX around the peak of the response (day 12 – 14) and later in the response (day 21 – 28) (data not shown).

Early termination of antigen expression affects magnitude and kinetics of antigen specific CD8+ T cell response.

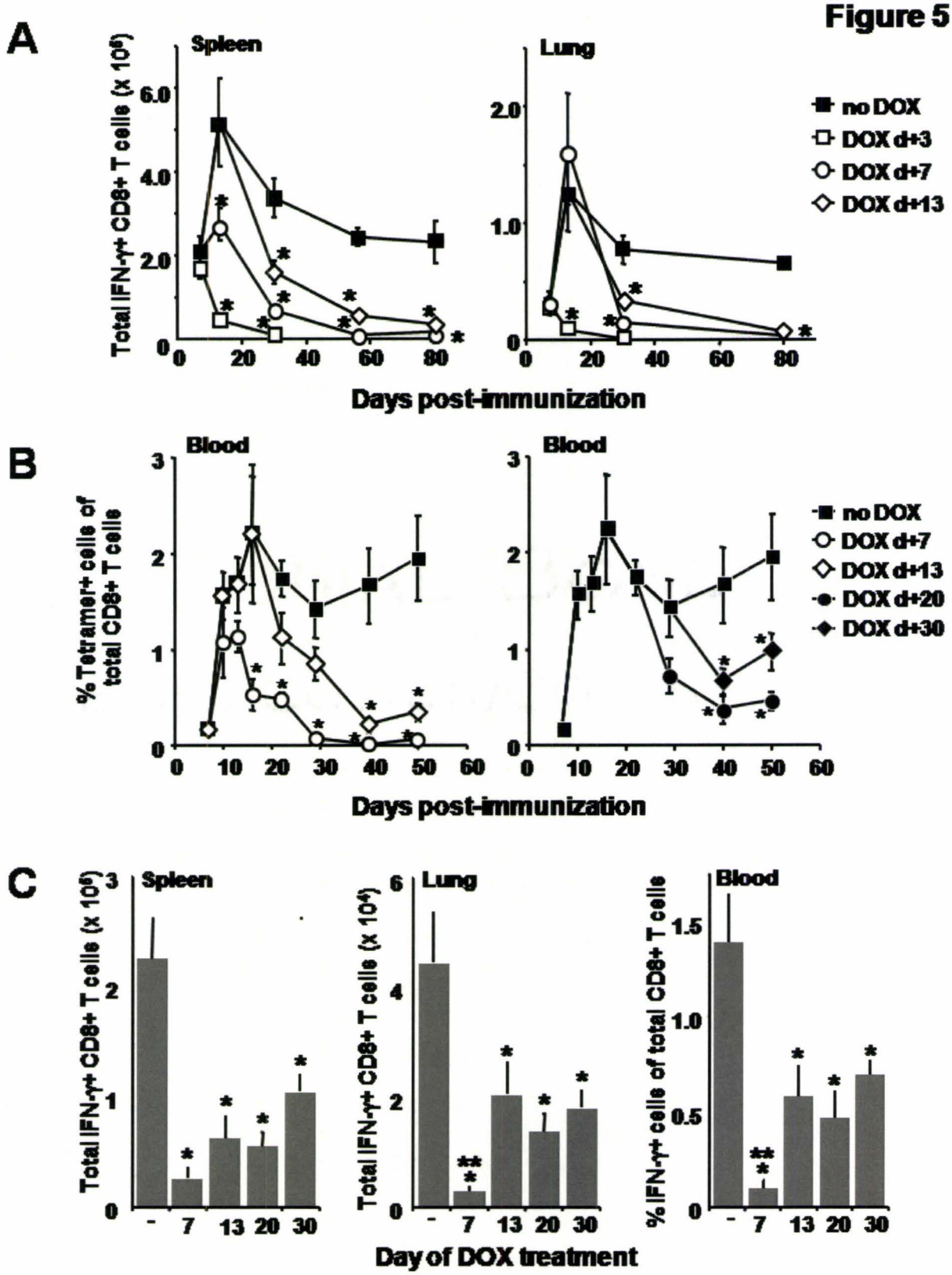
To determine a causal link between antigen expression and CD8+ T cell expansion/contraction, we employed the DOX-regulated vector to investigate the impact of premature termination of antigen expression on the CD8+ T cell response. Mice were immunized with 10^9 pfu of Ad-tTa-SIINFEKL-Luc and transgene expression was extinguished by initiating DOX treatment at 3 days (prior to the appearance of SIINFEKL-specific CD8+ T cells), 7 days (during the expansion phase), or 13 days (around the peak of the response) post-immunization. Mice were sacrificed at various

time points and the presence of SIINFEKL-specific CD8+ T cells was surveyed in the spleen, lung, blood and peritoneal lavage (Figure 5A and 5B and data not shown). When DOX was administered on day 3, SIINFEKL-specific CD8+ T cells could be measured in all tissues at day 7 (Figure 5, first time point) with frequencies similar to mice that did not receive DOX. This likely reflects the fact that several days are required to fully repress transgene expression (Figure 4D; triangles). What is most notable in the mice treated with DOX on day 3 (Figure 5; open squares) is the dramatic and rapid loss of SIINFEKL-specific CD8+ T cells such that we could barely detect any antigen-specific cells 30 days after immunization in this group. Similarly, the frequencies of SIINFEKL-specific CD8+ T cells in mice treated with DOX on day 7 continued to rise after DOX treatment was initiated but again, the population exhibited a dramatic decline (Figure 5, open circles). Strikingly, when DOX treatment was initiated at day 13, a point where the CD8+ T cell response was close to maximum (Figure 4B), the SIINFEKL-specific CD8+ T cell population again exhibited a marked decline demonstrating clearly that maintenance of the memory population was dependent upon continued transgene expression beyond the peak of the response.

Although our previous reports have demonstrated that the peak following intramuscular immunization with rHuAd5 occurs around 10 – 14 days post-immunization (15, 16, 46), it is possible that the peak response following immunization with Ad-tTA-SIINFEKL-Luc may occur at a later time point than our previous results. Therefore, we conducted a more thorough kinetic analysis of the SIINFEKL-specific CD8+ T cell response following

Figure 5. Premature suppression of transgene expression attenuates the magnitude of the primary responses and impairs memory maintenance. ***A.*** Mice were immunized with 10^9 pfu of Ad-tTA-SIINFEKL-Luc on d0 and were separated into 4 groups: Group 1 was started on DOX 3 days after immunization (DOX d+3, open squares), Group 2 was started on DOX 7 days after immunization (DOX d+7, open circles), Group 3 was started on DOX 13 days after immunization (DOX d+13, open diamonds), and Group 4 received no DOX (closed squares). At time points indicated mice were sacrificed and SIINFEKL-specific CD8+ T cells were enumerated in the spleen (***left***) and lung (***right***), Each data points represent the average of 8-12 mice +/- SEM. ***B.*** Mice were immunized with 10^9 pfu of Ad-tTA-SIINFEKL-Luc on d0 and treated with DOX 7 days (DOX d+7, open circles), 13 days (DOX d+13, open diamonds), 20 days (DOX d+20, closed circles) or 30 days (DOX d+30, closed diamonds) later. The control group received no DOX (closed squares). SIINFEKL-specific CD8+ T cells were identified in the peripheral blood by tetramer staining. Each data point represents the average of 5 mice +/- SEM. ***C.*** Mice were treated as described above. Ninety days after immunization, mice were sacrificed and SIINFEKL-specific CD8+ T cells were enumerated in the spleen (***left***), lung (***centre***) and blood (***right***). Each bar represents the average of 5 mice +/- SEM. *, significantly different from No DOX ($p<0.05$); **, significantly different from the groups receiving DOX on days 13 - 30 ($p<0.05$).

Figure 5


A
Spleen
Lung
Total IFN-γ+ CD8+ T cells (x 10⁶)
no DOX
DOX d+3
DOX d+7
DOX d+13
Days post-immunization
B
Blood
Blood
% Tetramer+ cells of total CD8+ T cells
no DOX
DOX d+7
DOX d+13
DOX d+20
DOX d+30
Days post-immunization
C
Spleen
Lung
Blood
Total IFN-γ+ CD8+ T cells (x 10⁶)
Total IFN-γ+ CD8+ T cells (x 10⁴)
% IFN-γ+ cells of total CD8+ T cells
Day of DOX treatment

AdtTA-SIINFEKL-Luc immunization and found that the response peaked around day 16 (Figure 5B). To determine whether transgene expression was required beyond the peak of the response, transgene expression was extinguished at either day 20 or day 30 post-immunization (Figure 5B, left-hand panel). Interestingly, the memory population was found to diminish under both conditions. Examination of the memory populations present 90 days following immunization revealed significant reduction in the numbers of SIINFEKL-specific CD8+ T cells in the spleen, lung and blood when transgene expression was extinguished as late as 30 days following immunization (Figure 5C). Termination of transgene expression at day 7 had the most pronounced effect. Interestingly, the frequencies of SIINFEKL-specific CD8+ T cells were similar in the groups treated with DOX on either day 13, day 20 or day 30.

Since extinction of transgene expression 30 days after immunization resulted in loss of memory CD8+ T cells, we examined a later time point, day 60, to determine whether we could identify a point where transgene expression was no longer required for maintenance of the CD8+ T cell population (Figure 6). In this case, we found that termination of transgene expression did not produce a significant decline in CD8+ T cell population 2 months after the onset of DOX treatment (120 days post-immunization). Thus, it appears that although long-term maintenance of the transgene-specific CD8+ T cell population produced by rHuAd5 is dependent upon persistent transgene expression for at least 30 days following immunization, the population becomes independent of transgene

Figure 6

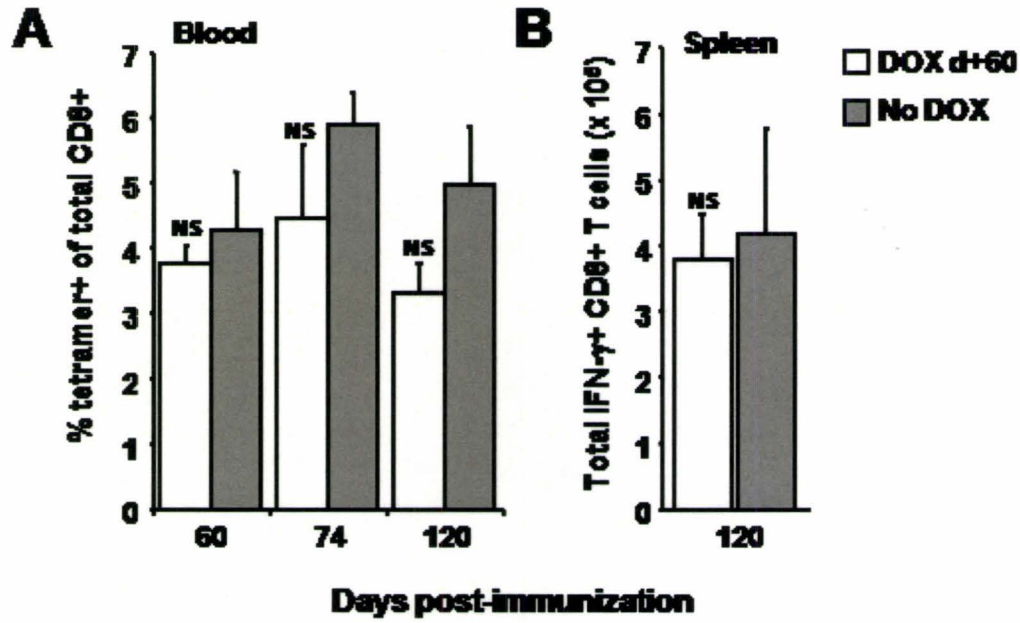
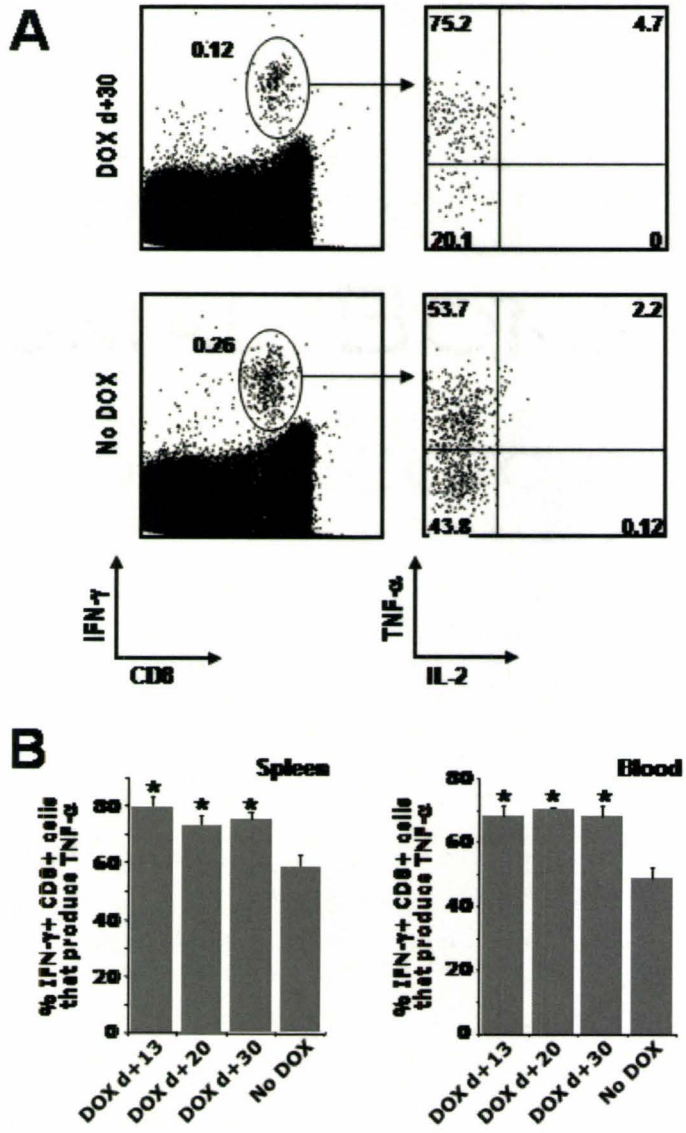


Figure 6. Termination of transgene expression 60 days following immunization does not affect the maintenance of the memory response. Mice were immunized with 10^9 pfu of Ad-tTA-SIINFEKL-Luc on d0. On day 60, half of the mice were put on DOX. ***A.*** The frequency of SIINFEKL-specific cells was assessed on the day DOX treatment began (day 60), 2 weeks later (day 74) and 2 months later (day 120). ***B.*** The number of antigen-specific CD8+ T cells in the spleen was assessed 2 months after the onset of DOX treatment. White bars reflect mice that received DOX on day 60 (DOX d+60) and the gray bars reflect mice that did not receive DOX. Each bar reflects 5 mice +/- SEM.

Figure 7. Premature termination of transgene expression yields memory cells with greater capacity for TNF-α production. Spleens and peripheral blood were harvested from these mice 90 days post-immunization and SIINFEKL-specific CD8+ T cells were identified using ICS. ***A.*** Representative flow cytometry data. Left-hand dot plots reflect total lymphocytes following stimulation with SIINFEKL. The numbers in the ellipses represent the percentage of IFN-γ-+ cells out of the entire lymphocyte population. The right-hand plots were gated on IFN-γ-+ cells. These plots are representative of mice that were treated with DOX 30 days after immunization (DOX d+30) and mice that did not receive DOX (No DOX). ***B*** The percentage of IFN-γ-+ cells that co-produce TNF-α in the spleen (left) and blood (right). Each histogram represents the mean percentage ±SEM for 5 mice. *, significantly different from No DOX ($p<0.05$)

Figure 7


A
DOX d+30
0.12
75.2
4.7
20.1
0
No DOX
0.26
53.7
2.2
43.8
0.12
IFN-γ
CD8
TNF-α
IL-2
B
Spleen
Blood
% IFN-γ+ CD8+ cells that produce TNF-α
0
20
40
60
80
DOX d+13
DOX d+20
DOX d+30
No DOX

expression around 60 days post-immunization. However, if the memory population has become independent of antigen remains to be determined.

Replication-defective rHuAd5 vectors, such as Ad-tTA-SIINFEKL-Luc, can persist within the muscle tissue for months (24), so it is possible that the loss in circulating SIINFEKL-specific CD8+ T cells may be the result of ongoing inflammation due to persistent expression of viral proteins. Therefore, we repeated the experiments where transgene expression was extinguished at day 7 or day 13 post-immunization using a helper-dependent adenovirus which lacks all virus genes (hdAd-tTA-SIINFEKL-Luc). These vectors have been shown to produce substantially less inflammation than E1-deleted Ad vectors such as Ad-tTA-SIINFEKL-Luc (53). Consistent with the results described above, we observed that extinction of transgene expression 7 or 13 days following immunization with hdAd-tTA-SINFEKL-Luc resulted in a pronounced loss of SIINFEKL-specific CD8+ T cells at later times post-immunization (data not shown) indicating that loss of CD8+ T cells following transgene extinction was not due to on-going inflammation produced by proteins expressed from the vector backbone.

Early termination of antigen expression augments TNF-α production by IFN-γ-secreting CD8+ T cells.

We have previously observed that only 50% – 70% of the CD8+ T cells produced by our rHuAd5 vectors also produce TNF-α (15, 54) and speculated that it may be related to the prolonged availability of antigen following immunization. Examination of the antigen-

specific CD8+ T cells present in the spleen, lungs and blood of mice 90 days post-immunization revealed that, indeed, extinction of transgene expression prior to day 30 yielded a CD8+ T cell population with significantly improved capacity for TNF-α production (Figure 7); however the effect was modest. It should be noted that we did not include samples from mice that received DOX on day 7 because the frequencies of SIINFEKL-specific CD8+ T cells were too low to conduct detailed multi-parametric analyses. We had also previously observed that the memory population produced by rHuAd5 fails to produce much IL-2 (15, 54). Although we observed that extinction of transgene expression between day 13 and day 30 post-immunization yielded approximately 2-fold greater numbers of IL-2-producing SIINFEKL-specific CD8+ T cells, the frequencies of IL-2-producing cells remained less than 10% of the total antigen-specific population defined by IFN-γ production (Figure 7A and data not shown). Thus, although premature termination of transgene expression does have an impact on the cytokine profile of the memory population, the effect is modest.

Early termination of antigen expression augments the proportion of central memory CD8+ T cells

We, and others, have previously reported that the CD8+ T cell population evoked by rHuAd5 immunization exhibits a sustained effector memory phenotype (15, 16, 24). This sustained effector memory phenotype was associated with prolonged antigen presentation and we have corroborated the importance of sustained transgene expression in this report. KLRG1 is a natural killer cell marker which serves as a marker for T cells which have

been chronically exposed to antigen and has more recently been associated with short-lived effector CD8+ T cells (SLECs). Indeed, consistent with the requirement for sustained expression of antigen to maintain the CD8+ T cell memory population, we observed that >80% of SIINFEKL-specific CD8+ T cells present at 90 days post-immunization were KLRG1-positive (Figure 8). Termination of transgene expression prior to day 30 resulted in a small decrease in KLRG1 expression (Figure 9A). Interestingly, the KLRG1-positive SIINFEKL-specific CD8+ T cells displayed a corresponding decrease in the expression of CD27, a marker associated with replicative competence and T cell fitness (Figure 8). Consistent with the modest decrease in KLRG1 expression when transgene expression was extinguished prior to day 30, we observed a reciprocal increase in CD27 expression (Figure 9B). We also examined the expression of CD62L and CD127 as these markers have been employed to separate memory cells into effector ($CD62L^-$ $CD127^-$), effector memory ($CD62L^-$ $CD127^+$) and central memory ($CD62L^+$ $CD127^+$) (55) (Figure 8B).

Figure 8

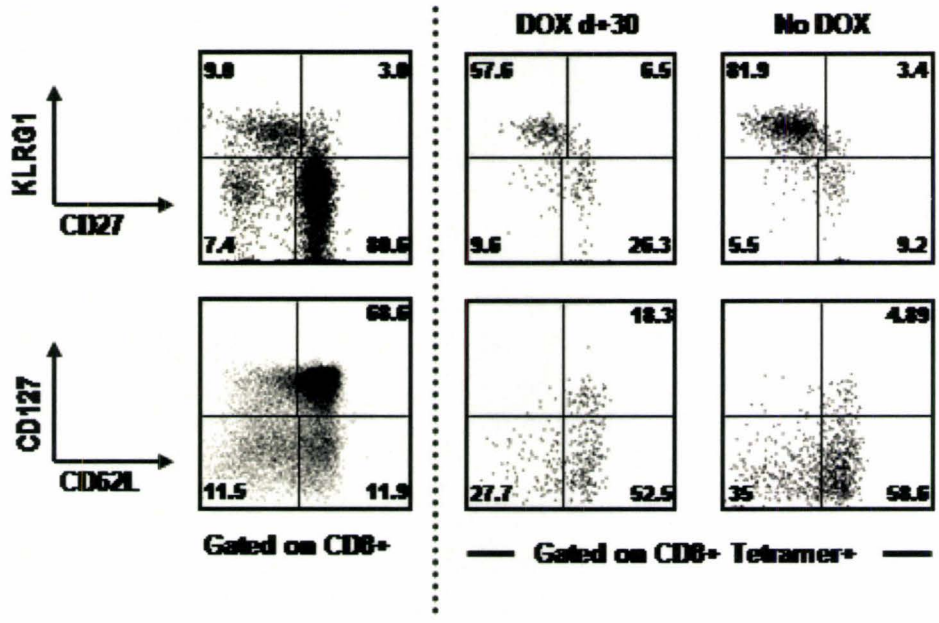
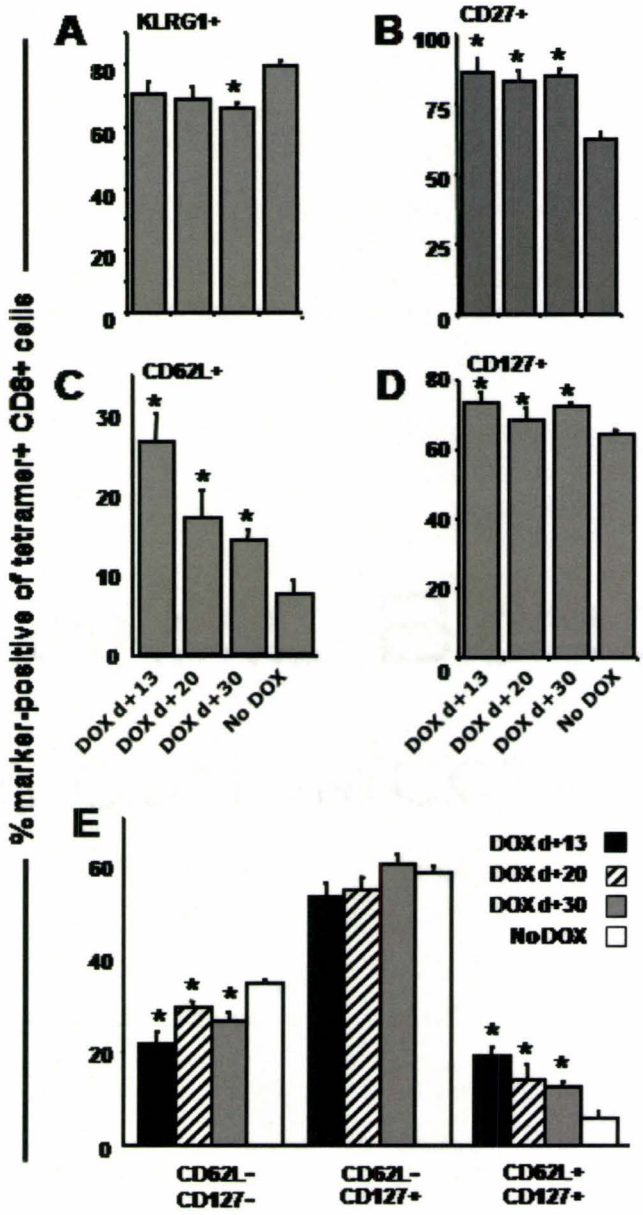


Figure 8. Examples of memory marker expression. Splenocytes were obtained 90 days following immunization, stained with K^b/SIINFEKL tetramer and stained for the expression of CD8, KLRG1, CD27, CD127 and CD62L. The left-hand panels a representative of the total CD8+ T cell population while the other panels are reflective of tetramer-specific CD8+ T cells. Representative plots are shown for mice that received DOX on day 30 post-immunization (DOX d+30) and mice that were not given DOX (No DOX).

Figure 9. Premature extinction of transgene expression results in increased frequencies of central memory cells. Mice were immunized with Ad-tTA-SIINFEKL-Luc and put on DOX 13, 20 and 30 days later. Splenocytes were obtained 90 days after immunization and examined for the expression of KLRG1, CD27, CD62L and CD127. Antigen-specific CD8+ T cells were identified by tetramer-staining. The histograms represent SIINFEKL-specific CD8+ T cells. ***A.*** Frequencies of cells expressing KLRG1. ***B.*** Frequencies of cells expressing CD27. ***C.*** Frequencies of cells expressing CD62L. ***D.*** Frequencies of cells expressing CD127. ***E.*** Frequencies of effector (CD127- CD62L-), effector memory (CD127+ CD62L-) and central memory (CD127+ CD62L+) cells within the tetramer+ CD8+ population. Data reflect the mean ±SEM for 5 mice. *, significantly different from No DOX ($p<0.05$)

Figure 9


A
KLRG1+
B
CD27+
C
CD62L+
D
CD127+
% marker-positive of tetramer+ CD8+ cells
DOX d+13
DOX d+20
DOX d+30
No DOX
E
DOX d+13
DOX d+20
DOX d+30
NoDOX
CD62L-
CD127-
CD62L-
CD127+
CD62L+
CD127+

Premature termination of transgene expression promoted an increase in both CD62L and CD127 expression (Figure 9A and 9B) which resulted in significant increases in central memory cells and a decrease in the frequencies of effector cells (Figure 9E). For the most part, however, the changes in phenotype were modest with the exception of CD62L expression and the majority of the cells retained an effector memory phenotype with high level expression of KLRG1, even in the DOX d+13 group.

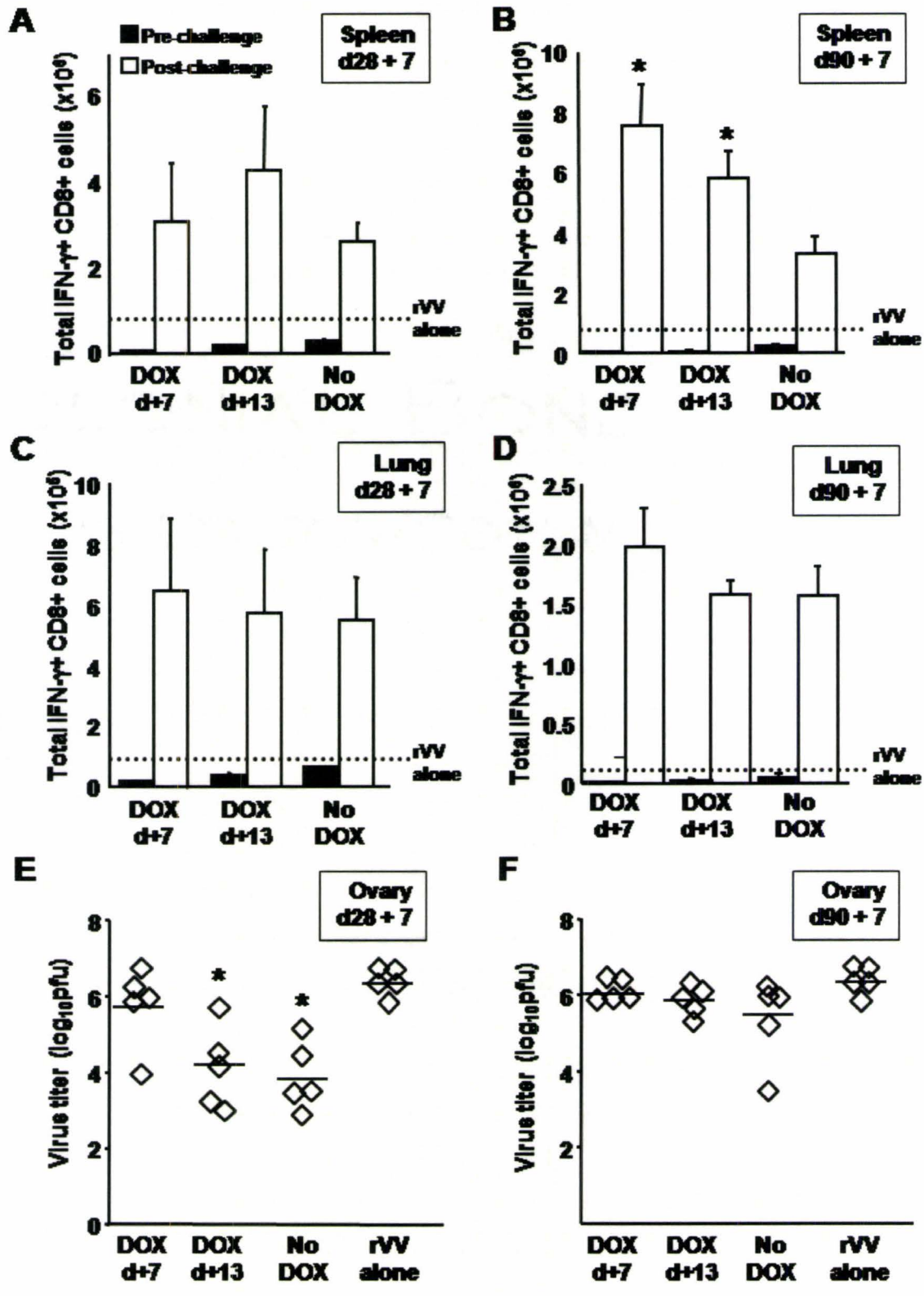
Long-term gene expression does not influence the magnitude of the CD8+ T cell population following secondary challenge.

Since CD62L expression has been associated with CD8+ T cells that have high-proliferative capacity (55), it was of interest to determine whether the memory populations in the mice where DOX was given at early time points would display greater secondary expansion than the populations in mice that did not receive DOX. Therefore, mice were immunized with Ad-tTA-SIINFEKL-Luc and started on DOX either 7 or 13 days after immunization. Thirty and ninety days after immunization, the mice were challenged with 5×10^6 pfu of rVV-ESOVA, which shares only the SIINFEKL epitope in common with Ad-tTA-SIINFEKL-Luc. Mice were sacrificed 7 days post challenge and the frequencies of SIINFEKL-specific CD8+ T cells were assessed in the spleen, blood, lung and peritoneal cavity. Interestingly, although premature termination of transgene expression had a marked impact upon the frequencies of SIINFEKL-specific CD8+ T cells prior to challenge (Figure 10, panels A-D, black bars; Figure 5, open symbols), all groups of mice displayed similar numbers of SIINFEKL-specific CD8+ T cells following

challenge (Figure 10, panels A-D, open bars). Thus, with regard to secondary expansion, the antigen-specific CD8+ T cells in the mice where transgene expression was terminated on day 7 or day 13 expanded considerably more than the CD8+ T cells in mice where transgene expression was not terminated. While these observations could be suggestive of enhanced proliferative capacity in the DOX-treated groups, it may also reflect more rapid clearance of antigen in the untreated group due to the higher frequency of SIINFEKL-specific effectors as described in other reports (56, 57). This may be true for the challenge at day 30 as we observed that levels of rVV-ESOVA in the challenged mice were inversely related to the magnitude of secondary expansion such that the mice treated with DOX on day 7 had the highest virus load and the greatest secondary expansion while the mice that never received DOX had the lowest virus load and smallest secondary expansion (Figure 10E). However, the challenge at day 90 resulted in similar virus loads in all mice, yet the groups that were treated with DOX still displayed greater expansion than the untreated group (Figure 9B and 9D) suggesting that the increased expansion in the DOX d+7 and DOX d+13 groups may be reflective of a memory population with increased capacity to expand consistent with the increased frequencies of CD62L+ memory CD8+ T cells (Figure 9).

Figure 10. Premature termination of transgene expression does not impair the magnitude of the secondary response. Mice were immunized with 10^9 pfu of Ad-tTA-SIINFEKL-Luc on d0 and were separated into 3 groups: Group 1 was started on DOX 7 days after immunization (DOX d+7), Group 2 was started on DOX 13 days after immunization (DOX d+13), and Group 3 received no DOX (closed squares). Five mice from each group were challenged with 10^7 pfu rVV-ESOVA either 28 or 90 days after immunization. SIINFEKL-specific CD8+ T cells were enumerated in the spleen (***A and C***) or lung (***B and D***) before rVV-ESOVA challenge (closed bars) and 7 days following rVV-ESOVA challenge (open bars). The dotted line represents the number of antigen-specific CD8+ T cells produced by immunization with rVV-ESOVA alone. Ovaries from the rVV-ESOVA challenged mice were also harvested following virus challenge and assayed for infectious vaccinia virus (***E and F***). Each point represents a single mouse.

Figure 10


A
Pre-challenge
Post-challenge
Spleen d28 + 7
Total IFN-γ+ CD8+ cells (x10⁶)
rVV alone
DOX d+7
DOX d+13
No DOX
B
Spleen d90 + 7
C
Lung d28 + 7
D
Lung d90 + 7
E
Ovary d28 + 7
Virus titer (log10pfu)
F
Ovary d90 + 7
rVV alone

Discussion

RHuAd5 vectors have emerged as a highly promising platform for immunization based on studies in rodents and non-human primates using a variety of pathogens, including: SHIV, HCV, H5N1 influenza, malaria and *M. tuberculosis* (2, 58-61) and these vectors have also proven to be effective agents for breaking tolerance to self-antigens in cancer immunotherapy strategies (62, 63). Therefore, understanding the mechanisms by which these vectors imprint and maintain the memory T cell response will provide important insights for future vaccine design. The results of the study described herein offer important new insight into the relationship between the longevity of transgene and maintenance of effector CD8+ T cell memory following immunization with rHuAd5. Furthermore, the temporal relationship between antigen expression and the development of CD8+ T cell immunity remains a nebulous area and the data in this manuscript also provides novel information regarding the role for long-term, low-level antigen in the maintenance of CD8+ T cell memory.

The observation that transgene expression was required throughout the expansion phase following rHuAd5 immunization contrasts considerably with the "auto-pilot" hypothesis that suggests CD8+ T cells undergo an intrinsic program of expansion and differentiation that is executed following a short period of stimulation in vivo (estimated to be on the order of 24 hours) (64). Consistent with the auto-pilot model, it has been demonstrated that antigen presentation following infection with several acute agents (*P. yoelii*, *L. monocytogenes*, LCMV) persists for only a brief period (2-4 days) following infection

(33, 42, 65-68) yet the CD8+ T cell population continues to expand beyond the point where antigen presentation ceases and subsequently contracts in a manner which appears to reflect an intrinsic program (65). This short-lived antigen presentation has been linked to a negative-feedback mechanism where recently activated CTL kill antigen-loaded DCs and ultimately remove the antigen reservoir (42, 68). In contrast, multiple reports support the need for antigen presentation beyond the first 3-4 days after immunization to achieve maximal CD8+ T cell expansion. Premature removal of the antigen depot following HSV-1 infection or plasmid vaccination diminished the magnitude of the primary response (38, 39). Thus, although only a brief exposure of antigen is required to engage individual CD8+ T cells, maximal activation of CD8+ T cell populations may require a longer period of antigen exposure likely as a result of the asynchronous nature of APC-T cell interactions *in vivo*. However, extending the availability of antigen does not always increase the magnitude of the primary response or delay the rate of contraction (41, 65), thus other factors must also be at play.

The need for continued transgene expression beyond the peak of the expansion phase to sustain CD8+ T cell levels may either reflect a situation where naïve T cells are continuously recruited into the memory population to replace dying cells or it may reflect a memory population which has become antigen-dependent. We believe that the data in our previous manuscript (15) and the present report support the latter hypothesis for the following reasons: 1) priming of naïve CD8+ T cells only occurs for a few days following intramuscular immunization, 2) thymectomy had no impact upon the magnitude of the

memory population, 3) induction of partial hematopoietic chimerism failed to demonstrate the recruitment of naïve CD8+ T cells into the circulating pool of antigen-specific cells at later time points following immunization (> 30 days) and 3) the majority of the memory cells elicited by rHuAd5 expressed high levels of KLRG1, which is an indicator of recent and repetitive antigenic stimulation (69). With regard to our hypothesis that the memory population is generated by continual stimulation of antigen-experienced cells, it is interesting to note that the memory population produced by intramuscular immunization with rHuAd5 resembles the phenotype of CD8+ T cells that have been expanded by multiple rounds of immunization. Following secondary and tertiary stimulations, the CD8+ T cells that were originally $CD62L^{hi}$ preferentially retain an effector memory phenotype, they are slow to regain CD62L expression, only a small fraction of the cells produced IL-2 and they remain KLRG1-positive for a prolonged period (70, 71). The need for prolonged transgene expression is an important observation and supports the use of robust, high-level, sustained promoters in the context of rAd vaccines to sustain maximal CD8+ T cell levels. Furthermore, it suggests that the mechanism of CD8+ T cell memory maintenance following intramuscular immunization with rHuAd5 vectors is distinct from other infectious agents, where a sustainable memory population is generated following brief antigenic stimulation (33, 42, 65-68).

Although transgene expression does not appear to be required to sustain the memory population beyond day 60 following intramuscular immunization, it remains unclear whether the memory CD8+ T cells produced by rHuAd5 ultimately become antigen-

independent. It is possible that a depot of processed antigen develops over time which ultimately sustains the CD8+ T cell population. In the case of chronic LCMV infection, the resultant CD8+ T cell population is dependent upon antigen even at very late time points (>100 days after immunization) (72). A key difference between the memory CD8+ T cells that develop during chronic LCMV infection and those produced by rHuAd5 is the emergence of CD127+ CD8+ T cells. As shown in this report and our previous report, a high fraction of the memory CD8+ T cells express CD127 by 60 days following intramuscular immunization with rHuAd5 and this frequency continues to rise at later time points where most of the cells express CD127 and increased levels of Bcl-2 (unpublished results). By contrast, the memory population that develops under conditions of chronic LCMV infection fails to express high levels of CD127 and is poorly-responsive to homeostatic survival cytokines like IL-7 and IL-15 (72). These data suggest that at some point in the memory phase, these rHuAd5-induced CD8+ T cells may be eventually "weaned" off antigen. However, the mechanisms and timing of this process are still under investigation.

Our observations are similar to two recent reports investigating temporal regulation of transgene expression following immunization with plasmid DNA where short-term transgene expression produced higher levels of central memory CD8+ T cells and a more robust secondary response (38, 73). It is interesting that in both studies, premature termination of antigen expression resulted in higher absolute levels following secondary stimulation whereas we did not observe a similar enhancement in the secondary response

in our studies, with the exception of the response in the spleen when the secondary stimulation was provided 90 days post-immunization. The data presented herein are consistent with previous reports from our lab, and others, where the magnitude of the secondary response to either vaccinia challenge or influenza challenge appears to be independent of the magnitude of the primary response produced by rAd (15, 16, 74-76); however we do not currently have an explanation for these observations.

Overall, this report demonstrates for the first time a causal link between the duration of transgene expression following immunization with a rHuAd5 vaccine and maintenance of the CD8+ T cell population. These data have important implication for future design of rAd vaccines. Our observations have also provided novel information regarding the importance of low-level persistent antigen in the maintenance of functional CD8+ T cell memory.

References

1. Tatsis, N., and H. C. Ertl. 2004. Adenoviruses as vaccine vectors. *Mol Ther.* 10:616-629.
2. Barouch, D. H., and G. J. Nabel. 2005. Adenovirus vector-based vaccines for human immunodeficiency virus type 1. *Hum Gene Ther.* 16:149-156.
3. Catanzaro, A. T., R. A. Koup, M. Roederer, R. T. Bailer, M. E. Enama, Z. Moodie, L. Gu, J. E. Martin, L. Novik, B. K. Chakrabarti, B. T. Butman, J. G. Gall, C. R. King, C. A. Andrews, R. Sheets, P. L. Gomez, J. R. Mascola, G. J. Nabel, and B. S. Graham. 2006. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. *J Infect Dis.* 194:1638-1649.
4. Stone, D., S. Ni, Z. Y. Li, A. Gaggar, N. DiPaolo, Q. Feng, V. Sandig, and A. Lieber. 2005. Development and assessment of human adenovirus type 11 as a gene transfer vector. *J Virol.* 79:5090-5104.
5. Barouch, D. H., M. G. Pau, J. H. Custers, W. Koudstaal, S. Kostense, M. J. Havenga, D. M. Truitt, S. M. Sumida, M. G. Kishko, J. C. Arthur, B. Korioth-Schmitz, M. H. Newberg, D. A. Gorgone, M. A. Lifton, D. L. Panicali, G. J. Nabel, N. L. Letvin, and J. Goudsmit. 2004. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol.* 172:6290-6297.
6. Tatsis, N., L. Tesema, E. R. Robinson, W. Giles-Davis, K. McCoy, G. P. Gao, J. M. Wilson, and H. C. Ertl. 2005. Chimpanzee-origin adenovirus vectors as vaccine carriers. *Gene Ther.*
7. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J Virol.* 78:5535-5545.
8. Zaph, C., J. Uzonna, S. M. Beverley, and P. Scott. 2004. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. *Nat Med.* 10:1104-1110.
9. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol.* 4:225-234.
10. Zammit, D. J., L. S. Cauley, Q. M. Pham, and L. Lefrancois. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity.* 22:561-570.
11. Bachmann, M. F., P. Wolint, K. Schwarz, and A. Oxenius. 2005. Recall proliferation potential of memory CD8+ T cells and antiviral protection. *J Immunol.* 175:4677-4685.
12. Jackson, S. S., J. E. Schmitz, M. J. Kuroda, P. F. McKay, S. M. Sumida, K. L. Martin, F. Yu, M. A. Lifton, D. A. Gorgone, and N. L. Letvin. 2005. Evaluation of CD62L expression as a marker for vaccine-elicited memory cytotoxic T lymphocytes. *Immunology.* 116:443-453.
13. Klebanoff, C. A., L. Gattinoni, P. Torabi-Parizi, K. Kerstann, A. R. Cardones, S. E. Finkelstein, D. C. Palmer, P. A. Antony, S. T. Hwang, S. A. Rosenberg, T. A. Waldmann, and N. P. Restifo. 2005. Central memory self/tumor-reactive CD8+ T

cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci U S A.* 102:9571-9576.

14. Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts, and D. L. Woodland. 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J Exp Med.* 204:1625-1636.
15. Yang, T. C., J. Millar, T. Groves, N. Grinshtein, R. Parsons, S. Takenaka, Y. Wan, and J. L. Bramson. 2006. The CD8+ T Cell Population Elicited by Recombinant Adenovirus Displays a Novel Partially Exhausted Phenotype Associated with Prolonged Antigen Presentation That Nonetheless Provides Long-Term Immunity. *J Immunol.* 176:200-210.
16. Yang, T., J. Millar, T. Groves, W. Zhou, N. Grinshtein, R. Parsons, C. Evelegh, Z. Xing, Y. Wan, and J. L. Bramson. 2007. On the role of CD4+ T cells in the CD8+ T cell response elicited by recombinant adenovirus vaccines. *Mol Ther.* Mar 20; [Epub ahead of print]
17. Kemball, C. C., E. D. Lee, V. Vezys, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2005. Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. *J Immunol.* 174:7950-7960.
18. Obar, J. J., S. Fuse, E. K. Leung, S. C. Bellfy, and E. J. Usherwood. 2006. Gammaherpesvirus persistence alters key CD8 T-cell memory characteristics and enhances antiviral protection. *J Virol.* 80:8303-8315.
19. Bachmann, M. F., P. Wolint, K. Schwarz, P. Jager, and A. Oxenius. 2005. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol.* 175:4686-4696.
20. Gold, M. C., M. W. Munks, M. Wagner, C. W. McMahon, A. Kelly, D. G. Kavanagh, M. K. Slifka, U. H. Koszinowski, D. H. Raulet, and A. B. Hill. 2004. Murine cytomegalovirus interference with antigen presentation has little effect on the size or the effector memory phenotype of the CD8 T cell response. *J Immunol.* 172:6944-6953.
21. Harrington, L. E., R. Most Rv, J. L. Whitton, and R. Ahmed. 2002. Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. *J Virol.* 76:3329-3337.
22. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science.* 291:2413-2417.
23. Kedzierska, K., N. L. La Gruta, S. J. Turner, and P. C. Doherty. 2006. Establishment and recall of CD8+ T-cell memory in a model of localized transient infection. *Immunol Rev.* 211:133-145.
24. Tatsis, N., J. C. Fitzgerald, A. Reyes-Sandoval, K. C. Harris-McCoy, S. E. Hensley, D. Zhou, S. W. Lin, A. Bian, Z. Q. Xiang, A. Iparraguirre, C. Lopez-Camacho, E. J. Wherry, and H. C. Ertl. 2007. Adenoviral vectors persist in vivo and maintain activated CD8+ T cells: implications for their use as vaccines. *Blood.* 110:1916-1923.

25. Kianizad, K., L. A. Marshall, D. Bernard, N. Grinshtein, F. Beermann, S. Zhang, Y. Wan, and J. L. Bramson. 2007. Elevated freuencies of self-reactive CD8+ T cells following immunization with a xenoantigen is due to the presence of heteroclitic CD4+ T cell helper epitopes. *Cancer Res.* 67:6459 - 6467.
26. Krebs, P., E. Scandella, B. Odermatt, and B. Ludewig. 2005. Rapid functional exhaustion and deletion of CTL following immunization with recombinant adenovirus. *J Immunol.* 174:4559-4566.
27. Pascual, D. W., N. Walters, and P. Hillemeyer. 1998. Repeated intratracheal instillations of nonreplicating adenovirus 2 vector attenuate CTL responses and IFN-gamma production. *J Immunol.* 160:4465-4472.
28. Turner, D. L., L. S. Cauley, K. M. Khanna, and L. Lefrancois. 2007. Persistent antigen presentation after acute vesicular stomatitis virus infection. *J Virol.* 81:2039-2046.
29. Zammit, D. J., D. L. Turner, K. D. Klonowski, L. Lefrancois, and L. S. Cauley. 2006. Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity.* 24:439-449.
30. Jelley-Gibbs, D. M., J. P. Dibble, D. M. Brown, T. M. Strutt, K. K. McKinstry, and S. L. Swain. 2007. Persistent depots of influenza antigen fail to induce a cytotoxic CD8 T cell response. *J Immunol.* 178:7563-7570.
31. van Stipdonk, M. J., G. Hardenberg, M. S. Bijker, E. E. Lemmens, N. M. Droin, D. R. Green, and S. P. Schoenberger. 2003. Dynamic programming of CD8+ T lymphocyte responses. *Nat Immunol.* 4:361-365.
32. Kaech, S. M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol.* 2:415-422.
33. Mercado, R., S. Vijh, S. E. Allen, K. Kerksiek, I. M. Pilip, and E. G. Pamer. 2000. Early programming of T cell populations responding to bacterial infection. *J Immunol.* 165:6833-6839.
34. Curtsinger, J. M., C. M. Johnson, and M. F. Mescher. 2003. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J Immunol.* 171:5165-5171.
35. Gett, A. V., F. Sallusto, A. Lanzavecchia, and J. Geginat. 2003. T cell fitness determined by signal strength. *Nat Immunol.* 4:355-360.
36. Storni, T., C. Ruedl, W. A. Renner, and M. F. Bachmann. 2003. Innate immunity together with duration of antigen persistence regulate effector T cell induction. *J Immunol.* 171:795-801.
37. Miller, M., G. Rekas, K. Dayball, Y. Wan, and J. Bramson. 2004. Effiency of electroporated plasmid vaccines correlates with long-term antigen production in-vivo. *Vaccine.* 22:5200 - 5205.
38. Hovav, A. H., M. W. Panas, S. Rahman, P. Sircar, G. Gillard, M. J. Cayabyab, and N. L. Letvin. 2007. Duration of antigen expression in vivo following DNA immunization modifies the magnitude, contraction, and secondary responses of CD8+ T lymphocytes. *J Immunol.* 179:6725-6733.

39. Stock, A. T., S. N. Mueller, A. L. van Lint, W. R. Heath, and F. R. Carbone. 2004. Cutting Edge: Prolonged Antigen Presentation after Herpes Simplex Virus-1 Skin Infection. *J Immunol.* 173:2241-2244.
40. Prlic, M., G. Hernandez-Hoyos, and M. J. Bevan. 2006. Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. *J Exp Med.* 203:2135-2143.
41. Hafalla, J. C., G. Sano, L. H. Carvalho, A. Morrot, and F. Zavala. 2002. Short-term antigen presentation and single clonal burst limit the magnitude of the CD8(+) T cell responses to malaria liver stages. *Proc Natl Acad Sci U S A.* 99:11819-11824.
42. Wong, P., and E. G. Pamer. 2003. Feedback regulation of pathogen-specific T cell priming. *Immunity.* 18:499-511.
43. Addison, C. L., M. Hitt, D. Kunsken, and F. L. Graham. 1997. Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. *J Gen Virol.* 78 (Pt 7):1653-1661.
44. Ng, P., R. J. Parks, D. T. Cummings, C. M. Evelegh, and F. L. Graham. 2000. An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. *Human Gene Therapy.* 11:693-699.
45. Palmer, D., and P. Ng. 2003. Improved system for helper-dependent adenoviral vector production. *Mol Ther.* 8:846-852.
46. Yang, T. C., K. Dayball, Y. H. Wan, and J. Bramson. 2003. Detailed analysis of the CD8+ T-cell response following adenovirus vaccination. *J Virol.* 77:13407-13411.
47. Bramson, J. L., M. Hitt, J. Gauldie, and F. L. Graham. 1997. Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. *Gene Ther.* 4:1069-1076.
48. Restifo, N. P., I. Bacik, K. R. Irvine, J. W. Yewdell, B. J. McCabe, R. W. Anderson, L. C. Eisenlohr, S. A. Rosenberg, and J. R. Bennink. 1995. Antigen processing in vivo and the elicitation of primary CTL responses. *J Immunol.* 154:4414-4422.
49. Vezys, V., D. Masopust, C. C. Kemball, D. L. Barber, L. A. O'Mara, C. P. Larsen, T. C. Pearson, R. Ahmed, and A. E. Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J Exp Med.* 203:2263-2269.
50. Badovinac, V. P., J. S. Haring, and J. T. Harty. 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity.* 26:827-841.
51. Mintern, J. D., G. M. Davey, G. T. Belz, F. R. Carbone, and W. R. Heath. 2002. Cutting edge: precursor frequency affects the helper dependence of cytotoxic T cells. *J Immunol.* 168:977-980.
52. Furth, P. A., L. St Onge, H. Boger, P. Gruss, M. Gossen, A. Kistner, H. Bujard, and L. Hennighausen. 1994. Temporal control of gene expression in transgenic

mice by a tetracycline-responsive promoter. *Proc Natl Acad Sci U S A.* 91:9302-9306.

53. Chen, H. H., L. M. Mack, S. Y. Choi, M. Ontell, S. Kochanek, and P. R. Clemens. 1999. DNA from both high-capacity and first-generation adenoviral vectors remains intact in skeletal muscle. *Hum Gene Ther.* 10:365-373.
54. Yang, T. C., J. Millar, T. Groves, W. Zhou, N. Grinshtein, R. Parsons, C. Evelegh, Z. Xing, Y. Wan, and J. Bramson. 2007. On the role of CD4+ T cells in the CD8+ T-cell response elicited by recombinant adenovirus vaccines. *Mol Ther.* 15:997-1006.
55. Huster, K. M., V. Busch, M. Schiemann, K. Linkemann, K. M. Kerksiek, H. Wagner, and D. H. Busch. 2004. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A.* 101:5610-5615.
56. Cockburn, I. A., S. Chakravarty, M. G. Overstreet, A. Garcia-Sastre, and F. Zavala. 2008. Memory CD8+ T cell responses expand when antigen presentation overcomes T cell self-regulation. *J Immunol.* 180:64-71.
57. Wong, P., M. Lara-Tejero, A. Ploss, I. Leiner, and E. G. Pamer. 2004. Rapid development of T cell memory. *J Immunol.* 172:7239-7245.
58. Capone, S., A. Meola, B. B. Ercole, A. Vitelli, M. Pezzanera, L. Ruggeri, M. E. Davies, R. Tafi, C. Santini, A. Luzzago, T. M. Fu, A. Bett, S. Colloca, R. Cortese, A. Nicosia, and A. Folgori. 2006. A novel adenovirus type 6 (ad6)-based hepatitis C virus vector that overcomes preexisting anti-ad5 immunity and induces potent and broad cellular immune responses in rhesus macaques. *J Virol.* 80:1688-1699.
59. Xing, Z., M. Santosuosso, S. McCormick, T. C. Yang, J. Millar, M. Hitt, Y. Wan, J. Bramson, and H. M. Vordermeier. 2005. Recent advances in the development of adenovirus- and poxvirus-vectored tuberculosis vaccines. *Curr Gene Ther.* 5:485-492.
60. Gao, W., A. C. Soloff, X. Lu, A. Montecalvo, D. C. Nguyen, Y. Matsuoka, P. D. Robbins, D. E. Swayne, R. O. Donis, J. M. Katz, S. M. Barratt-Boyes, and A. Gambotto. 2006. Protection of Mice and Poultry from Lethal H5N1 Avian Influenza Virus through Adenovirus-Based Immunization. *J Virol.* 80:1959-1964.
61. Li, S., E. Locke, J. Bruder, D. Clarke, D. L. Doolan, M. J. Havenga, A. V. Hill, P. Liljestrom, T. P. Monath, H. Y. Naim, C. Ockenhouse, D. C. Tang, K. R. Van Kampen, J. F. Viret, F. Zavala, and F. Dubovsky. 2006. Viral vectors for malaria vaccine development. *Vaccine.*
62. Gallo, P., S. Dharmapuri, B. Cipriani, and P. Monaci. 2005. Adenovirus as vehicle for anticancer genetic immunotherapy. *Gene Ther.* 12 Suppl 1:S84-91.
63. Lane, C., J. Leitch, X. Tan, J. Hadjati, J. L. Bramson, and Y. Wan. 2004. Vaccination-induced autoimmune vitiligo is a consequence of secondary trauma to the skin. *Cancer Res.* 64:1509-1514.
64. Bevan, M. J., and P. J. Fink. 2001. The CD8 response on autopilot. *Nat Immunol.* 2:381-382.
65. Badovinac, V. P., B. B. Porter, and J. T. Harty. 2002. Programmed contraction of CD8(+) T cells after infection. *Nat Immunol.* 3:619-626.

66. Corbin, G. A., and J. T. Harty. 2004. Duration of infection and antigen display have minimal influence on the kinetics of the CD4+ T cell response to Listeria monocytogenes infection. *J Immunol.* 173:5679-5687.
67. Porter, B. B., and J. T. Harty. 2006. The onset of CD8+-T-cell contraction is influenced by the peak of Listeria monocytogenes infection and antigen display. *Infect Immun.* 74:1528-1536.
68. Hafalla, J. C., A. Morrot, G. Sano, G. Milon, J. J. Lafaille, and F. Zavala. 2003. Early self-regulatory mechanisms control the magnitude of CD8+ T cell responses against liver stages of murine malaria. *J Immunol.* 171:964-970.
69. Thimme, R., V. Appay, M. Koschella, E. Panther, E. Roth, A. D. Hislop, A. B. Rickinson, S. L. Rowland-Jones, H. E. Blum, and H. Pircher. 2005. Increased expression of the NK cell receptor KLRG1 by virus-specific CD8 T cells during persistent antigen stimulation. *J Virol.* 79:12112-12116.
70. Masopust, D., S. J. Ha, V. Vezys, and R. Ahmed. 2006. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J Immunol.* 177:831-839.
71. Jabbari, A., and J. T. Harty. 2006. Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype. *J Exp Med.* 203:919-932.
72. Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A.* 101:16004-16009.
73. Radcliffe, J. N., J. S. Roddick, F. K. Stevenson, and S. M. Thirdborough. 2007. Prolonged Antigen Expression following DNA Vaccination Impairs Effector CD8+ T Cell Function and Memory Development. *J Immunol.* 179:8313-8321.
74. Bertram, E. M., W. Dawicki, B. Sedgmen, J. L. Bramson, D. H. Lynch, and T. H. Watts. 2004. A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza in vivo. *J Immunol.* 172:981-988.
75. Pinto, A. R., J. C. Fitzgerald, W. Giles-Davis, G. P. Gao, J. M. Wilson, and H. C. Ertl. 2003. Induction of CD8+ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol.* 171:6774-6779.
76. Bruna-Romero, O., G. Gonzalez-Aseguinolaza, J. C. Hafalla, M. Tsuji, and R. S. Nussenzweig. 2001. Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen. *Proc Natl Acad Sci U S A.* 98:11491-11496.

- Chapter 4 –

CD8+ and CD4+ T cell responses produced by adenovirus vaccines are differentially influenced by antigen design

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Prologue

In this chapter we have evaluated CD4+ T cell immunity following rAd immunization and how antigen targeting influences both CD8+ T cell and CD4+ T cell responses. The primary findings of this study are as follows:

- Following RHuAd5 immunization, the CD4+ T cell response was found to be more polyfunctional and acquired a central memory phenotype more quickly than CD8+ T cells.
- Antigen targeting influenced the kinetics of the CD8+ T cell response but did not have a significant impact on their functional or phenotypic characteristics.
- Unlike CD8+ T cells, the CD4+ T cell response was not influenced by antigen targeting elements.

The works contained within this study, where planned, executed and assembled by myself, with technical assistance from Teng Chih Yang, Natalie Grinshtein, Jennifer Bassett, Dana Nyholt, Jonathan Finn, Robin Parsons and Carole Evelegh. Dr. Jonathan Bramson provided general supervision and along with Dr Yonghong Wan assisted with experimental design and interpretation of the results. The data described in this chapter will be submitted to the Journal of Immunology.

Title: CD8+ and CD4+ T cell responses produced by adenovirus vaccines are differentially influenced by antigen design.

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Running title: Antigen design influences on T cell immunity

Keywords: T lymphocyte, antigen, adenovirus, vaccine

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Abstract

Recent studies have suggested that effective protective immunity relies upon T cells which elaborate multiple cytokines and display a central memory phenotype. We have observed that the CD8+ T cell population elicited by recombinant human adenovirus type 5 (rHuAd5) displays some functional limitations and a phenotype consistent with effector memory T cells. To determine whether the CD4+ T cell response produced by rHuAd5 displays functional and phenotypic properties similar to the CD8+ T cell population, we generated a series of synthetic antigens that were composed of well-defined epitopes from the lymphocytic choriomeningitis. These antigens incorporated various targeting elements to direct the epitopes to the cytosol, the ER and the endocytic compartment. The CD4+ T cell population elicited by rHuAd5 was found to be more polyfunctional than the CD8+ T cell population. Additionally, the CD4+ T cells acquired a central memory phenotype more rapidly than the CD8+ T cells. Targeting elements directing the epitopes to the endocytic pathway did not augment the magnitude of the T cell response. Although the kinetics of the CD4+ T cell response were similar for all of the antigens used for immunization, CD8+ T cell expansion and contraction was accelerated following immunization with synthetic antigens that were targeted to the ER. Nonetheless, CD8+ T cells produced by all antigens displayed comparable functionality and phenotype. These data demonstrate that CD4+ and CD8+ T cell responses are differentially influenced by rHuAd5 and offer new insight into the immunobiology of rHuAd5 vaccines.

Introduction

CD4+ and CD8+ T cells play a central role in the adaptive immune response and host defence against infectious agents. As such, a great deal of attention has been directed towards the development of vaccine technologies that generate protective T cell memory. CD8+ T cells have the capacity to directly recognize and lyse virus-infected cells and tumors. As a result, CD8+ T cells have traditionally been considered the key effector cell in the cellular immune response. CD4+ T cells have classically been considered "helper" cells that are required to optimally engage CD8+ T cell and B cell immunity. However, further investigation has also revealed a direct effector role of CD4+ T cells in models of virus infection and tumor challenge. Thus, to provide optimal T cell memory, a vaccine should effectively engage both CD8+ and CD4+ T cells.

Recombinant adenovirus (rAd) vaccines have garnered considerable attention as a vaccination platform due to the strong protective immunity produced by rAd vaccines in multiple infectious disease models and tumor challenge studies . Given the robust protective cellular immunity generated by rAd vaccines in murine models and non-human primates, further investigation of the biology of the immune response produced by these vaccines should yield important insights for vaccine design. The prototypic rAd vectors based on human serotype 5 (rHuAd5) are rendered replication-deficient by removal of the E1 region and are generally thought to produce an acute infection. However, the CD8+ T cell response produced by rAd vaccines exhibits a phenotypic and functional signature more consistent with CD8+ T cells produced by persistent infectious agents like polyoma

virus [2], murine herpesvirus-68 and murine cytomegalovirus [4, 5] rather than acute agents like LCMV , vaccinia [7] , VSV [8], or influenza . Indeed work from our group, and others, has revealed that antigen presentation and transgene expression can persist for months after immunization with rAd [10, 11]. Thus, the rHuAd5 seems to produce a persistent infection, which yields a long-term transgene-specific CD8+ T cell population that is composed primarily of effector-memory cells that retain long-term cytolytic ability.

In contrast to the detailed information available regarding the CD8+ T cell response produced rHuAd5, less is known about the CD4+ T cell response. Our previous work has demonstrated that CD4+ T cells are required for maximal expansion and maintenance of the CD8+ T cell population following immunization with rHuAd5 vaccines . However, we did not directly examine CD4+ T cells directly in those studies. In several reports, the CD4+ T cell population produced by rHuAd5 appeared to display evidence of some functional impairments [13-15] consistent with our previous report that rHuAd5-induced CD8+ T cells had some functional limitations . However, those previous reports did not directly compare the polyfunctionality of rHuAd5-induced CD8+ T cells to rHuAd5-induced CD4+ T cells. In the current manuscript, we have directly compared the CD8+ T cell and CD4+ T cell response produced by rHuAd5 in terms of kinetics, phenotype and functionality and investigated how antigen structure influenced these parameters. To this end, we monitored the response to the well-defined CD8+ and CD4+ T cell epitopes of the lymphocytic choriomeningitis virus glycoprotein (LCMV GP) using a number of

different antigen configurations where mice were either immunized with the full-length LCMV GP or synthetic antigens where the dominant CD8+ and CD4+ T cell epitopes were fused to cytosolic proteins or directed to the endocytic pathway to enhance MHC class II loading [16, 17]. Functionality and phenotype did not appear to be influenced by these various configurations. Interestingly, while the kinetics and magnitude of the CD8+ T cell response was strikingly influenced by antigen, the CD4+ T cell response was not similarly affected. These results have direct implications for vaccine design and our understanding of T cell immunity.

Materials and Methods

Replication-deficient adenoviruses (rHuAd5):

All the rHuAd5 vectors used in these studies were constructed using the E1,E3-deleted backbone described by Ng et al . Transcription was initiated by the MCMV immediate early promoter and terminated by the SV40 polyadenylation signal. A number of vectors were created that present immunodominant class-I and class-II epitopes from the LCMV-GP protein [amino acids 33-43 (GP_{33-43}) and 61-80 (GP_{61-80}), respectively] (schematics are shown in Figure 1). rHuAd5-LCMV-GP expresses the full-length glycoprotein from LCMV Armstrong (a kind gift from P. Ohashi, OCI, Toronto, ON). rHuAd5-GP33/61-Luc, expresses a modified version of luciferase bearing the GP_{33-43} and GP_{61-80} epitopes fused tagged to the N-terminus. rHuAd5-GP33/61-LAMP expresses the epitopes from rHuAd5-GP33/61-Luc flanked by the endoplasmic reticulum targeting sequence and transmembrane domain/sorting signals from LAMP-1 using the strategy described by Wu et al. [16]. rHuAd5-GP33/61-MTS expresses the epitopes from AdGP33/61-Luc flanked by the endoplasmic reticulum targeting sequence from adenovirus E3gp19K and the melanosomal targeting sequence from TRP-1 . rHuAd5-GP33-ER encodes a modified version of GP_{33-41} epitope (KAVYNFATM where the C-terminal residue was modified to improve binding to D^b) linked to the adenovirus E3gp19K ER targeting sequence. All rHuAd5 vectors were propagated using 293 cells and purified using CsCl gradient centrifugation as previously described .

Animals and immunizations:

Female C57BL/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). P14 transgenic mice which harbour CD8+ T cells specific for the D^b restricted LCMV GP33-41 epitope were obtained from Taconic Farms (Germantown, NY). SMARTA transgenic mice which harbour CD4+ T cells specific for the IA^b restricted LCMV GP61-80 epitope were a kind gift of Pamela Ohashi. Both mouse strains were backcrossed with B6.PL-*Thy1*^{a}/CyJ mice purchased from Jackson Laboratories (Bar Harbor, ME) to produce congenic P14-Thy1.1 and SMARTA-Thy1.1 mice (both colonies were bred at McMaster). For immunizations, 10^6-10^8 pfu rHuAd5 was diluted to 100µl in sterile PBS and subsequently injected intramuscularly (IM) in both rear thighs.

Flow cytometry:

The following reagents were purchase from BD Biosciences (San Jose, CA): anti-CD4 (clone RM4-5), anti-CD8α (clone 53-6.7), anti-Thy1.1 (clone OX-7), anti-CD62L (clone MEL-14), anti-IL-2 (clone JE56-5H4), anti-IFN-γ (clone XMG1.2), and anti-TNF-α (clone 53-2.1). Anti-Granzyme B (clone GB11) was purchased from Caltag Laboratories (Burlingame, CA). The remaining antibodies were obtained from eBiosciences (San Diego, CA): anti-CD127 (clone A7R34), anti-CD27 (clone LG.7F9), KLRG-1-biotin (clone 2F1), CD62L (clone MEL-14) and CD90.1 (clone HIS51). Data was acquired using either an LSRII equipped with 3 lasers (405nm, 488nm, and 633nm) or a

FACSCanto equipped with 2 lasers (488nm and 633nm laser). All flow cytometry data was analyzed using Flowjo software (Tree Star Inc, Ashland, OR).

Preparation of tissues and T cell analysis:

Preparation of tissues and staining methodologies have been extensively described by our group in previous publications [12, 20]. The D^b/GP_{33-41} tetramer used for these studies was produced by the Protein Core at Baylor College of Medicine and labelled with APC. The peptides used for stimulation (GP_{33-41}, GP_{34-42} and GP_{61-80}) were produced by Biomer Technologies (Hayward CA).

In vivo antigen presentation assay:

CD8+ T cells and CD4+ T cells were isolated from lymph node preparations from P14-Thy1.1 and SMARTA-Thy1.1 mice respectably. T cells were purified using CD8 or CD4 negative selection kits from Miltenyi biotech (Auburn, CA). CD8+ and CD4+ T cell purity was > 90% as assessed by flow cytometry. Following purification, cells were labelled with 5uM CFSE and $5x10^5$ cells were adoptively transferred to naïve or previously immunized C57Bl/6 or $Kb^{-/-}$ by intravenous injection. Draining lymph nodes were harvested 72 hours post P14-Thy1.1 transfer and 84 hours after SMARTA-Thy1.1 transfer. Samples were stained with anti-Thy1.1 and analyzed for T cell proliferation (determined by CFSE dilution) and expansion (based on total numbers of Thy1.1 positive cells).

Results and Discussion

Our previous work focused primarily on CD8+ T cell immunity produced by rHuAd5. To examine both CD8+ and CD4+ T cell responses simultaneously, we chose to immunize mice with an rHuAd5 vector expressing LCMV GP which carries well defined immunodominant MHC class I and MHC class II epitopes [GP33 (amino acids 33-43) and GP61 (amino acids 61-80), respectively]. Following immunization with 10^8 pfu rHuAd5-LCMVGP, we only observed modest levels of CD4+ T cell immunity ($1.3x10^4 \pm 0.2x10^4$ cells/spleen at the peak of the response; data not shown), which was too low for further biological evaluation. Similarly low GP61 responses following immunization with a rHuAd5 vector expressing LCMV GP were reported by another group (21).

Previous reports have demonstrated that CD4+ T cell immunity could be improved by targeting antigen to the endocytic compartment using the transmembrane domain and sorting signal from LAMP-1. We created a synthetic antigen where the GP33 and GP61 epitopes were linked together and targeted using LAMP-1 sequences (rHuAd5-GP33/61-LAMP; Figure 1). Robust CD4+ T cell immunity following immunization with rHuAd5-GP33/61-LAMP over a range of doses, 10^6-10^8pfu was observed (Figure 2A). Regardless of the dose employed we observed the peak CD4+ T cell response eight days post immunization. However, dose did impact the magnitude of the response, 10^8 pfu elicited the highest response, while 10^7 pfu generated a response that was 2-fold lower and 10^6 pfu generated a response that was approximately 10 fold lower. Following the peak response

Figure 1

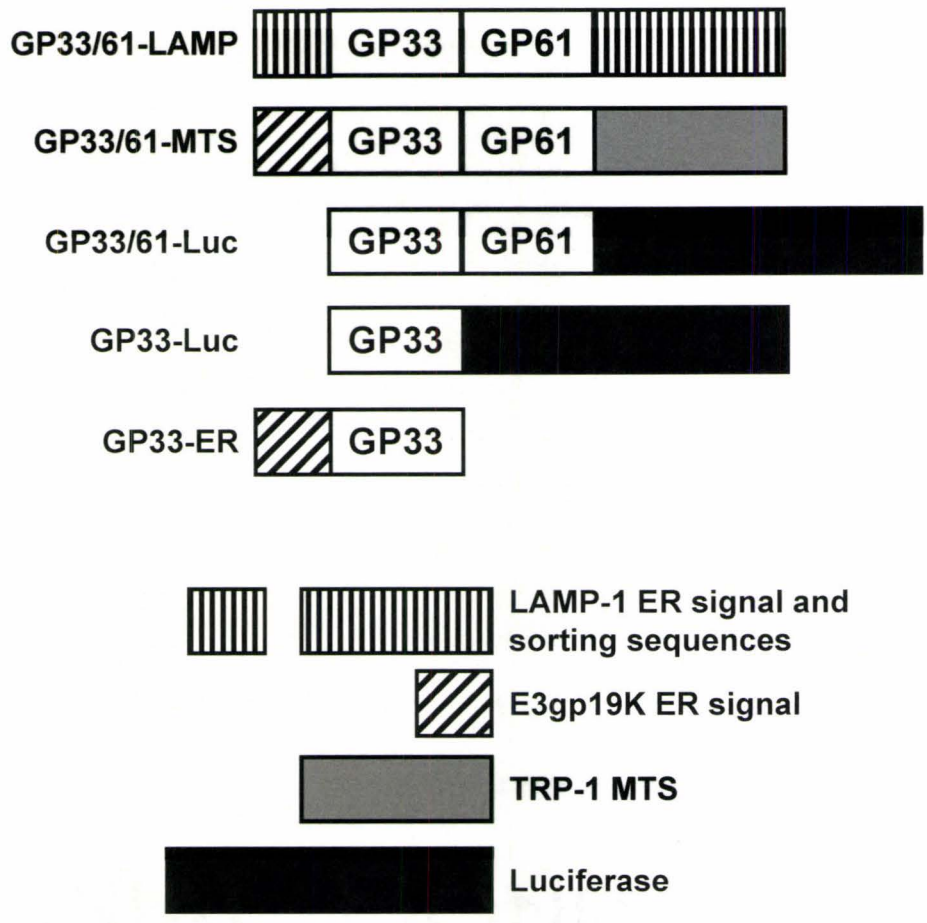


Figure 1. Schematic of synthetic antigens employed for these investigations. Five synthetic antigens were constructed for these experiments and the structure of the antigens is presented here. The CD8+ and CD4+ T cell epitopes from LCMV GP are depicted as boxes labelled with GP33 and GP61, respectively. The ER signal peptide, transmembrane domain and sorting signal of LAMP1 are presented as boxes with vertical bars. The ER signal peptide from the Ad5 E3gp19K protein is shown as a box with diagonal bards. The transmembrane domain and sorting signal from TRP-1 is displayed as a gray box. The full-length luciferase protein is depicted as a black box.

the CD4+ T cell response contracted markedly to 10-25% of the peak magnitude by day 35 post-immunization depending on the dose used for immunization. We also investigated the CD8+ T cell response produced by rHuAd5-GP33/61-LAMP. The magnitude of the CD8+ T cell response was 10 – 20 fold greater than the CD4+ T cell response (Figure 2B), similar to reports for other recombinant virus vaccines [7]. A similar dose relationship for the CD8+ T cell response was observed. We were surprised to find that the kinetics of the CD8+ T cell response following immunization with 10^7 and 10^8 pfu of rHuAd5-GP33/61-LAMP were markedly different from our previous studies (Figure 2B and 3A). Whereas we had previously observed that the CD8+ T cell response produced by rHuAd5 exhibited a delay in expansion and peaked between day 10 and day 14 [11, 12], the GP33-specific response produced by 10^8 pfu rHuAd5-GP33/61-LAMP expanded rapidly and peaked at day 8. Another striking difference between these results and our previous work was the pronounced contraction of the CD8+ T cell population. The population rapidly contracted between day 8 and day 12 and continued to contract to day 25 where approximately 20% of the peak T cell number remained. Interestingly, at lower doses of rHuAd5-GP33/61-LAMP (i.e. 10^6 pfu), the kinetics of the GP33-specific CD8+ T cell response following immunization with 10^6 pfu rHuAd5-GP33/61-LAMP was similar to our previous results where the peak occurred around day 12 and the contraction was less pronounced with 50% of the peak cells surviving to memory. Thus, the enhanced kinetics observed following immunization with rHuAd5-GP33/61-LAMP appears to be dose-dependent although the mechanism remains to be determined. To

Figure 2

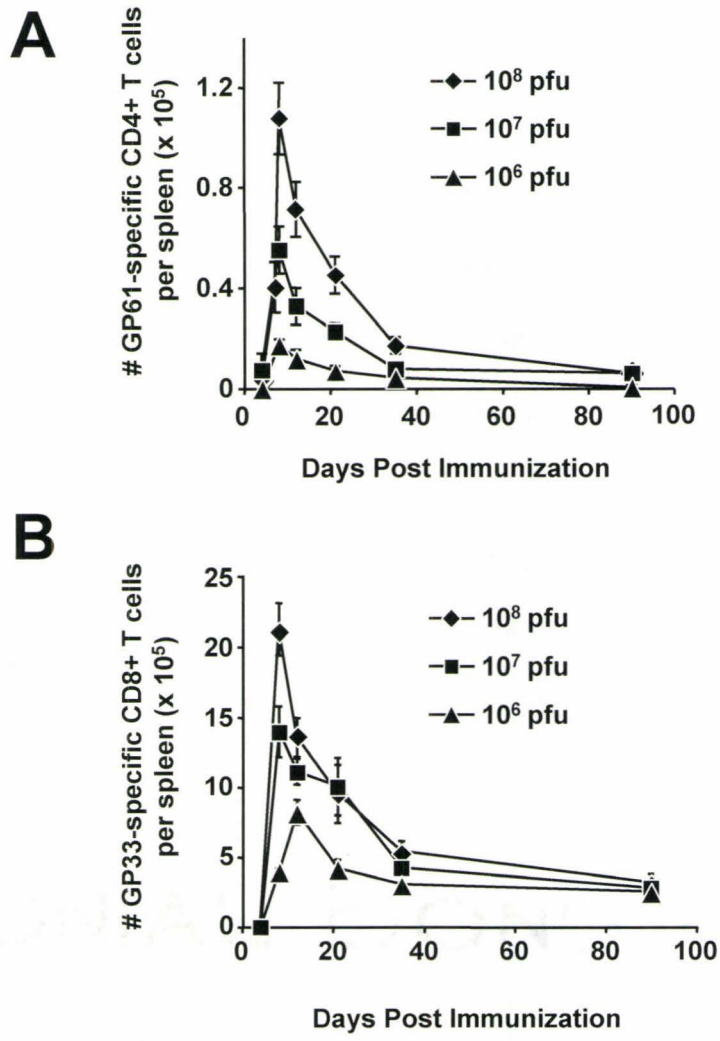


Figure 2. Enumeration of GP61- and GP33-specific T cells following immunization with rHuAd5-GP33/61-LAMP. C57BL/6 mice were immunized with varying doses of rHuAd5-GP33/61-LAMP. Mice were sacrificed at various time points post-immunization and the frequency of antigen-specific CD8+ T cells in the spleen was assessed by intracellular cytokine staining following stimulation with specific peptide. ***A***, results from GP61 stimulation; ***B***, results from GP33 stimulation; ***Diamonds***, 10^8 pfu; ***squares***, 10^7 pfu,; ***triangles***, 10^6 pfu; ***diamonds***. Each point represents the mean ± sem for 8-15 mice.

facilitate further experimentation, we chose to employ 10^8 pfu of rHuAd5 for immunization as this dose generated the most robust CD4+ and CD8+ T cell responses. We speculated that the distinct CD8+ T cell kinetics produced by rHuAd5-GP33/61-LAMP may have resulted from presentation of CD4+ and CD8+ T cell epitopes on the same antigen presenting cell. Therefore, we constructed a vector that expressed the GP61 and GP33 epitopes linked to a cytosolic antigen with the logic that the CD4+ T cell epitopes will only be presented by antigen presenting cells that engulfed antigen from dying cells following infection, whereas the CD8+ T cell epitope will be presented by the cells that have been directly infected by the virus. The two epitopes were tagged to the N-terminus of luciferase similar to our previous strategy to yield rHuAd5-GP33/61-Luc. Mice immunized with rHuAd5-GP33/61-Luc exhibited robust immunity against both the GP33 and GP61 epitopes and the ratio of the GP33-specific CD8+ T cells to GP61-specific CD4+ T cells was similar to AdGP33/61-LAMP (Figure 3A). Likewise, the magnitude of the T cell response was similar for both vaccines (Figure 3A). In accordance with our hypothesis, the CD8+ T cell response produced by rHuAd5-GP33/61-Luc displayed a kinetic similar to our previous studies which peaked around day 12 and displayed a slow contraction (Figure 3B). By contrast, the kinetics of the CD4+ T cell response produced by rHuAd5-GP33/61-Luc were comparable to the kinetics of the CD4+ T cell response produced by rHuAd5-GP33/61-LAMP (Figure 3B). Thus, it appears that the kinetics of the CD8+ T cell response are influenced by the antigen structure but the kinetics of the CD4+ T cell response are not.

CD4+ T cells elicited by rHuAd5 have greater capacity for production of IFN-γ, TNF-α and IL-2 than CD8+ T cells

We conducted further analysis of the CD8+ and CD4+ T cell response produced by these vectors. Interestingly, the antigen-specific CD8+ T cells and CD4+ T cells (identified by IFN-γ production following stimulation with specific peptide) displayed distinct distribution patterns following immunization with rHuAd5-GP33/61-LAMP (Figure 4). Whereas the highest frequencies of GP33-specific CD8+ T cells were found within peripheral sites like the lungs and blood following immunization (Figure 4B), GP61-specific CD4+ T cells were primarily located within the draining lymph nodes and spleens. At later time points, while transgene-specific CD8+ T cells could be found throughout the host, GP61-specific CD4+ T cells could only be identified consistently in the spleen (data not shown). Identical patterns were observed with rHuAd5-GP33/61-Luc, so these distinct distribution patterns reflect differences in the migration of effector CD8+ and CD4+ T cells rather than effects related to antigen configuration (data not shown).

Further examination of the functional properties of the GP33- and GP61-specific T cell populations revealed that the CD4+ T cells expressed greater functionality in terms of cytokine production than the CD8+ T cells (Figure 4). Almost all of the IFN-γ-producing CD4+ T cells produced TNF-α (Figure 4A) whereas only about half of the IFN-γ-producing CD8+ T cells produced TNF-α (Figure 3B). Additionally, greater than 50% of the GP61-specific CD4+ T cells produced IL-2 (Figure 4A) while less than 10% of the

Figure 3. Characterization of the T cell response kinetics following immunization with rHuAd5-GP33/61-LAMP and rHuAd5-GP33/61-Luc. C57BL/6 mice were immunized with either 10^8 pfu of rHuAd5-GP33/61-LAMP or rHuAd5-GP33/61-Luc. Mice were sacrificed at various time points post-immunization and the frequency of antigen-specific CD8+ T cells in the spleen was assessed by intracellular cytokine staining following stimulation with specific peptide. ***A***. GP61- and GP33-reactive T cells were enumerated in the spleens of immunized mice. ***left panel***, mice immunized with rHuAd5-GP33/61-LAMP; ***B right panel***, mice immunized with rHuAd5-GP33/61-Luc; ***open squares***, results from GP33 stimulations, ***closed triangles***, results from GP61 stimulations. ***B***. The results shown in ***A*** were normalized to the peak of the response and presented as a percentage of the peak response. ***left panel***, results from GP61 stimulation; ***B right panel***, results from GP33 stimulation; ***closed squares***, mice immunized with rHuAd5-GP33/61-LAMP, ***closed triangles***, mice immunized with rHuAd5-GP33/61-Luc. Each point represents the mean ± sem for 8-15 mice.

Figure 3

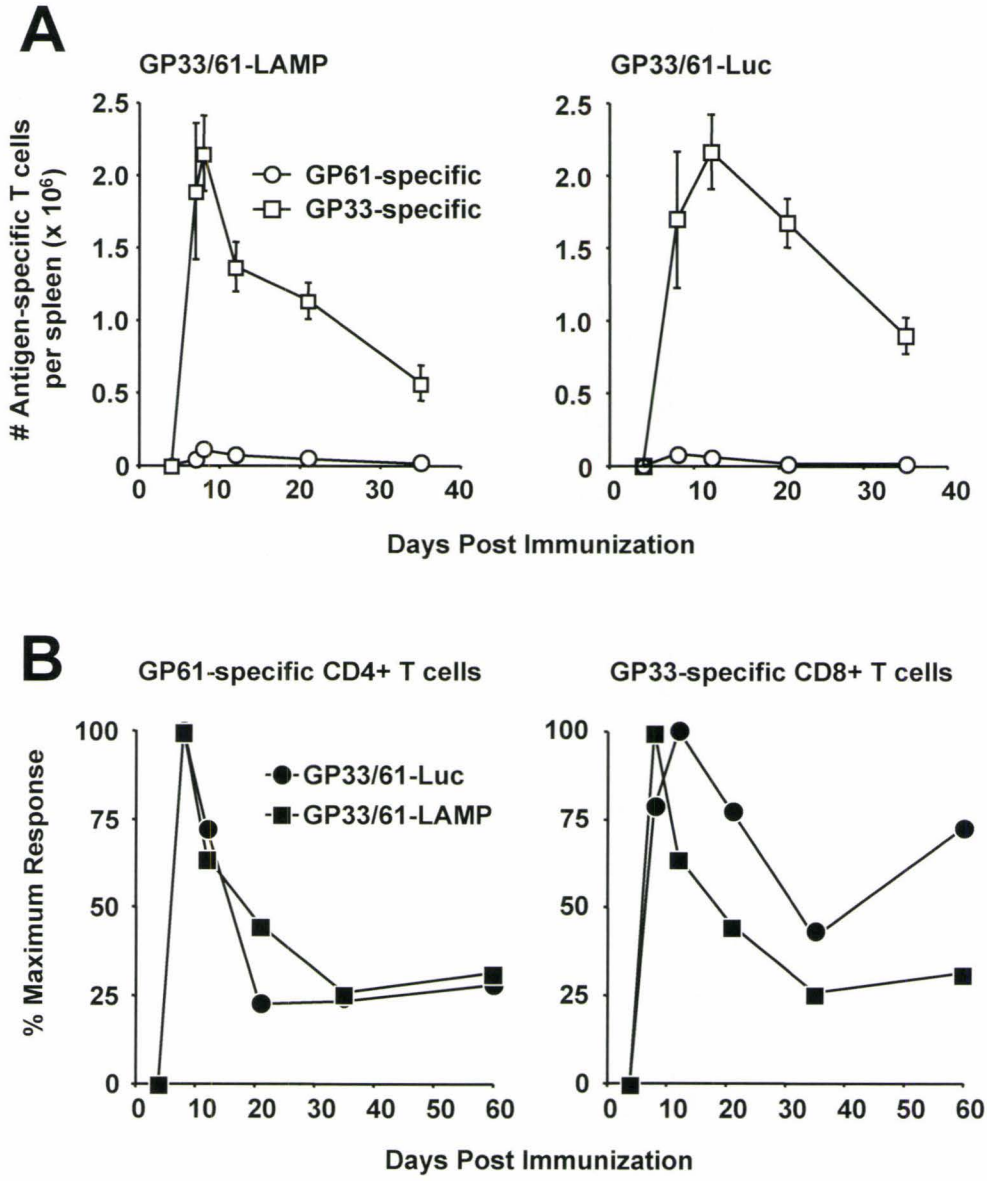
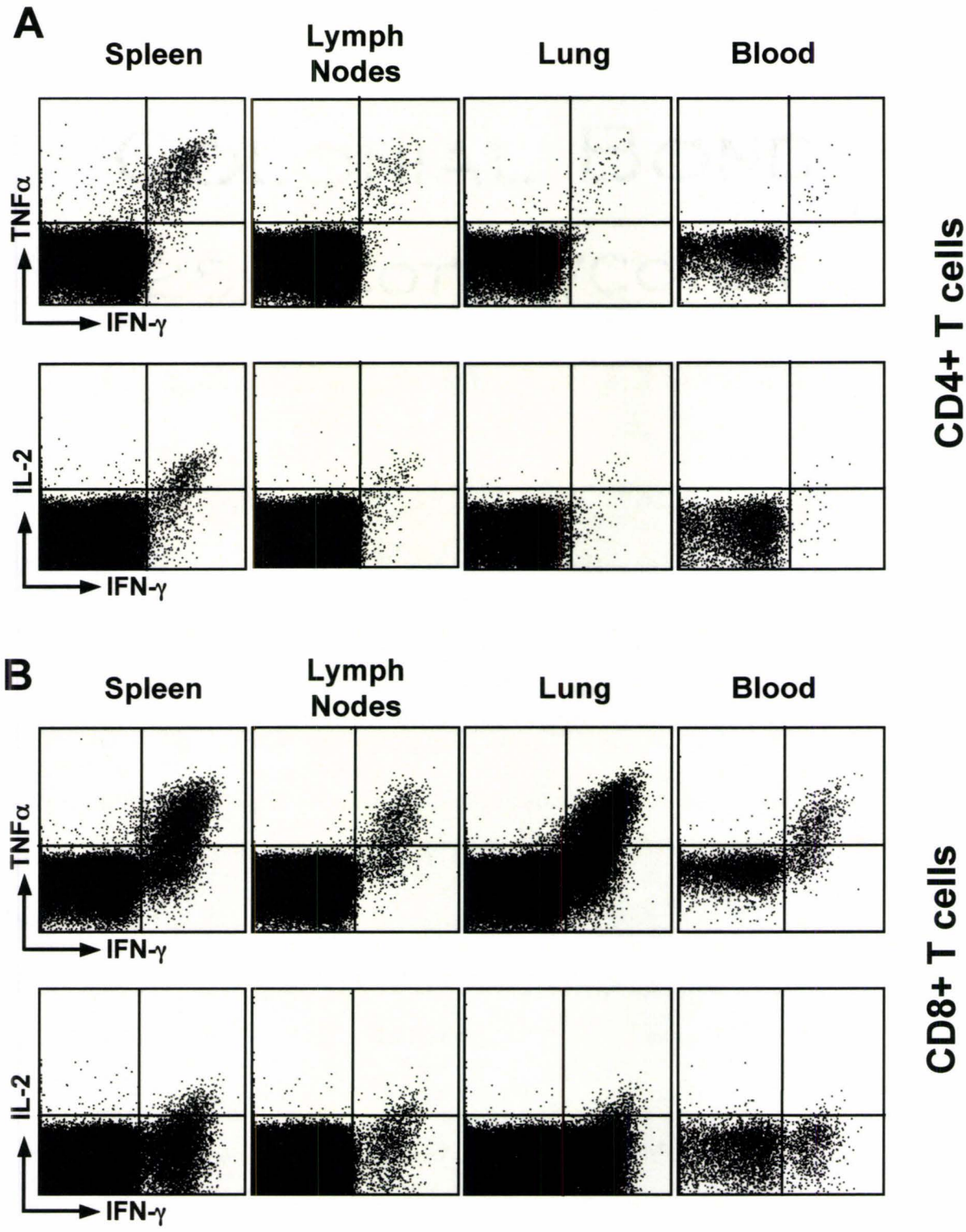

A
GP33/61-LAMP
GP33/61-Luc
Antigen-specific T cells per spleen (x 10^6)
GP61-specific
GP33-specific
2.5
2.0
1.5
1.0
0.5
0
0 10 20 30 40
Days Post Immunization
B
GP61-specific CD4+ T cells
GP33-specific CD8+ T cells
% Maximum Response
GP33/61-Luc
GP33/61-LAMP
100
75
50
25
0
0 10 20 30 40 50 60
Days Post Immunization

Figure 4. Tissue distribution and cytokine profile of GP61- and GP33-reactive T cells following immunization with rHuAd5-GP33/61-LAMP. C57BL/6 mice were immunized with 10^8 pfu of rHuAd5-GP33/61-LAMP. Lymphocytes were harvested from spleen, lymph nodes, lung and blood to enumerate antigen-specific T cells and characterize their cytokine profile. ***A.*** Samples were stimulated with GP61 and stained for expression of IFN-γ, TNF-α and IL-2. ***B.*** Samples were stimulated with GP33 and stained for expression of IFN-γ, TNF-α and IL-2.

Figure 4


A
Spleen
Lymph Nodes
Lung
Blood
TNFα
IFN-γ
IL-2
IFN-γ
CD4+ T cells
B
Spleen
Lymph Nodes
Lung
Blood
TNFα
IFN-γ
IL-2
IFN-γ
CD8+ T cells

GP33-specific CD8+ T cells produced this cytokine (Figure 4B). These results were the same for both AdGP33/61-LAMP (Figure 4) and AdGP33/61-Luc (data not shown) suggesting that the kinetics of the CD8+ T cell response do not influence functionality.

The CD4+ T cell population produced by rHuAd5 progresses more rapidly to a central memory phenotype than the CD8+ T cell population

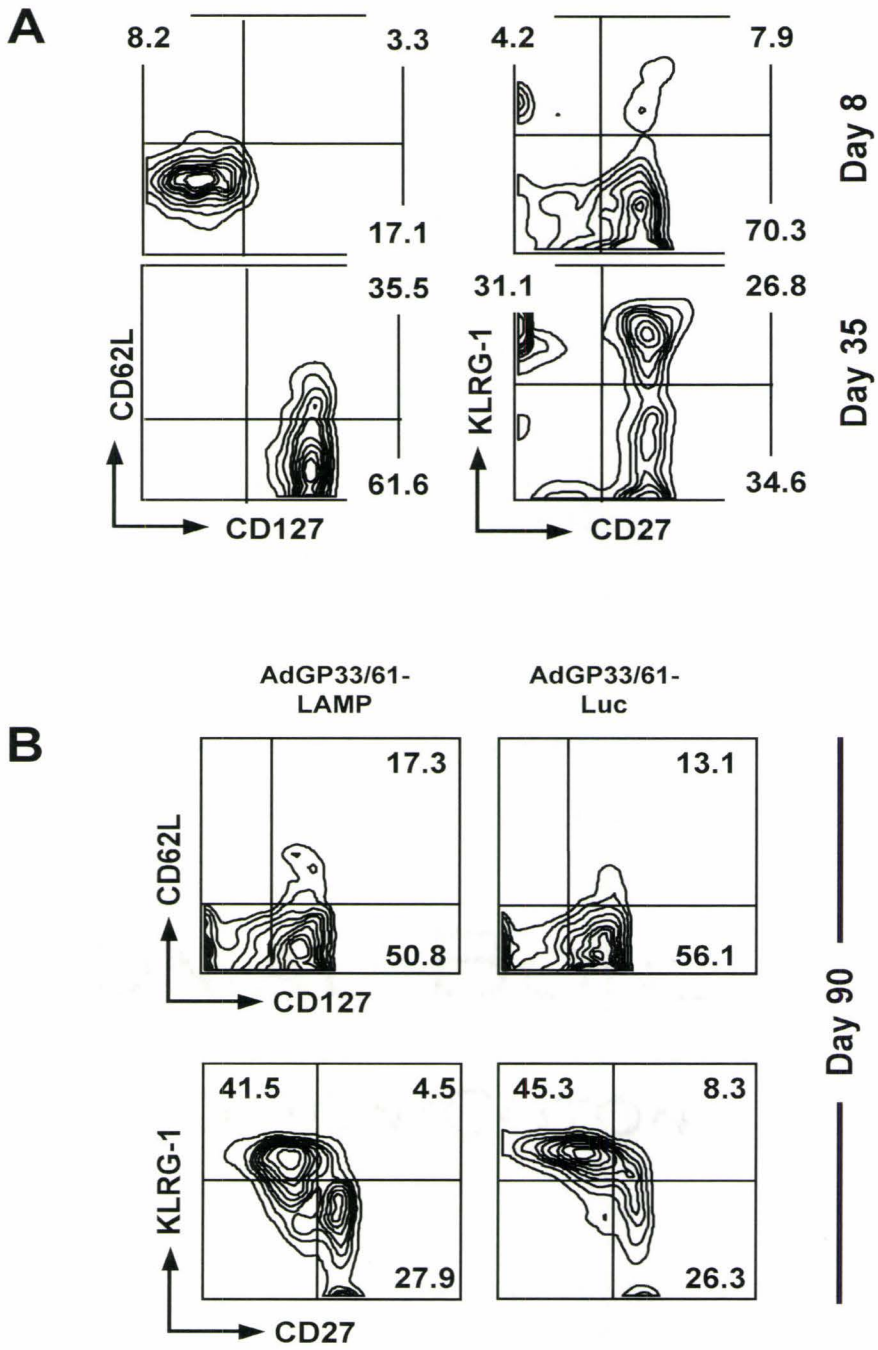
Based on these functional differences between the CD4+ T cells and CD8+ T cells produced by rHuAd5, we extended our analyses to the surface phenotype. Previously, we determined that the CD8+ T cell population produced by rHuAd5 displays a sustained effector memory phenotype [11, 12]. We employed surface expression of CD154 following stimulation with GP61 peptide as a measure of all antigen-specific CD4+ T cells. It is notable that all CD154+ cells were also IFN-γ+ (data not shown) supporting the use of either marker as a tool for measuring GP61-specific immunity. We examined the expression of CD62L, CD127, KLRG1 and CD27. Whereas the GP61-specific CD4+ T cell population at the peak of the response display low-level expression of CD62L and CD127, these markers are rapidly regained by day 35 (Figure 5A and 5B). Interestingly, the GP61-specific CD4+ T cells display increased levels of KLRG1 at day 35 (Figure 5A and 5B). KLRG1 is a marker associated with persistently activated T cells which suggests that the CD4+ T cell population may be exposed to persistent antigen from the rHuAd5 vaccine. Similar results were obtained for both AdGP33/61-Luc and AdGP33/61-LAMP (data not shown). These results reveal that the early memory CD4+ T cell population has already progressed towards a central memory phenotype.

Unfortunately, the memory CD4+ T cell population continued to decline beyond day 35 and we were not able to reliably identify GP61-reactive CD4+ T cells for phenotyping at day 60 or beyond.

We did examine the memory CD8+ T cell population at days 60 and 90 post-immunization with either AdGP33/61-LAMP or AdGP33/61-Luc. We found no significant differences in the phenotype of the CD8+ T cells produced by the 2 vectors (Figure 5C and data not shown). With regard to memory development, even at day 90, a substantial fraction (30%) retained an effector phenotype (CD62L-, CD127-) and few (<20%) had adopted a central memory phenotype (CD62L+, CD127+). This differs markedly from the CD4+ T cell response where the majority of the cells were CD127+ by day 35 post-immunization and a substantial fraction (approx. 35%) expressed CD62L. Notably, KLRG1 levels were elevated on both CD4+ and CD8+ T cell populations which is suggestive of persistent antigen exposure and consistent with our previous observations of prolonged antigen presentation following immunization with rHuAd5 [11, 12]. These results reveal that, although antigen structure may influence the kinetics of the CD8+ T cell response, the antigen structure does not appear to influence functionality or memory phenotype. These data further demonstrate that the memory CD4+ T cell population produced by rHuAd5 appears to progress more rapidly to central memory than the CD8+ T cell population. This difference in memory development may explain the differential pattern of CD4+ and CD8+ T cell distribution as central memory T cells traffic

Figure 5. Phenotypic analysis of GP61- and GP33-reactive T cells following immunization with rHuAd5-GP33/61-LAMP. C57BL/6 mice were immunized with 10^8 pfu of rHuAd5-GP33/61-LAMP. Splenocytes were obtained 8, 35 and 90 days after immunization and examined for the expression of KLRG1, CD27, CD62L and CD127. Antigen-specific CD4+ T cells were identified by CD154 mobilization following stimulation with GP61. Antigen-specific CD8+ T cells were identified by tetramer-staining. ***A.*** Frequencies of GP61-specific CD4+ T cells expressing CD27, CD127, KLRG1 and CD62L at day 8 and day 35 post-immunization. ***B.*** Frequencies of GP33-specific CD8+ T cells expressing CD27, CD127, KLRG1 and CD62L at day 90 post-immunization.

Figure 5


A
8.2
3.3
17.1
4.2
7.9
70.3
Day 8
35.5
61.6
31.1
26.8
34.6
Day 35
CD62L
CD127
KLRG-1
CD27
B
AdGP33/61-LAMP
AdGP33/61-Luc
17.3
50.8
13.1
56.1
41.5
4.5
27.9
45.3
8.3
26.3
Day 90
CD62L
CD127
KLRG-1
CD27

LAMP or rHuAd5-GP33/61-Luc. Labelled TCR-transgenic T cells (SMARTA T cells are specific for GP61 and P14 T cells are specific for GP33) were adoptively transferred into mice at various times before or after immunization. TCR-transgenic T cells which were activated by the presence of antigen *in vivo* were identified as being $CFSE^{lo}$. Since the frequency of $CFSE^{lo}$ cells would be directly related to the amount of antigen present, we have used this value as a semi-quantitative measure of the antigen present *in vivo* similar to our previous work.

We found that antigen presentation persisted for a longer period following immunization with rHuAd5-GP33/61-Luc compared to rHuAd5-GP33/61-LAMP (Figure 6). The increased duration of antigen presentation was observed for both CD4+ T cells (SMARTA; Fig. 6A) and CD8+ T cells (P14; Fig. 6B). Thus, we are left to conclude that either the kinetics of the T cell response are not related to the duration of antigen presentation or that CD8+ and CD4+ T cells are differentially influence by the duration of antigen availability. It is interesting to note that studies with *L. monocytogenes* found that the duration of antigen presentation was more important for CD4 than CD8 [26]. Whether the same is true for rHuAd5 remains to be determined.

Figure 6

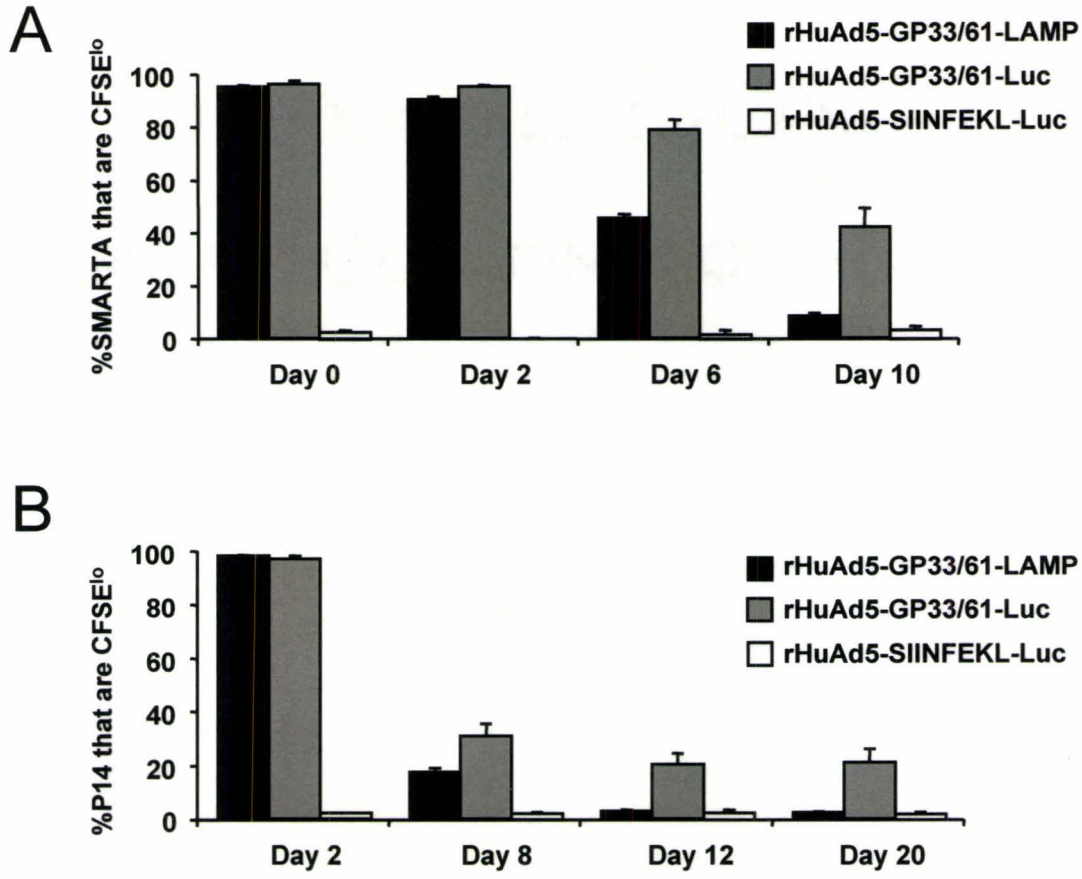


Figure 6. Characterization of antigen persistence in vivo following immunization with rHuAd5-GP33/61-LAMP and rHuAd5-GP33/61-Luc. C57BL/6 mice were immunized with 10^8 pfu of rHuAd5-GP33/61-LAMP (***black bars***) or rHuAd5-GP33/61-Luc (***grey bars***). At various times post-immunization (indicated on the X-axis) 5 x 10^5 CFSE-labelled TCR-transgenic T cells were adoptively transferred into the immunized mice. As a control, mice were immunized with rHuAd5-SIINFEKL-Luc which does not express either GP33 or GP61. SMARTA cells were used to monitor the GP61 expression (***panel A***) and P14 cells were used to monitor GP33 expression (***panel B***). The data represent the percentage of TCR transgenic cells

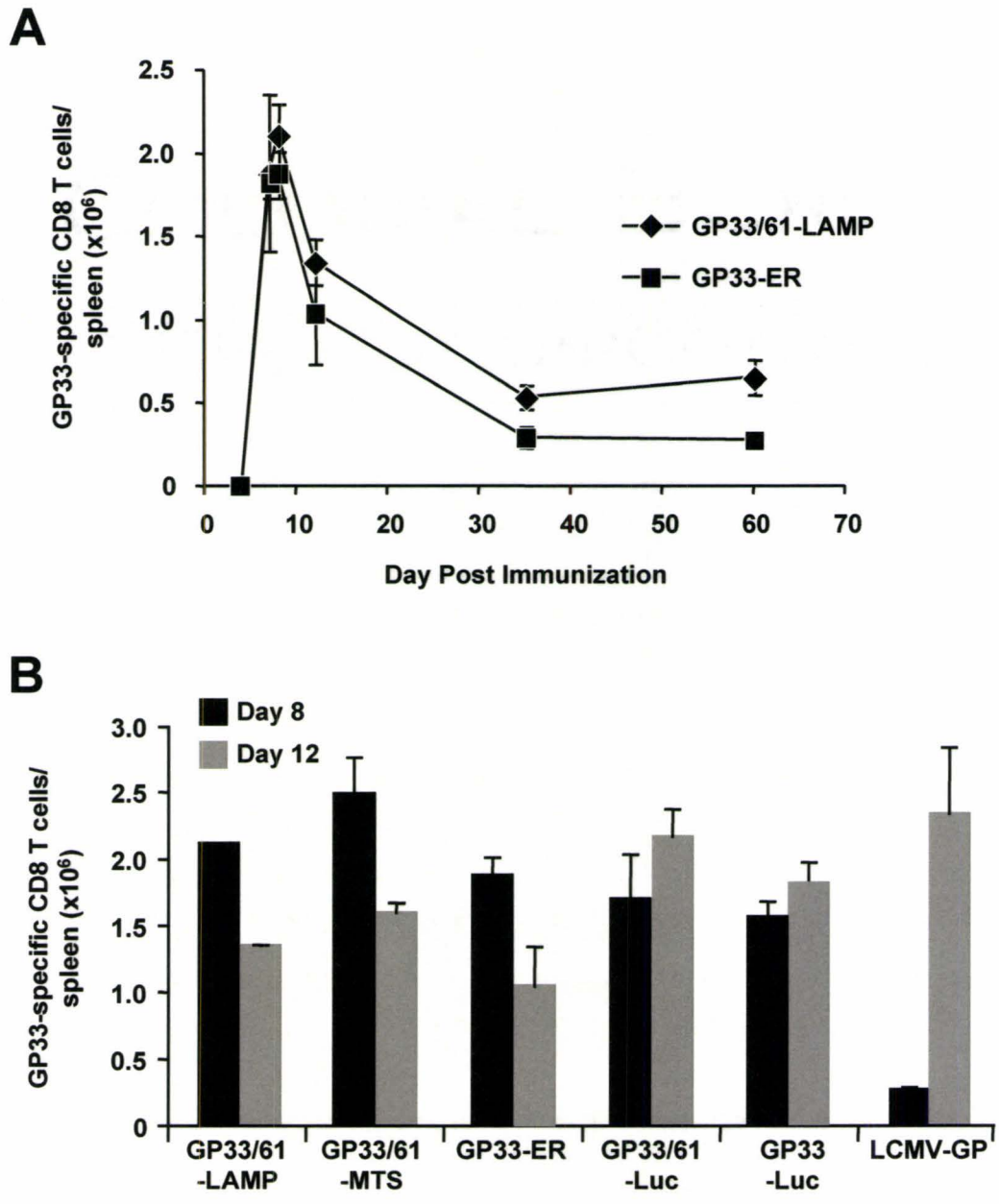
The effect of antigen configuration on the kinetics of T cell immunity following rHuAd5 immunization

As stated earlier, the kinetics of the CD8+ T cell response produced by the rHuAd5GP33/61-LAMP immunization was quite different from our previous work. To determine whether this effect was a unique property of the LAMP-targeted vectors, we constructed another vector, rHuAd5-GP33/61-MTS, where the GP33 and GP61 epitopes were targeted to the endocytic compartment using the melanosome transport signal. This vector evoked a CD8+ and CD4+ T cell response that displayed similar kinetics, tissue distribution and cytokine profile as rHuAd5-GP33/61-LAMP (data not shown). Thus, the increased rapidity of the CD8+ T cell expansion and contraction appeared to correlate with antigens where GP33 was linked to GP61 and targeted to the endosomes. We reasoned that concomitant presentation of robust CD8+ and CD4+ T cell epitopes on the same APC may influence the kinetics of the CD8+ T cell response. Therefore, we employed a construct where the GP33 epitope was solely expressed (rHuAd5-GP33-ER). To efficiently load MHC class I, the GP33 epitope was linked to the adenovirus E3gp19K ER signal peptide. Strikingly, the kinetics of the GP33-specific CD8+ T cell response produced by rHuAd5-GP33-ER were identical to rHuAd5-GP33/61-LAMP, although the overall response was slightly lower in magnitude (Figure 7A). These data reveal that the altered kinetics of the GP33 response in these vectors was not due to the presence of the CD4+ T cell epitope but rather it appeared to be due to the ER targeting element. In Figure 7B, we present a direct comparison of the CD8+ T cell response at day 8 and day

12 following immunization with vectors expressing antigens carrying ER signal peptides (rHuAd5-GP33/61-LAMP, rHuAd5-GP33-ER, and rHuAd5-GP33/61-MTS) and vectors expressing antigens that do not (rHuAd5-GP33/61-Luc and rHuAd5-GP33-Luc). All 5 vectors elicit a similar peak magnitude of the CD8+ T cell response; however the levels of GP33-reactive CD8+ T cells do not change much between day 8 and day 12 for the antigens that are expressed in the cytosol, whereas the population contracts significantly (approximately 30-40%) for the antigens containing the ER-signal peptides. Interestingly, rHuAd5-LCMV-GP, which expresses a viral glycoprotein that is processed through the ER also displays a delayed expansion phase, which peaks around day 12. Similarly, immunization with melanosomal proteins which are synthesized in the ER also did not result in an accelerated CD8+ T cell expansion ([23]and data not shown). Thus, merely synthesizing antigen in the ER was not sufficient to provoke an accelerated CD8+ T cell response. We suspect that synthetic antigens which are targeted to the ER produce ER stress because they do not fold appropriately. By contrast, natural proteins which traffic through the ER contain appropriate glycosylation signals to enable protein folding and limit ER stress. In this regard, it is of interest to note that the unfolded protein response, which is associated with ER stress, has been shown to influence inflammatory pathways and the induction of T cell responses (24). Furthermore, molecular chaperones in the ER, which bind up unfolded proteins, have been shown to play an important role in cross-presentation to CD8+ T cells (25).

Figure 7 Enumeration of GP33-specific T cells following immunization with rHuAd5 expressing different synthetic antigens. ***A.*** C57BL/6 mice were immunized with 10^8 pfu of rHuAd5-GP33/61-LAMP (***diamonds***) or rHuAd5-GP33-ER (***squares***). Mice were sacrificed at various time points post-immunization and the frequency of GP33-specific CD8+ T cells in the spleen was assessed by intracellular cytokine staining following stimulation with specific peptide. Each point represents the mean ± sem for 8-15 mice. ***B.*** C57BL/6 mice were immunized with 10^8 pfu of rHuAd5 expressing either GP33/61-LAMP, GP33/61-MTS, GP33-ER, GP33/61-Luc, GP33-Luc or LCMV GP. Mice were sacrificed at day 8 (***black bars***) and day 12 (***grey bars***) post-immunization and the frequency of GP33-specific CD8+ T cells in the spleen was assessed by intracellular cytokine staining following stimulation with specific peptide. Each point represents the mean ± sem for 8-15 mice

Figure 7


A
GP33-specific CD8 T cells/ spleen (x10⁶)
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
70
Day Post Immunization
GP33/61-LAMP
GP33-ER
B
GP33-specific CD8 T cells/ spleen (x10⁶)
Day 8
Day 12
3.0
2.5
2.0
1.5
1.0
0.5
0
GP33/61 -LAMP
GP33/61 -MTS
GP33-ER
GP33/61 -Luc
GP33 -Luc
LCMV-GP

Clearly, antigen design can influence CD8+ T cell kinetics but it does not appear to have much influence on the phenotype or function of the CD8+ T cells. We were surprised to find that targeting antigen to the endosomal compartment using sorting signals from either LAMP-1 or TRP-1 had no impact on the CD4+ T cell response compared to the antigen configuration where the epitope was fused to luciferase. However, results in the literature employing such targeting elements have been variable and inclusion of the LAMP-1 sorting signal does not always enhance immunogenicity [16, 27-31]. This may reflect the need for additional elements, such as

the luminal domain, to increase the fidelity of trafficking for certain proteins [31]. Further investigation will be required to understand the mechanistic reasons for the differential kinetics of the CD8+ T cell response. We speculate that ER stress associated with the delivery of unnatural proteins to the ER may have influenced the CD8+ T cell response. Holst et al. found that linking GP33 epitope to β2-microglobulin increased the rapidity of the CD8+ T cell response. It was unclear whether this was due to improved loading of MHC I or the presence of an ER-targeting element in the N-terminus of the synthetic antigen [32]. In light of our results, we would predict that the SS sequence was the reason. Overall, our data demonstrate that the functionality of CD4+ T cells produced by rHuAd5 vectors is higher than CD8+ T cells with regard to cytokine production. Furthermore, the CD4+ T cell population produced by rHuAd5 shows greater evidence of progression towards a central memory phenotype than the CD8+ T cell population. Finally, the CD4+ T cell response produced by rHuAd5 does not appear to be influenced

by antigen structure whereas the antigen can have an effect on the kinetics of the CD8+ T cell response. These results offer new insight into the immunobiology of rHuAd5 vectors and can be applied to future vaccine design.

Acknowledgment

This work was supported by a grant to Jonathan Bramson from the Canadian Institutes for Health Research (MOP-42433). Natalie Grinshtein and Jennifer Bassett were supported by studentships from the National Cancer Institute of Canada and the Canadian Institutes for Health Research, respectively.

References

1. Yang, T. C., Millar, J. B., Grinshtein, N., Bassett, J., Finn, J., and Bramson, J. L. (2007). T-cell immunity generated by recombinant adenovirus vaccines. *Expert review of vaccines* 6: 347-356.
2. Kemball, C. C., Lee, E. D., Vezys, V., Pearson, T. C., Larsen, C. P., and Lukacher, A. E. (2005). Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. *J Immunol* 174: 7950-7960.
3. Obar, J. J., Fuse, S., Leung, E. K., Bellfy, S. C., and Usherwood, E. J. (2006). Gammaherpesvirus persistence alters key CD8 T-cell memory characteristics and enhances antiviral protection. *Journal of virology* 80: 8303-8315.
4. Bachmann, M. F., Wolint, P., Schwarz, K., Jager, P., and Oxenius, A. (2005). Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol* 175: 4686-4696.
5. Gold, M. C., et al. (2004). Murine cytomegalovirus interference with antigen presentation has little effect on the size or the effector memory phenotype of the CD8 T cell response. *J Immunol* 172: 6944-6953.
6. Wherry, E. J., and Ahmed, R. (2004). Memory CD8 T-cell differentiation during viral infection. *Journal of virology* 78: 5535-5545.
7. Harrington, L. E., Most Rv, R., Whitton, J. L., and Ahmed, R. (2002). Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. *Journal of virology* 76: 3329-3337.
8. Masopust, D., Vezys, V., Marzo, A. L., and Lefrancois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. *Science (New York, N.Y* 291: 2413-2417.
9. Kedzierska, K., La Gruta, N. L., Turner, S. J., and Doherty, P. C. (2006). Establishment and recall of CD8+ T-cell memory in a model of localized transient infection. *Immunological reviews* 211: 133-145.
10. Tatsis, N., et al. (2007). Adenoviral vectors persist in vivo and maintain activated CD8+ T cells: implications for their use as vaccines. *Blood* 110: 1916-1923.
11. Yang, T. C., et al. (2006). The CD8+ T cell population elicited by recombinant adenovirus displays a novel partially exhausted phenotype associated with prolonged antigen presentation that nonetheless provides long-term immunity. *J Immunol* 176: 200-210.
12. Yang, T. C., et al. (2007). On the role of CD4+ T cells in the CD8+ T-cell response elicited by recombinant adenovirus vaccines. *Mol Ther* 15: 997-1006.
13. Liu, J., et al. (2008). Magnitude and phenotype of cellular immune responses elicited by recombinant adenovirus vectors and heterologous prime-boost regimens in rhesus monkeys. *Journal of virology* 82: 4844-4852.
14. Darrah, P. A., et al. (2007). Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nature medicine* 13: 843-850.

15. Wille-Reece, U., et al. (2006). Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *The Journal of experimental medicine* 203: 1249-1258.

16. Wu, T. C., et al. (1995). Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. *Proceedings of the National Academy of Sciences of the United States of America* 92: 11671-11675.
17. Wang, S., et al. (1999). A role for a melanosome transport signal in accessing the MHC class II presentation pathway and in eliciting CD4+ T cell responses. *J Immunol* 163: 5820-5826.
18. Ng, P., Parks, R. J., Cummings, D. T., Evelegh, C. M., and Graham, F. L. (2000). An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. *Human gene therapy* 11: 693-699.
19. Bramson, J. L., Hitt, M., Addison, C. L., Muller, W. J., Gauldie, J., and Graham, F. L. (1996). Direct intratumoral injection of an adenovirus expressing interleukin-12 induces regression and long-lasting immunity that is associated with highly localized expression of interleukin-12. *Human gene therapy* 7: 1995-2002.
20. Millar, J., et al. (2007). The magnitude of the CD8+ T cell response produced by recombinant virus vectors is a function of both the antigen and the vector. *Cell Immunol* 250: 55-67.
21. Holst, P. J., Sorensen, M. R., Mandrup Jensen, C. M., Orskov, C., Thomsen, A. R., and Christensen, J. P. (2008). MHC Class II-Associated Invariant Chain Linkage of Antigen Dramatically Improves Cell-Mediated Immunity Induced by Adenovirus Vaccines. *J Immunol* 180: 3339-3346.
22. Yang, T. C., Dayball, K., Wan, Y. H., and Bramson, J. (2003). Detailed analysis of the CD8+ T-cell response following adenovirus vaccination. *Journal of virology* 77: 13407-13411.
23. Kianizad, K., et al. (2007). Elevated frequencies of self-reactive CD8+ T cells following immunization with a xenoantigen are due to the presence of a heteroclitic CD4+ T-cell helper epitope. *Cancer research* 67: 6459-6467.
24. Todd, D. J., Lee, A. H., and Glimcher, L. H. (2008). The endoplasmic reticulum stress response in immunity and autoimmunity. *Nat Rev Immunol* 8: 663-674.
25. Srivastava, P. (2002). Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annual review of immunology* 20: 395-425.
26. Corbin, G. A., and Harty, J. T. (2004). Duration of infection and antigen display have minimal influence on the kinetics of the CD4+ T cell response to Listeria monocytogenes infection. *J Immunol* 173: 5679-5687.
27. Vidalin, O., Tanaka, E., Spengler, U., Trepo, C., and Inchauspe, G. (1999). Targeting of hepatitis C virus core protein for MHC I or MHC II presentation does not enhance induction of immune responses to DNA vaccination. *DNA Cell Biol* 18: 611-621.

28. Dobano, C., Rogers, W. O., Gowda, K., and Doolan, D. L. (2007). Targeting antigen to MHC Class I and Class II antigen presentation pathways for malaria DNA vaccines. *Immunol Lett* 111: 92-102.
29. Deng, H., et al. (2002). A modified DNA vaccine to p53 induces protective immunity to challenge with a chemically induced sarcoma cell line. *Cell Immunol* 215: 20-31.
30. Chen, C. H., Ji, H., Suh, K. W., Choti, M. A., Pardoll, D. M., and Wu, T. C. (1999). Gene gun-mediated DNA vaccination induces antitumor immunity against human papillomavirus type 16 E7-expressing murine tumor metastases in the liver and lungs. *Gene therapy* 6: 1972-1981.
31. Marques, E. T., Jr., et al. (2003). HIV-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid vaccine chimera is highly expressed, traffics to the major histocompatibility class II compartment, and elicits enhanced immune responses. *J Biol Chem* 278: 37926-37936.
32. Holst, P. J., Bartholdy, C., Stryhn, A., Thomsen, A. R., and Christensen, J. P. (2007). Rapid and sustained CD4(+) T-cell-independent immunity from adenovirus-encoded vaccine antigens. *The Journal of general virology* 88: 1708-1716.

- Chapter 5 –

On the role of CD4+ T cells in the CD8+ T-cell response elicited by recombinant adenovirus vaccines

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Prologue

In this chapter we have evaluated the role of CD4+ T cell help in CD8+ T cell immunity elicited by recombinant adenovirus immunization. Between different models there is considerable discourse in the timing and mechanism of CD4+ T cell help and the role of CD4+ T cell help was not known in the rAd system. Our results have demonstrated the following:

- CD4+ T cell help is required during priming to maximize the magnitude of primary CD8+ T cell expansion and memory development.
- CD4+ T cell help was not required for secondary CD8+ T cell expansion
- Helpless CD8+ T cells exhibited only minor functional defects compared to helped CD8+ T cells

In this study I was involved in experimental development, implementation and execution. In particular I contributed to data displayed in figures 2-6. The data contained within this chapter has been published in:

Yang, T.C., Millar, J., Groves, T., Zhou, W., Grinshtein, N., Xing, Z., Wan, Y. and Bramson, J.L. On the role of CD4+ T cells in the CD8+ T cell response elicited by recombinant adenovirus vaccines. Mol. Ther. 15: 997-1006, 2007.

original article

On the Role of CD4+ T Cells in the CD8+ T-Cell Response Elicited by Recombinant Adenovirus Vaccines

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We have investigated the role of CD4+ T cells in the development of the CD8+ T-cell response after immunization with recombinant adenovirus (rAd). In the absence of CD4+ T cells, the "unhelped" CD8+ T-cell population exhibited a reduction in primary expansion and long-term survival that appeared to be due to inadequate priming of naïve T cells. There were few functional or phenotypic differences between the helped and unhelped CD8+ T-cell populations with the exception of O-glycosylated CD43, a marker of effector cells, which was augmented on the unhelped CD8+ T-cell population. In some cases, the unhelped CD8+ T-cell population exhibited reduced ability to control virus infection; however, this appeared to be a function of the reduced frequency of antigen-specific CD8+ T cells. Most notably, the unhelped CD8+ T-cell population exhibited no defect in secondary expansion. These results provide insight into the role of CD4+ T cells during the primary CD8+ T-cell response generated by rAd vaccines and identify potential benefits and issues that must be considered when using adenovirus vaccines under conditions where CD4+ T-cell function may be limiting, such as vaccination of human immunodeficiency virus patients.

Received 5 April 2006; accepted 7 February 2007; published online 20 March 2007. doi:10.1038/mt.sj.6300130

INTRODUCTION

Recombinant adenovirus (rAd) vaccines have garnered considerable attention as platforms for eliciting CD8+ T-cell immunity owing to the strong immunogenicity they have shown in numerous studies, including simian models and preliminary human trials.[1,2] Although the commonly used Ad5 vectors may not represent the optimal serotype for use in humans, because of the high prevalence of pre-existing immunity, vectors of different serotypes and species have been developed that should overcome this.[3–5] In preparation for the use of these vectors in human trials, we have been investigating their immunobiology in pre-clinical rodent models as a means of optimizing the vectors and identifying potential limitations.

CD8+ T cells play an important role in host defense against tumors and viral infections. During the primary phase of the CD8+ T-cell response, the activated precursors undergo a rapid and dramatic expansion phase followed by a period of contraction where 80–90% of the antigen-specific population dies off, leaving the remaining cells to constitute the memory population.[6] CD8+ T cells mature over the course of the primary response and acquire the ability to produce interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and, to a lesser degree, interleukin-2 (IL-2). Memory T cells can be divided into central-memory and effector-memory T cells based on phenotype and anatomical location.[6] These phenotypic differences have also been linked to functional differences; however, the existence of such relationships remains controversial.[7–12]

Our studies have revealed some unexpected qualities of the CD8+ T-cell response generated by rAd immunization. The CD8+ T-cell response exhibited a protracted contraction phase where 40–60% of the peak number of CD8+ T cells persisted 3 weeks after the peak response in multiple compartments (spleen, lung, blood, bone marrow).[13] The rAd-induced memory CD8+ T-cell population was composed primarily of effector and effector-memory cells; only a fraction of the population produced TNF-α, and very few cells produced IL-2. Various stages of CD8+ T-cell functional impairment have been described that can be identified according to cytokine production.[14] Whereas a healthy memory CD8+ T-cell population can secrete IFN-γ, TNF-α, and some IL-2, progressive exhaustion is characterized by a hierarchical loss of IL-2, TNF-α, and, ultimately, IFN-γ production. On the basis of the cytokine profile of the rAd-induced CD8+ T-cell population, we suggested that this population exhibited partial exhaustion that may have resulted from prolonged exposure to antigen *in vivo*.[13] It is difficult to appreciate fully the implications of these observations at this time because rAd vectors have been used successfully to elicit protective immunity in many models of pathogen infection and tumor challenge.[1,2] Moreover, the partially exhausted population in our model provided protective immunity against

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virus challenge for at least 90 days after immunization.[13] These data must, however, be considered in light of reports from other groups showing that rAd vectors can lead to dysfunctional $CD8^+$ T-cell immunity.[15,16]

$CD8^+$ T cells require "help" from $CD4^+$ T cells for maximal expansion and function. In various models, $CD8^+$ T cells elicited in the absence of $CD4^+$ T-cell help were shown to exhibit defects in primary and/or secondary expansion, functional maturation, and survival;[17] however, the exact defects varied with the immunization method. As rAd is currently being considered as a candidate vector for therapeutic human immunodeficiency virus vaccines, it is important to investigate the functionality of $CD8^+$ T cells elicited by rAd in the absence of functional $CD4^+$ T-cell help. Previous work on gene transfer determined that $CD4^+$ T-cell depletion at the time of rAd injection could increase the longevity of gene expression.[18–21] Although the primary goal of those experiments was to suppress the development of neutralizing antibody responses to permit vector re-administration, it was also shown that transient $CD4^+$ T-cell depletion resulted in reduced levels of anti-adenovirus and anti-transgene cytotoxic T lymphocyte as measured by chromium release assays.[20,21] The chromium release assay does not provide an adequate assessment of $CD8^+$ T-cell immunity because the assay is dependent upon secondary expansion *in vitro* and it has been demonstrated that $CD8^+$ T cells generated in the absence of $CD4^+$ T cells mount a defective secondary expansion.[22] Therefore, it remained unclear whether rAds were unable to elicit $CD8^+$ T cells in the absence of $CD4^+$ T-cell help or whether those $CD8^+$ T cells that were generated manifested defects in secondary expansion. To gain further insight into the mechanisms that regulate $CD8^+$ T cells after rAd immunization, we have performed a more detailed analysis of the $CD8^+$ T-cell response elicited by rAd under conditions of sufficient and insufficient $CD4^+$ T-cell help.

RESULTS

Reduced expansion of anti-transgene $CD8^+$ T cells after rAd immunization in the absence of $CD4^+$ T cells

To determine the importance of $CD4^+$ T cells to the $CD8^+$ T-cell response elicited by rAd, we examined the kinetics of the primary $CD8^+$ T-cell response in major histocompatibility complex class II–deficient (C2D) hosts, which lack $CD4^+$ T cells, and wild-type (WT) mice. A striking defect in the magnitude of primary $CD8^+$ T-cell expansion in the peripheral blood of C2D mice was observed after immunization with 10^8 plaque-forming units (pfu) AdSIINFEKL-Luc (**Figure 1a**). A similar defect in expansion was observed in the spleens and lungs of the C2D mice (**Supplementary Figure S1**). To verify that the lower magnitude of $CD8^+$ T-cell expansion was not antigen specific, WT and C2D mice were also immunized with 10^8 pfu Adβgal. As with AdSIINFEKL-Luc, we observed a defect in primary expansion of β-gal-specific $CD8^+$ T cells (**Figure 1b**) and a marked absence of antigen-specific $CD8^+$ T cells from multiple sites at day 45 after immunization (**Supplementary Figure S1**). To assess the impact of vector dose on the requirement for $CD4^+$ T cells, WT and C2D mice were immunized with one-tenth the amount of AdSIINFEKL-Luc (10^7 pfu). Again, we observed a striking absence of SIINFEKL-specific $CD8^+$ T cells in the C2D mice relative to the frequency of SIINEKL-specific $CD8^+$ T cells in the WT mice (**Figure 1c**).

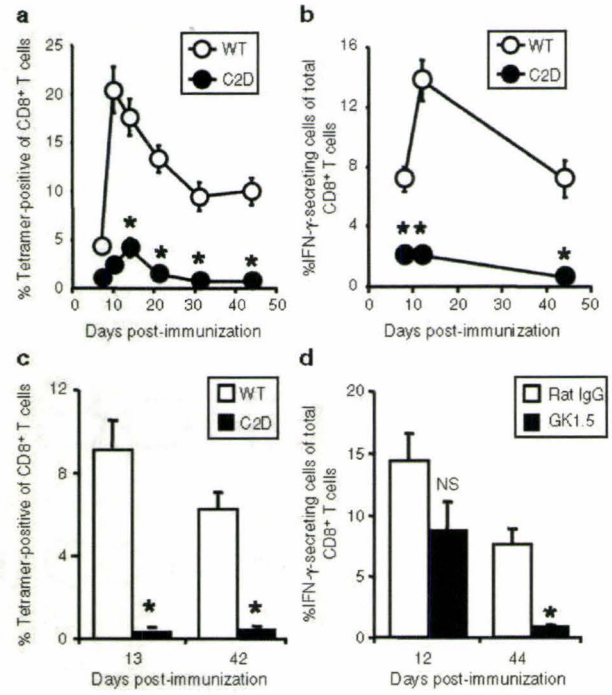

Figure 1 The kinetics of $CD8^+$ T-cell expansion and contraction after immunization of wild-type (WT) and major histocompatibility complex (MHC) class II–deficient (C2D) mice. (**a**) WT (open circles) and C2D (closed circles) mice were immunized with 10^8 plaque-forming units (pfu) AdSIINFEKL-Luc, and antigen-specific $CD8^+$ T cells were enumerated in the peripheral blood. The results represent the mean ± SEM for eight mice at each time point. (**b**) WT (open circles) and C2D (closed circles) mice were immunized with 10^8 pfu Adβgal, and antigen-specific $CD8^+$ T cells were quantified in the blood at days 8, 12, and 45. The results represent the mean ± SEM for six mice at each time point. (**c**) WT (open bars) and C2D (closed bars) mice were immunized with 10^7 pfu AdSIINFEKL-Luc, and antigen-specific $CD8^+$ T cells were enumerated in the peripheral blood 13 and 42 days after immunization. The data represent the mean ± SEM for five mice per group. (**d**) BALB/c mice treated with total rat immunoglobulin G (IgG) WT (open bars) or GK1.5 (closed bars) were immunized with 10^8 pfu Adβgal, and antigen-specific $CD8^+$ T cells were enumerated in the peripheral blood 12 and 44 days after immunization. The data represent the mean ± SEM for five mice per group. N.S., not significant, $^*P < 0.01$.

To verify that the defect in the primary $CD8^+$ T-cell response was not specific to the C2D mice, we also examined the $CD8^+$ T-cell response in WT mice that were depleted of $CD4^+$ T cells using the GK1.5 antibody. In C57Bl/6 mice depleted of $CD4^+$ T cells using the GK1.5 antibody, we observed a reduced frequency of antigen-specific $CD8^+$ T cells relative to non-depleted mice (**Supplementary Figure S1**). As all of this work has used C57Bl/6 mice, we also examined the $CD8^+$ T-cell response in GK1.5-depleted BALB/c mice to determine whether the requirement for $CD4^+$ T cells was strain dependent. Under these conditions, we observed that the frequency of antigen-specific $CD8^+$ T cells was reduced twofold in the $CD4^+$ T-cell-depleted animals at day 12, although this difference did not achieve statistical significance.

Most striking, there was an eightfold difference in the $CD8^+$ T-cell frequencies at day 44 ($P < 0.001$).

Thus, it appears that $CD4^+$ T cells are required for expansion and maintenance of the primary $CD8^+$ T-cell response elicited by rAd immunization. For the sake of stringency and simplicity, all further experiments reported in this article were conducted in the C2D mice.

The defect in primary expansion is due to insufficient priming of naïve T cells

The failure of expansion during the primary response to rAd immunization may be due to a number of factors: (i) reduced proliferation, (ii) increased apoptosis, or (iii) insufficient antigen presentation. Given the reduced rate of expansion observed in **Figure 1a** and **b**, we suspected that the $CD8^+$ T-cell population might not be proliferating at the same rate in both strains of mice. Proliferation of effector $CD8^+$ T cells was assessed by measuring deoxybromouridine (BrdU) incorporation over a 48-hour period starting from day 7 after immunization. As a second measure of proliferation, we examined the fraction of cells positive for Ki-67 on day 9 after immunization. We did not observe any difference in BrdU incorporation or Ki-67 staining between the antigen-specific $CD8^+$ T-cell populations from WT and C2D mice in either the draining lymph nodes or the spleen (**Supplementary Figure S2**). We also examined annexin V staining as a measure of apoptosis. Again, no significant differences were observed between the antigen-specific $CD8^+$ T-cell populations in WT and C2D mice (**Supplementary Figure S2**). These data suggest that the reduced expansion of antigen-specific $CD8^+$ T cells in the C2D mice was not due to differential proliferative rates or increased apoptosis.

In a recent study, we demonstrated that the $CD8^+$ T-cell response elicited by rAd in WT mice was associated with prolonged antigen presentation.[13] Therefore, another explanation for the lower $CD8^+$ T-cell expansion might be reduced duration of antigen presentation in the absence of $CD4^+$ T cells. To address this possibility, C2D mice were immunized with AdSIINFEKL-Luc or AdLuc. At various times after immunization (days 2, 6, 12, and 21), congenic $CD8^+$ OT-I cells were transferred into the immunized hosts. Three days after transfer, the draining lymph nodes were harvested and the expansion of OT-I cells during that period was measured and used as an indicator of antigen presentation as we described previously.[13] Based on OT-I expansion in the AdSIINFEKL-Luc-immunized C2D mice relative to the AdLuc-immunized mice, antigen presentation in the C2D strain was maintained for at least 21 days (**Figure 2a**). High-level OT-I proliferation was observed at days 2 and 6 after immunization and the degree of expansion abated at later time points, similar to our previous observations.[13] To compare the priming of naïve T cells in WT mice and C2D mice after rAd immunization directly, $CD44^{lo}$ $CD62L^{hi}$ congenic $CD8^+$ T cells were purified from lymph nodes of OT-I mice by flow-sorting and transferred into WT and C2D mice 24 hours before immunization with AdSIINFEKL-Luc or AdLuc. No difference in the degree of OT-I expansion between WT and C2D mice was observed with a dose of 5×10^5 OT-I T cells per mouse (**Figure 2b**). As previous reports demonstrated that $CD8^+$ T cells can license antigen-presenting cells (APCs) in the absence of $CD4^+$ T cells

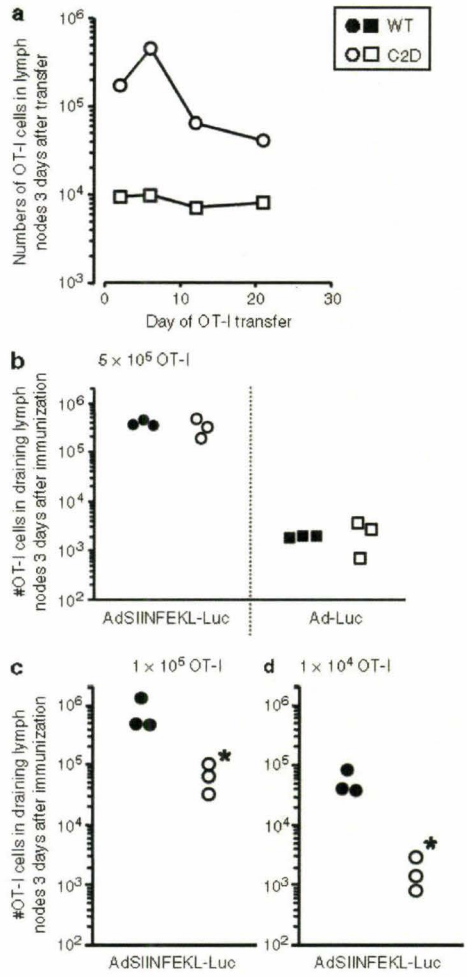

Figure 2 Measurement of antigen presentation after AdSIINFEKL-Luc immunization. (**a**) Major histocompatibility complex (MHC) class II–deficient (C2D) mice were immunized with either 10^8 plaque-forming units (pfu) AdSIINFEKL-Luc (open circles) or 10^8 pfu AdLuc (open squares). At various times after immunization, 5×10^5 $CD8^+$ OT-I cells were adoptively transferred into the immunized mice. Three days later, the number of OT-I cells was enumerated in the draining lymph nodes. The data represent the mean ± SEM for three mice per group. (**b–d**) Sorted, phenotypically naïve $CD8^+$ OT-I cells were adoptively transferred into WT (closed symbols) and C2D (open symbols) mice at 5×10^5 cells/mouse (panel **b**), 10^5 cells/mouse (panel **c**), or 10^4 cells/mouse (panel **d**). The next day, the mice were immunized with either AdSIINFEKL-Luc (circles) or AdLuc (squares). Three days later, the number of OT-I cells was enumerated in the draining lymph nodes. Each symbol represents a single mouse. $^*P < 0.01$.

when the frequency of $CD8^+$ T cells is high enough,[23,24] defects in priming of naïve $CD8^+$ T cells resulting from insufficient $CD4^+$ T-cell help may be masked in this experiment if the precursor frequency is too high. Therefore, the experiment was repeated with limiting amounts of OT-I cells transferred before immunization. When only 10^5 OT-I T cells were transferred before immunization, we observed a 12-fold reduction in OT-I expansion in the C2D mice relative to WT mice (**Figure 2c**). When 10^4 OT-I T cells were transferred, a 32-fold reduction in OT-I expansion was observed

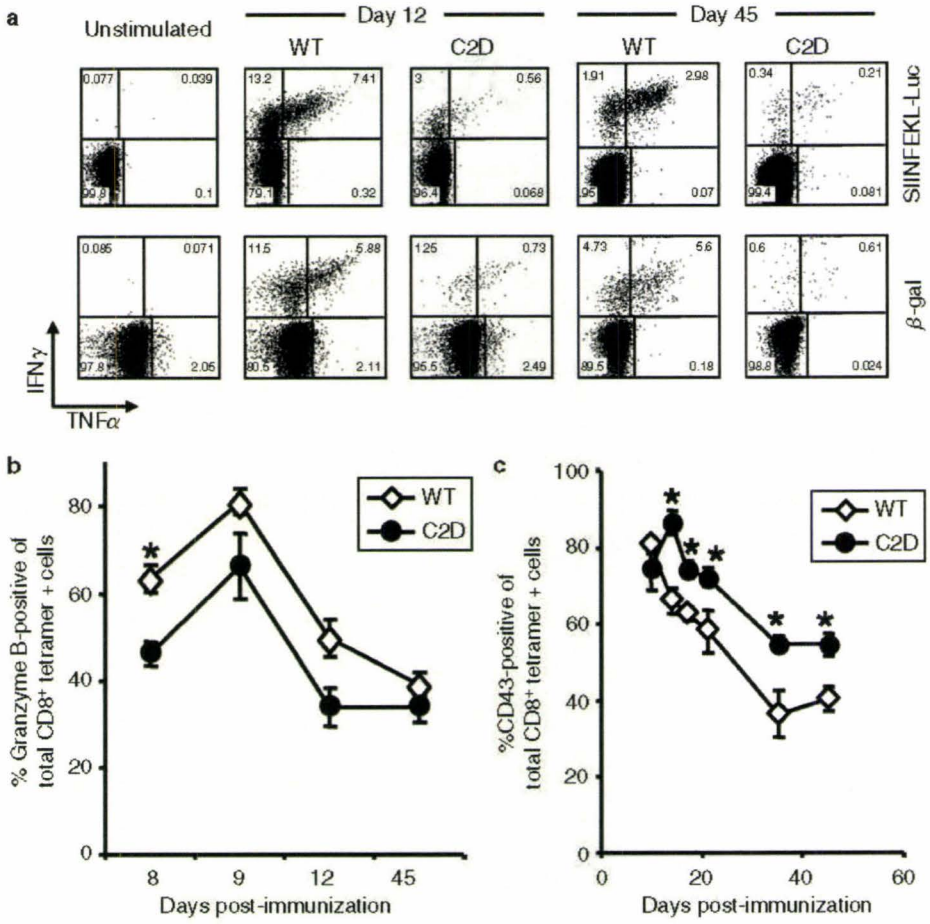

Figure 3 Functional and phenotypic differences in the antigen-specific $CD8^+$ T cells elicited by recombinant adenovirus (rAd) in wild-type (WT) and major histocompatibility complex (MHC) class II–deficient mice (C2D). WT and C2D mice were immunized with 10^8 plaque-forming units (pfu) AdSIINFEKL-Luc or 10^8 pfu Adβgal. (**a**) Intracellular interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production in splenocytes harvested 12 and 45 days after immunization was measured after a 5-hour stimulation with specific peptide. Each dot plot was gated on $CD8^+$ T cells. (**b**) Pre-formed intracellular granzyme B was measured in $CD8^+$ T cells from the lungs of WT (open diamonds) and C2D (closed circles) mice at various times after immunization. Each point represents the mean ± SEM for three to five mice. (**c**) O-glycosylated CD43 was measured on the surface of tetramer-positive $CD8^+$ T cells in WT (open diamonds) and C2D (closed circles) mice. Each point represents the mean ± SEM of five mice per time point. $^*P < 0.05$.

(**Figure 2d**). Thus, when responding $CD8^+$ T-cell precursors were limiting, we observed a defect in priming of naïve $CD8^+$ T cells after rAd immunization in C2D mice. These combined results suggest that the failure of the antigen-specific $CD8^+$ T-cell population to expand in the absence of $CD4^+$ T cells is due to inadequate priming of naïve T cells.

Functionality of the $CD8^+$ T-cell population elicited by rAd in the absence of $CD4^+$ T cells

Previous reports have demonstrated that $CD4^+$ T-cell depletion can extend transgene expression after intramuscular injection of rAd.[21] Such prolonged antigen persistence is a concern because chronic exposure to antigen may negatively affect the $CD8^+$ T-cell population, as has been seen in other models.[25,26] We observed that the antigen levels in the muscle of C2D mice remained largely unchanged over a period of 20 days after Adsiinfekl-Luc injection, and expression levels dropped only tenfold in the draining lymph nodes over the same period (**Supplementary Figure S3**). In contrast, the majority of the antigen was cleared from the muscle and lymph nodes of WT mice within the first 15 days.[13] To determine whether the increased antigen load in the C2D mice impaired $CD8^+$ T-cell function, we examined cytokine production, functional avidity, levels of preformed granzyme B, and degranulation (**Figure 3** and **Supplementary Figure S4**). With regard to cytokine production, 50–60% of the $CD8^+$ T-cell effectors elicited by immunization of WT mice with either AdSIINFEKL-Luc or Adβgal secreted both IFN-γ and TNF-α. This was true of all the sites that we sampled (blood, spleen, lung, peritoneal cavity, and bone marrow), so only data from the spleen are shown here (**Figure 3a**). At early time points, the SIINFEKL-specific $CD8^+$ T-cell population in C2D mice had a noticeably reduced ratio of IFN-γ/TNF-α double-positive cells to IFN-γ single-positive cells;

however, this was not the case at later time points after immunization (days 33 and days 45), nor was this the case for the β-gal-specific $CD8^+$ T cells (**Figure 3a** and data not shown). Examination of functional responsiveness to SIINFEKL at day 12 demonstrated that the populations were equally responsive to peptide (**Supplementary Figure S4**). Measurement of degranulation, as evidenced by CD107a staining, showed that all of the IFN-γ-positive $CD8^+$ T cells in both populations degranulated equally (**Supplementary Figure S4**). The $CD8^+$ T-cell population in the C2D mice had lower levels of pre-formed granzyme B than $CD8^+$ T cells from WT mice at early time points (days 8–12); however, this difference was found to be significant only at day 8 ($P < 0.05$) and, similar to the cytokine production, this difference was corrected at later time points (day 45) (**Figure 3b**). Thus, the $CD8^+$ T-cell population generated in C2D mice exhibited some modest defects in functionality at early time points after infection but appeared functionally comparable to WT mice at later times.

The phenotype of the $CD8^+$ T-cell population elicited by rAd in the absence of $CD4^+$ T cells

$CD4^+$ T-cell help plays an important role in licensing APC to prime $CD8^+$ T cells; $CD4^+$ T cells can also play a role in $CD8^+$ T-cell maturation.[17,27] At a phenotypic level, we found no difference in the expression of CD11a, CD44, Ly-6C, CD127, or CD62L at days 8, 12, 33, and 45 after immunization (an example of the phenotype at day 33 after immunization is given in **Supplementary Figure S4**). The relative distribution of effector, effector-memory, and central-memory $CD8^+$ T cells, as defined by the combined expression of CD62L and CD127 ($CD127^{lo}/CD62L^{lo}$, $CD127^{hi}/CD62L^{lo}$, and $CD127^{hi}/CD62L^{hi}$, respectively[28]), was also comparable between the two strains (**Supplementary Figure S4**). Thus, the $CD8^+$ T cells elicited in the C2D mice do not appear phenotypically impaired relative to the population in the WT hosts. Interestingly, the O-glycosylated form of CD43, a molecule that is associated with effector $CD8^+$ T cells, was expressed on a higher fraction of the $CD8^+$ T-cell population in the C2D mice at all time points with the exception of the peak of the response (day 14; **Figure 6c**; see **Supplementary Figure S4** for an example of the raw data). The implications of the up-regulated expression of O-glycosylated CD43 are presently unclear.

$CD8^+$ T-cell-mediated protective immunity is impaired in the absence of $CD4^+$ T cells

To examine the efficacy of the $CD8^+$ T-cell population elicited by rAd, we immunized WT and C2D mice with 10^8 pfu AdSIINFEKL-Luc and challenged the mice with recombinant vaccinia virus (rVV)-ESOVA 14 and 45 days later (**Figure 4a** and **b**). The only common epitope between the immunization virus and the challenge virus is the $CD8^+$ T-cell epitope, SIINFEKL. As a negative control, similarly immunized mice were challenged with rVV-βgal. Consistent with our previous results, we observed 100% protection against rVV-ESOVA in WT mice at both 14 and 45 days after immunization.[13] In the case of the C2D mice, we did not observe complete protection when mice were challenged 14 days after immunization, although there was a 3-log reduction in virus titers. Most striking, no protection was observed when C2D mice were challenged 45 days after immunization.

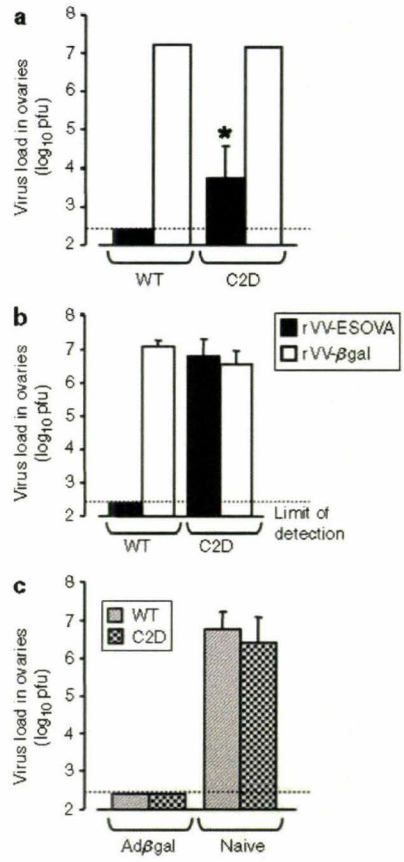

Figure 4 Protective immunity generated by recombinant adenovirus (rAd) in wild-type (WT) and major histocompatibility complex (MHC) class II–deficient (C2D) mice. (**a**) Mice were immunized with 10^8 plaque-forming units (pfu) AdSIINFEKL-Luc and challenged 14 days later with recombinant vaccinia virus (rVV)-ESOVA (closed bars) to measure protective immunity. As a control for antigen specificity, comparably immunized mice were challenged with rVV-βgal (open bars). Each bar represents the mean ± SEM for five mice. (**b**) Mice were immunized with 10^8 pfu AdSIINFEKL-Luc and challenged 45 days later with either rVV-ESOVA (closed bars) or rVV-βgal (open bars). Each bar represents the mean ± SEM for ten mice. (**c**) WT (striped bars) and C2D (checkered bars) mice were immunized with 10^8 pfu Adβgal and challenged 45 days later with rVV-βgal to measure protective $CD8^+$ T-cell immunity. As a control for protection, groups of naïve mice were challenged at the same time with rVV-βgal. Each bar represents the mean ± SEM for five mice. *$P < 0.01$.

The protection provided by AdSIINFEKL-Luc was dose dependent. Mice challenged with rVV-ESOVA 42 days after immunization with 10^7 pfu AdSIINEKL-Luc were not fully protected from virus challenge (mean virus titer in the ovaries = $3.6 \pm 2.4 \times 10^4$ pfu, $n = 5$; similar to the load in C2D mice after the day-14 challenge). At this dose of AdSIINFEKL-Luc, the mean virus titer in the ovaries of C2D mice was $2.6 \pm 0.2 \times 10^7$ pfu ($n = 5$), which was comparable to the titers observed in naïve mice.

Interestingly, the impairment in protective immunity was not observed in WT and C2D mice immunized with Adβgal and challenged with rVV-βgal. In this case, both strains demonstrated complete protection against rVV challenge (**Figure 4c**)

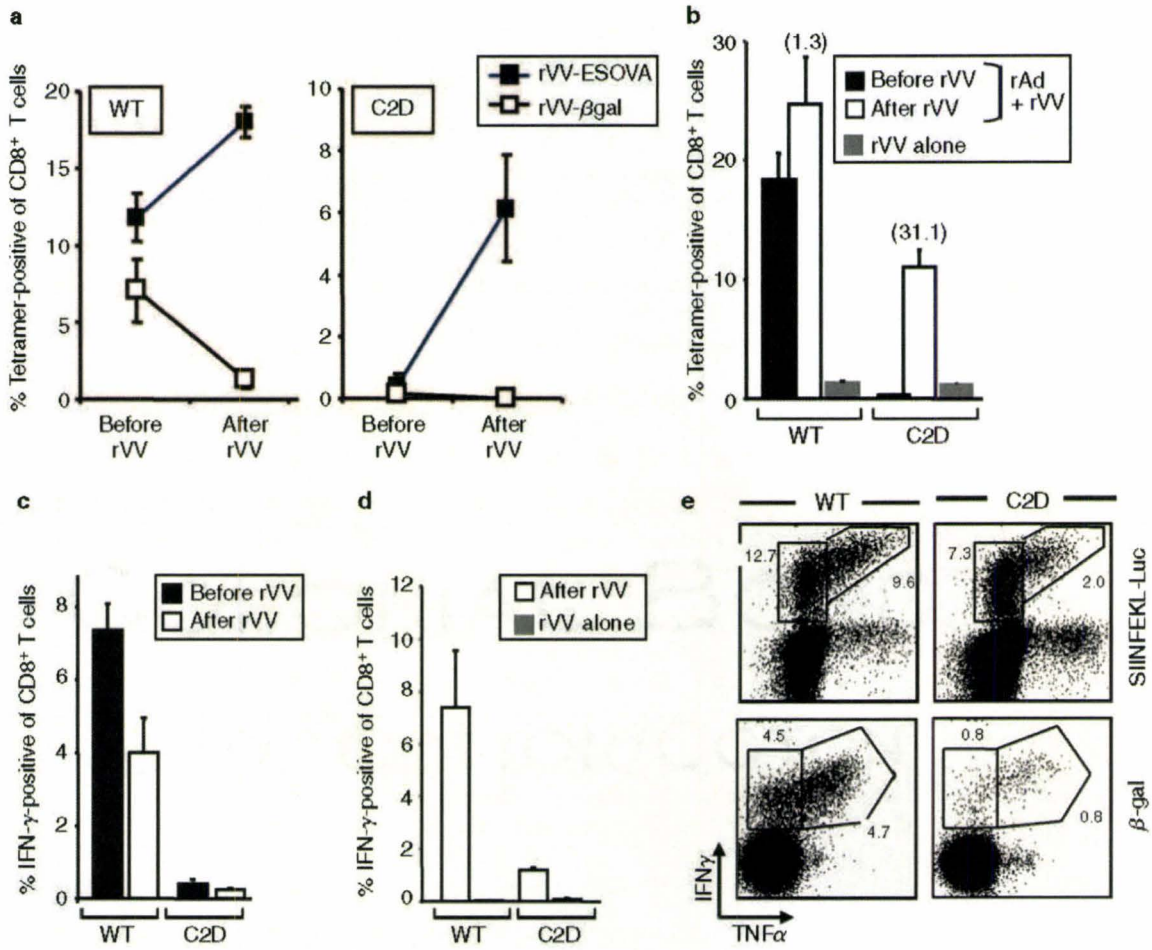

Figure 5 Enumeration and cytokine analysis of the antigen-specific CD8+ T cells after secondary stimulation *in vivo*. The antigen-specific CD8+ T-cell population was enumerated in wild-type (WT) and major histocompatibility complex MHC class II–deficient (C2D) mice immunized with recombinant adenovirus (rAd) and challenged 45 days later with recombinant vaccinia virus (rVV). (**a**) Mice were immunized with 10^8 plaque-forming units (pfu) AdSIINFEKL-Luc. The frequency of tetramer-positive CD8+ T cells in the peripheral blood was determined before and after challenge with rVV-ESOVA (black squares) or rVV-βgal (open squares). Each point represents the mean ± SEM for five mice. (**b**) Mice were immunized with 10^8 pfu AdSIINFEKL-Luc and boosted with rVV-ESOVA. Antigen-specific CD8+ T cells were enumerated in the peritoneal lavage before (closed bars) and after (open bars) rVV-ESOVA boosting. The numbers in parentheses reflect the fold expansion. The gray bars represent mice that received rVV-ESOVA alone. The data represent the mean ± SEM for five to ten mice. (**c**) WT and C2D mice were immunized with 10^8 pfu rAdβgal. The frequency of βgal-specific CD8+ T cells in the peripheral blood was determined by intracellular cytokine analysis before (closed bars) and after (open bars) boosting with rVV-βgal. Each bar represents the mean ± SEM for five mice. (**d**) βgal-specific CD8+ T cells were enumerated in the peritoneal lavage of mice that were immunized with Adβgal and boosted with rVV-βgal (open bars). The gray bars represent mice that received rVV-βgal alone. Each bar represents the mean ± SEM for five mice. (**e**) Intracellular interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production in peritoneal exudate cells harvested after rVV challenge was measured after a 5-hour stimulation with specific peptide. Each dot plot was gated on CD8+ T cells.

despite the large difference in antigen-specific CD8+ T-cell frequencies (**Figure 1c**).

Antigen-specific CD8+ T cells elicited in the absence of CD4+ T cells mount a vigorous secondary response

As previous work has demonstrated that CD8+ T cells elicited in the absence of CD4+ T cells can exhibit a defect in secondary expansion,[10,22,29] we investigated the possibility that reduced protective immunity in the C2D mice immunized with AdSIINFEKL-Luc may be due to a lack of secondary expansion. We examined the expansion of SIINFEKL-specific CD8+ T cells in the peripheral blood of the mice whose viral titers are shown in **Figure 4b**. Surprisingly, the SIINFEKL-specific CD8+ T-cell population in WT mice expanded minimally (less than twofold) after rVV-ESOVA challenge, whereas there was a sevenfold expansion in the frequency of CD8+ T cells measured in the blood of C2D mice (**Figure 5a**). The marked expansion in the C2D mice was not simply due to the rVV infection, because boosting with rVV-βgal actually resulted in attrition of the SIINFEKL-specific CD8+ T cells (**Figure 5a**). We were also surprised to observe that the number of SIINFEKL-specific CD8+ T cells in the spleens and lungs of C2D mice after boost with rVV-ESOVA was comparable to the number in WT

mice (**Supplementary Figure S5**). Most important, high frequencies of antigen-specific CD8$^+$ T cells were observed in the infection site (the peritoneal cavity), although the frequency in C2D mice was reduced relative to that in WT mice (**Figure 5b**). Thus, although the CD8$^+$ T-cell population in C2D mice exhibited a defect in primary expansion, there seemed to be no pronounced defect in secondary expansion in response to an rVV boost. Similar results were observed when WT and C2D mice were immunized with 10^7 pfu AdSIINFEKL-Luc (**Supplementary Figure S5**).

Starkly different results were obtained after immunization with 10^8 pfu Adβgal, as the population of βgal-specific CD8$^+$ T cells did not expand in the blood after challenge with rVV-βgal. In fact, we observed a small, but significant, decrease in the β-gal-specific CD8$^+$ T cells in the peripheral blood of WT mice and no significant change in the C2D mice (**Figure 5c**). β-gal-specific CD8$^+$ T cells were measurable in the peritoneum of both the WT and C2D mice, but there was a sixfold difference in frequencies between the WT and C2D groups. It should be noted that the frequencies of SIINFEKL-specific CD8$^+$ T cells elicited in naïve mice challenged with rVV-ESOVA (**Figure 5b**; "rVV alone") were on the order of 1–2% of total CD8$^+$ T cells in the peritoneal lavage, whereas we measured only very low levels of β-gal-specific CD8$^+$ T cells in the peritoneal lavage of mice immunized with rVV-βgal alone (<0.1% of total CD8$^+$ T cells; **Figure 5d**; "rVV alone"). Thus, the modest secondary response observed in these studies may be a function of the reduced immunogenicity of the rVV-βgal relative to rVV-ESOVA.

Phenotypically, the population that expanded within the site of infection of WT mice was enriched in central-memory cells (25–30% CD127^{hi} CD62L^{hi}) compared with the spleen, where very few central-memory cells were identified (<5%), and a similar enrichment was observed in C2D mice (**Supplementary Figure S5**). With regard to cytokine production, we found that there were fewer IFN-γ/TNF-α double-positive CD8$^+$ T cells in the peritoneal lavage of C2D mice immunized with AdSIINFEKL-Luc (26% ± 5.8%) compared to WT mice (47.9% ± 6.0%; $P = 0.035$) after the secondary challenge (**Figure 5e**). However, this loss in cytokine production seemed to be related to TNF-α production only because the ratio of tetramer-positive to IFN-γ-positive cells was 0.99 ± 0.18 for C2D and 1.27 ± 0.20 for WT ($P = 0.3$), indicating that the antigen-specific CD8$^+$ T cells in both strains of mice maintained equal capacity for IFN-γ production. The implications of this selective loss of TNF-α production are currently unknown. Again, the situation with the Adβgal-immunized mice was different; we observed no difference in the frequency of IFN-γ/TNF-α in the peritoneal lavage of C2D mice (50.6% ± 14.0%) compared with WT mice (52.9% ± 9.4%) after secondary challenge with rVV-βgal.

The modest secondary responses observed in the WT mice were unexpected. Although it was possible that the CD8$^+$ T cells elicited by AdSIINFEKL-Luc in C2D mice had greater capacity for secondary expansion, this did not seem very likely. Previous reports have demonstrated that a lack of secondary response can be due to high levels of effector cells, which rapidly control antigen production and limit the availability of antigen to drive the secondary response.[30] As there were significantly greater numbers of SIINFEKL-specific CD8$^+$ T cells circulating in the WT mice 45 days after immunization with 10^8 pfu AdSIINFEKL-Luc

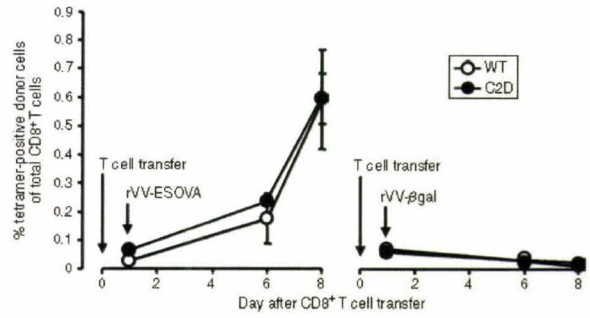

Figure 6 Antigen-specific CD8$^+$ T cells elicited by AdSIINFEKL-Luc in wild-type (WT) and major histocompatibility complex (MHC) class II–deficient (C2D) mice display similar capacity for secondary expansion. WT and C2D mice were immunized with 10^8 plaque-forming units (pfu) AdSIINFEKL-Luc. Thirty days later, CD8$^+$ T cells were isolated from the spleens of the immunized mice and equal numbers of tetramer-positive splenocytes were transferred to WT congenic (CD45.1) hosts. The next day, the mice were challenged with either recombinant vaccinia virus (rVV)-ESOVA to expand the SIINFEKL-specific T cells or rVV-βgal as a negative control. The frequency of tetramer + CD45.2$^+$ CD8$^+$ cells was enumerated in the blood on the day of challenge, 5 days after challenge, and 7 days after challenge. Each point represents the mean ± SEM for three mice.

compared with the C2D mice, we suspected the greater secondary expansion observed in C2D mice after rVV-ESOVA challenge (**Figure 5a** and **Supplementary Figure S5**) was due to rapid clearance of the rVV-ESOVA in the WT mice. Indeed, this explanation would be consistent with lack of secondary expansion in both WT and C2D mice immunized with rAdβgal and challenged with rVV-, because both strains displayed robust protective immunity (**Figure 4c**). To assess directly the ability of SIINFEKL-specific CD8$^+$ T cells from WT and C2D mice to expand in response to rVV-ESOVA challenge, CD8$^+$ T cells were isolated from WT and C2D mice 30 days after immunization with 10^8 pfu AdSIINFEKL-Luc and equal numbers of SIINFEKL-specific CD8$^+$ T cells (as assessed by tetramer staining) were transferred into naïve congenic hosts. To adjust for total differences in the number of CD8$^+$ T cells transferred into the congenic hosts owing to the reduced frequencies of SIINFEKL-specific CD8$^+$ T cells in the C2D mice, additional CD8$^+$ T cells isolated from naïve C2D mice were added to the WT CD8$^+$ T-cell population. The next day, the frequencies of SIINFEKL-specific CD8$^+$ T cells were assessed in the peripheral blood of all mice and the mice were challenged with either rVV-ESOVA or rVV-βgal (**Figure 6**). The SIINFEKL-specific CD8$^+$ T cells from both WT and C2D mice (identified by the congenic marker) displayed equal capacity to expand in response to rVV-ESOVA challenge, whereas the SIINFEKL-specific CD8$^+$ T cells were gradually lost from the mice challenged with rVV-βgal. Thus, the differences in secondary expansion between the WT and C2D hosts observed in **Figure 5** were not due to a differential ability of these cells to proliferate in a secondary fashion.

DISCUSSION

Depending on the model, the absence of CD4$^+$ T-cell help can influence the CD8$^+$ T-cell population in a number of ways, including impaired primary expansion, increased functional exhaustion,

diminished secondary responses, and reduced longevity.[17] Most reports demonstrate some degree of impaired primary $CD8^+$ T-cell expansion in the absence of $CD4^+$ T-cell help, but the extent is highly pathogen dependent.[27] Indeed, although we observed attenuated primary expansion of the rAd-induced $CD8^+$ T-cell population, the $CD8^+$ T cells elicited by rVV alone expanded to a comparable level in both WT and C2D hosts, similar to a previous report.[29] Both herpes simplex virus-1 and *Listeria monocytogenes* infection models also exhibited a marked defect in primary $CD8^+$ T-cell expansion in $CD4^+$ T-cell-deficient hosts.[31,32] In the case of herpes simplex virus-1, the impaired primary expansion was a result of insufficient cognate stimulation of APC, which is probably also the case in our model. The similarities between rAd, herpes simplex virus-1, and *L. monocytogenes* that underlie this strict dependence upon $CD4^+$ T-cell help for primary $CD8^+$ T-cell expansion remain to be determined.

Mice immunized with rAd in the absence of $CD4^+$ T cells also displayed some impairment in protective immunity against rVV challenge compared with WT mice, consistent with the reduced frequencies of memory $CD8^+$ T cells. Interestingly, this impairment was observed only with a challenge involving rVV-ESOVA, not with rVV-βgal. A recent publication has demonstrated that the number of $CD8^+$ T cells at the time of rVV challenge is key to controlling virus infection.[10] Therefore, our data suggest that fewer antigen-specific $CD8^+$ T cells are required to control the rVV-βgal infection relative to the rVV-ESOVA infection. Indeed, it would appear that protection against rVV-ESOVA requires a high frequency of circulating $CD8^+$ T cells, because protection from challenge was reduced even in WT mice immunized with a low dose (10^7 pfu) of AdSIINFEKL-Luc, despite a robust SIINFEKL-specific $CD8^+$ T-cell response. Furthermore, the protection against rVV-ESOVA in C2D mice challenged at 14 days after immunization with 10^8 pfu AdSIINFEKL-Luc was equivalent to the protection in WT mice challenged at 42 days after immunization with 10^7 pfu AdSIINFEKL-Luc. Under these conditions, the frequencies of circulating SIINFEKL-specific $CD8^+$ T cells were also equivalent (approximately 5% of total $CD8^+$ T cells) in WT and C2D mice. Therefore, our data support the likelihood that the reduced protective immunity observed in the C2D mice is a function of limiting numbers of antigen-specific $CD8^+$ T cells.

Perhaps the most striking observation from these experiments relates to the ability of the $CD8^+$ T cells elicited by rAd in the absence of $CD4^+$ T cells ("unhelped" $CD8^+$ T cells) to expand in a secondary fashion. Unlike in previous studies, where unhelped $CD8^+$ T cells elicited in the absence of $CD4^+$ T cells exhibited a defect in secondary expansion,[10,22,29] the unhelped $CD8^+$ T-cell population elicited by rAd undergoes robust secondary expansion comparable to helped $CD8^+$ T cells. Likewise, in at least one report, the unhelped $CD8^+$ T-cell population generated by *L. monocytogenes* infection also underwent normal secondary expansion.[31] These results challenge the concept that $CD4^+$ T-cell help is required during the primary $CD8^+$ T-cell response to program the secondary memory response and demonstrate that effective secondary responses can be generated under conditions of insufficient $CD4^+$ T-cell help when the appropriate immunogen is employed. It is curious to note that the $CD8^+$ T-cell population elicited by rAd exhibited only a modest secondary expansion in WT mice, similar to our previous observations in a model of influenza infection[33] and other reports.[34,35] On the basis of our observation that the unhelped $CD8^+$ T cells show the same capacity for secondary expansion as helped $CD8^+$ T cells, we suggest that the lack of secondary response is due to rapid control of the rVV infection by memory $CD8^+$ T cells limiting the availability of antigen to drive the secondary response, as in a recent report investigating memory responses after *L. monocytogenes* infection.[30] Indeed, this would be consistent with the increased secondary response observed in WT mice immunized with 10^7 pfu AdSIINFEKL-Luc compared with those immunized with 10^8 pfu, because the mice at the lower dose exhibited reduced capacity to control the rVV-ESOVA infection. Similarly, in the case of rVV-βgal, where very few antigen-specific $CD8^+$ T cells seem to be required to control infection, we observed no evidence of secondary expansion, presumably because of rapid control of the infection.

Another curious similarity between the observations of the current study and our previous work with influenza is that the size of the secondary population appears to be unconnected with the frequency of memory cells at the time of the boost. In the case of influenza, infection of WT mice with an rAd expressing influenza nucleoprotein yielded a splenocyte population with approximately eightfold higher frequency of nucleoprotein-specific $CD8^+$ T cells than mice immunized with influenza HKx31, yet after secondary immunization with a serologically distinct influenza strain (PR8), similar frequencies of nucleoprotein-specific $CD8^+$ T cells were observed in both groups of mice, which amounted to a twofold expansion in the rAd-primed mice but an approximately 20-fold expansion in the flu-primed mice.[33] A similar observation has been made using a model of adoptively transferred memory cells, where the magnitude of the secondary expansion was independent of the frequency of memory cells at the time of challenge.[36] The mechanisms that regulate this process remain to be determined.

We were concerned that the $CD8^+$ T-cell population elicited by rAd would be predisposed to functional exhaustion in the absence of $CD4^+$ T-cell help because the population in WT mice already exhibited evidence of partial exhaustion. However, the unhelped $CD8^+$ T cells elicited by rAd5 presented only subtle phenotypic and functional changes compared with the population in WT mice. The most striking phenotypic difference between the helped and unhelped $CD8^+$ T-cell populations in our study was the increased frequency of unhelped $CD8^+$ T cells bearing the O-glycosylated form of CD43 recognized by the 1B11 antibody. Similar up-regulation of CD43 has been seen in chronic virus and parasitic infections.[37-39] Although the role of CD43 is still being elucidated, it has been shown to be involved in the recruitment of $CD8^+$ T cells into inflamed tissues, and recent data have demonstrated that the 130 kda form CD43, recognized by 1B11, is a ligand for E-selectin.[40,41] This putative role of CD43 is consistent with the observation that 1B11 binding is increased on effector cells and diminishes as cells progress toward a memory phase.[42] In that regard, the prolonged up-regulation of this molecule may simply be due to the increased duration of antigen expression after rAd immunization in C2D mice and merely reflective of repeated antigenic stimulation. However, given the additional role for CD43 as a negative regulator of T-cell function, it is

possible that the up-regulation of the 1B11 reactive form may actually be a protective mechanism wherein the $CD8^+$ T-cell attempts to reduce its interaction with antigen-loaded cells to avoid functional exhaustion.[43] Aside from increased expression of CD43, we also observed reduced levels of pre-formed granzyme B and TNF-α secretion in the unhelped $CD8^+$ T-cell population elicited by AdSIINFEKL-Luc. These defects were observed only at early time points and resolved in the memory phase. These subtle defects also appear to be antigen-specific, because we did not observe any defect in TNF-α production by unhelped $CD8^+$ T cells elicited by Adβgal.

Our findings support the use of rAd vectors for prime–boost strategies in individuals with defects in $CD4^+$ T-cell helper function, as the unhelped $CD8^+$ T-cell population appears to maintain functional competence during the secondary phase. More important, similar levels of antigen-specific $CD8^+$ T cells could be achieved in WT and C2D mice using a prime–boost strategy, suggesting that rAd immunization may represent an excellent tool to prime $CD8^+$ T cells under conditions of insufficient helper T-cell function. However, the evidence of some mild functional defects in the unhelped population indicates that we should proceed cautiously, and further investigations will be required to validate this prime–boost strategy in relevant challenge models.

MATERIALS AND METHODS

Replication-deficient adenoviruses (rAds). All the rAd vectors used in these studies were constructed using the E1,E3-deleted adenovirus backbone described by Ng *et al.*[44] Transcription is initiated by the murine cytomegalovirus immediate-early promoter and terminated by the SV40 polyadenylation signal. The following rAd vectors have been described previously: (i) AdSIINFEKL-Luc, expressing a modified version of luciferase bearing the immunodominant class-I epitope from chicken egg ovalbumin (SIINFEKL) tagged to the N-terminus; (ii) AdLuc, expressing luciferase; (iii) Adβgal, expressing β-galactosidase.[45,46] All rAds were propagated using 293 cells and purified using CsCl gradient centrifugation as previously described.[46]

Replication-competent vaccinia viruses (rVV). An rVV expressing the SIINFEKL epitope linked to an ER-targeting signal (rVV-ESOVA) was graciously provided by Jonathan Yewdell (National Institute of Allergy and Infections Disease, Bethesda, MD).[47] An rVV expressing β-gal (rVV-β-gal) was kindly provided by Therion Biologics (Cambridge, MA).

Animals and immunizations. Female C57Bl/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Major histocompatibility complex C2D mice[48] on a C57Bl/6 background and OT-I transgenic mice[49] were bred in-house in a barrier facility. $CD45.1^+$ congenic mice (B6.SJL-*Ptprc*^{a}/BoAiTac) were purchased from Taconic (Germantown, NY). For immunizations, 10^8 pfu rAd was diluted to 100 µl in sterile phosphate-buffered saline and subsequently injected intramuscularly in both rear thighs. For rVV challenge, 10^7 pfu rVV was diluted to 200 µl in sterile phosphate-buffered saline and subsequently injected intraperitoneally. $CD4^+$ T cells were depleted from WT animals using the GKI.5 antibody as described previously.[50]

Flow cytometry reagents. All flow cytometry antibodies were purchased from BD Pharmingen (San Jose, CA) except anti-granzyme B-allophycocyanin and anti-CD127-fluorescein isothiocyanate, which were purchased from Caltag Laboratories (Burlingame, CA) and eBiosciences (San Diego, CA), respectively. Phycoerythrin- or allophycocyanin-labeled K^b/SIINFEKL tetramers were obtained from the Molecular Biology Core at the Trudeau Institute (Saranac Lake, NY) or the Protein Chemistry Core at the Baylor College of Medicine or produced in our laboratory at McMaster. Most staining conditions involved five flurochromes (fluorescein isothiocyanate, phycoerythrin, phycoerythrin-Cy5, phycoerythrin-Cy7, and allophycocyanin), and data were acquired using either an LSRII or a FACSCanto equipped with a 488 and 633 nm laser.

Preparation of tissues for flow cytometry. Spleens, lymph nodes (popliteal, inguinal, and ileac), femur, tibia, blood, lungs, and peritoneal exudate cells were harvested from mice killed at various times after immunization as described previously.[13]

Tetramer staining. This method has been described by our group previously.[13]

Staining of intracellular proteins. To measure the intracellular levels of Ki-67 and granzyme B, cells were stained with tetramer and surface markers as described above, permeabilized using cytofix/cytoperm solution (BD Pharmingen) and stained with antibodies against intracellular proteins diluted in 1× Perm/Wash buffer (BD Pharmingen).

BrdU incorporation assay. Mice received intraperitoneal injections of 1 mg BrdU at 48 and 24 hours before harvest. Lymphocytes were stained with tetramer as described previously[13] and subsequently stained for BrdU using the BrdU staining kit from BD Pharmingen, according to the manufacturer's instructions.

Intracellular cytokine staining. Intracellular cytokine was visualized using a protocol we described previously.[13] To measure cytokine production in lymphocytes isolated from blood samples, an aliquot of 50–100 µl of peripheral blood obtained from the orbital sinus was drawn into a Falcon 2058 polystyrene tube containing heparin. Red blood cells were lysed by two treatments with 0.15 M NH_4Cl lysis buffer. Each specimen was split into two wells of a 96-well round-bottomed plate containing 2×10^6 carboxyfluorescein-labeled syngeneic splenocytes. Cells were stimulated with peptide and processed for staining of intracellular cytokine. Cytokine production was measured in the carboxyfluorescein-negative, CD8-positive population.

Degranulation assay. The degranulation assay has been described by our group previously.[13]

In vivo antigen presentation assay. $CD44^{lo}$ $CD62L^{hi}$ Thy 1.1^+ OT-I $CD8^+$ T cells were isolated from lymph node preparations by flow cytometry using a FACSVantage (Becton Dickinson, San Jose, CA). Various amounts of OT-I cells (10^4–5×10^5) were adoptively transferred to congenic C57Bl/6 recipients by intravenous injection. Mice were immunized with AdSIINFEKL or AdLuc 24 hours later. Draining lymph nodes were harvested 72–96 hours after immunization to measure expansion of the OT-I population.

Statistical analysis. Data are presented as mean ± SEM. Statistical analysis was carried out using Microsoft Excel on log-transformed data to normalize variations. Differences were considered significant at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Figure S1. The $CD8^+$ T-cell response in various tissues after immunization of wild-type (WT), major histocompatibility complex class II–deficient (C2D), and GKI.5-depleted mice.
Figure S2. Example of Ki-67 and BrdU staining.
Figure S3. Transgene expression after injection of AdSIINFEKL-Luc.
Figure S4. Functional and phenotypic analysis of $CD8^+$ T cells.
Figure S5. Enumeration and phenotypic analysis of the antigen-specific $CD8^+$ T cells after secondary stimulation *in vivo*.

ACKNOWLEDGMENTS

We would like to thank Kelley Putzu (McMaster University) for assisting with some of the animal studies. Funding for this work was provided by the Canadian Institutes of Health Research (MOP-42433 and MGC-57082 to J.L.B.). J.L.B. is the recipient of an Rx & D–Health Research Foundation/CIHR Career Award in Health Research. Y.H.W. is a CIHR New Investigator. T.G., T.C.Y., and J.M. were supported through a Premier's Research Excellence Award (PREA) given to J.L.B. from the Ontario Ministry of Enterprise, Opportunity, and Innovation.

REFERENCES

1. Tatsis, N and Ertl, HC (2004). Adenoviruses as vaccine vectors. *Mol Ther* **10**: 616–629.
2. Barouch, DH and Nabel, GJ (2005). Adenovirus vector-based vaccines for human immunodeficiency virus type 1. *Hum Gene Ther* **16**: 149–156.
3. Stone, D, Ni, S, Li, ZY, Gaggar, A, DiPaolo, N, Feng, Q *et al.* (2005). Development and assessment of human adenovirus type 11 as a gene transfer vector. *J Virol* **79**: 5090–5104.
4. Barouch, DH, Pau, MG, Custers, JH, Koudstaal, W, Kostense, S, Havenga, MJ *et al.* (2004). Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol* **172**: 6290–6297.
5. Tatsis, N, Tesema, L, Robinson, ER, Giles-Davis, W, McCoy, K, Gao, GP *et al.* (2006). Chimpanzee-origin adenovirus vectors as vaccine carriers. *Gene Ther* **13**: 421–429.
6. Wherry, EJ and Ahmed, R (2004). Memory CD8 T-cell differentiation during viral infection. *J Virol* **78**: 5535–5545.
7. Zaph, C, Uzonna, J, Beverley, SM and Scott, P (2004). Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. *Nat Med* **10**: 1104–1110.
8. Wherry, EJ, Teichgraber, V, Becker, TC, Masopust, D, Kaech, SM, Antia, R *et al.* (2003). Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* **4**: 225–234.
9. Zammit, DJ, Cauley, LS, Pham, QM and Lefrancois, L (2005). Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity* **22**: 561–570.
10. Bachmann, MF, Wolint, P, Schwarz, K and Oxenius, A (2005). Recall proliferation potential of memory $CD8^+$ T cells and antiviral protection. *J Immunol* **175**: 4677–4685.
11. Jackson, SS, Schmitz, JE, Kuroda, MJ, McKay, PF, Sumida, SM, Martin, KL *et al.* (2005). Evaluation of CD62L expression as a marker for vaccine-elicited memory cytotoxic T lymphocytes. *Immunology* **116**: 443–453.
12. Klebanoff, CA, Gattinoni, L, Torabi-Parizi, P, Kerstann, K, Cardones, AR, Finkelstein, SE *et al.* (2005). Central memory self/tumor-reactive $CD8^+$ T cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci USA* **102**: 9571–9576.
13. Yang, TC, Millar, J, Groves, T, Grinshtein, N, Parsons, R, Takenaka, S *et al.* (2006). The $CD8^+$ T Cell population elicited by recombinant adenovirus displays a novel partially exhausted phenotype associated with prolonged antigen presentation that nonetheless provides long-term immunity. *J Immunol* **176**: 200–210.
14. Wherry, EJ, Blattman, JN, Murali-Krishna, K, van der Most, R and Ahmed, R (2003). Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* **77**: 4911–4927.
15. Krebs, P, Scandella, E, Odermatt, B and Ludewig, B (2005). Rapid functional exhaustion and deletion of CTL following immunization with recombinant adenovirus. *J Immunol* **174**: 4559–4566.
16. Pascual, DW, Walters, N and Hillemeyer, P (1998). Repeated intratracheal instillations of nonreplicating adenovirus 2 vector attenuate CTL responses and IFN-gamma production. *J Immunol* **160**: 4465–4472.
17. Bevan, MJ (2004). Helping the $CD8^{(+)}$ T-cell response. *Nat Rev Immunol* **4**: 595–602.
18. Poller, W, Schneider-Rasp, S, Liebert, U, Merklein, F, Thalheimer, P, Haack, A *et al.* (1996). Stabilization of transgene expression by incorporation of E3 region genes into an adenoviral factor IX vector and by transient anti-CD4 treatment of the host. *Gene Ther* **3**: 521–530.
19. Kolls, JK, Lei, D, Odom, G, Nelson, S, Summer, WR, Gerber, MA *et al.* (1996). Use of transient CD4 lymphocyte depletion to prolong transgene expression of E1-deleted adenoviral vectors. *Hum Gene Ther* **7**: 489–497.
20. DeMatteo, RP, Markmann, JF, Kozarsky, KF, Barker, CF and Raper, SE (1996). Prolongation of adenoviral transgene expression in mouse liver by T lymphocyte subset depletion. *Gene Ther* **3**: 4–12.
21. Yang, Y, Haecker, SE, Su, Q and Wilson, JM (1996). Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle. *Hum Mol Genet* **5**: 1703–1712.
22. Janssen, EM, Lemmens, EE, Wolfe, T, Christen, U, von Herrath, MG and Schoenberger, SP (2003). $CD4^+$ T cells are required for secondary expansion and memory in $CD8^+$ T lymphocytes. *Nature* **421**: 852–856.
23. Mintern, JD, Davey, GM, Belz, GT, Carbone, FR and Heath, WR (2002). Cutting edge: precursor frequency affects the helper dependence of cytotoxic T cells. *J Immunol* **168**: 977–980.
24. Wang, B, Norbury, CC, Greenwood, R, Bennink, JR, Yewdell, JW and Frelinger, JA (2001). Multiple paths for activation of naive $CD8^+$ T cells: CD4-independent help. *J Immunol* **167**: 1283–1289.
25. Dittmer, U, He, H, Messer, RJ, Schimmer, S, Olbrich, AR, Ohlen, C *et al.* (2004). Functional impairment of $CD8^{(+)}$ T cells by regulatory T cells during persistent retroviral infection. *Immunity* **20**: 293–303.
26. Wherry, EJ, Barber, DL, Kaech, SM, Blattman, JN and Ahmed, R (2004). Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci USA* **101**: 16004–16009.
27. Behrens, G, Li, M, Smith, CM, Beltz, GT, Mintern, J, Carbone, FR *et al.* (2004). Helper T cells, dendritic cells and CTL immunity. *Immunol Cell Biol* **82**: 84–90.
28. Bachmann, MF, Wolint, P, Schwarz, K, Jager, P and Oxenius, A (2005). Functional properties and lineage relationship of $CD8^+$ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol* **175**: 4686–4696.
29. Shedlock, DJ and Shen, H (2003). Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**: 337–339.
30. Wong, P, Lara-Tejero, M, Ploss, A, Leiner, I and Pamer, EG (2004). Rapid development of T cell memory. *J Immunol* **172**: 7239–7245.
31. Marzo, AL, Vezys, V, Klonowski, KD, Lee, SJ, Muralimohan, G, Moore, M *et al.* (2004). Fully functional memory CD8 T cells in the absence of CD4 T cells. *J Immunol* **173**: 969–975.
32. Smith, CM, Wilson, NS, Waithman, J, Villadangos, JA, Carbone, FR, Heath, WR *et al.* (2004). Cognate $CD4^{(+)}$ T cell licensing of dendritic cells in $CD8^{(+)}$ T cell immunity. *Nat Immunol* **5**: 1143–1148.
33. Bertram, EM, Dawicki, W, Sedgmen, B, Bramson, JL, Lynch, DH and Watts, TH (2004). A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza *in vivo*. *J Immunol* **172**: 981–988.
34. Pinto, AR, Fitzgerald, JC, Giles-Davis, W, Gao, GP, Wilson, JM and Ertl, HC (2003). Induction of $CD8^+$ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol* **171**: 6774–6779.
35. Bruna-Romero, O, Gonzalez-Aseguinolaza, G, Hafalla, JC, Tsuji, M and Nussenzweig, RS (2001). Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen. *Proc Natl Acad Sci USA* **98**: 11491–11496.
36. Badovinac, VP, Messingham, KA, Hamilton, SE and Harty, JT (2003). Regulation of $CD8^+$ T cells undergoing primary and secondary responses to infection in the same host. *J Immunol* **170**: 4933–4942.
37. Fuller, MJ, Khanolkar, A, Tebo, AE and Zajac, AJ (2004). Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. *J Immunol* **172**: 4204–4214.
38. Gallego, MD, Aguado, E, Kindelan, JM, Pena, J, Santamaria, M and Molina, IJ (2001). Altered expression of CD43-hexasaccharide isoform on peripheral T lymphocytes from HIV-infected individuals. *Aids* **15**: 477–481.
39. Martin, DL and Tarleton, RL (2005). Antigen-specific T cells maintain an effector memory phenotype during persistent *Trypanosoma cruzi* infection. *J Immunol* **174**: 1594–1601.
40. Matsumoto, M, Atarashi, K, Umemoto, E, Furukawa, Y, Shigeta, A, Miyasaka, M *et al.* (2005). CD43 functions as a ligand for E-Selectin on activated T cells. *J Immunol* **175**: 8042–8050.
41. Woodman, RC, Johnston, B, Hickey, MJ, Teoh, D, Reinhardt, P, Poon, BY *et al.* (1998). The functional paradox of CD43 in leukocyte recruitment: a study using CD43-deficient mice. *J Exp Med* **188**: 2181–2186.
42. Harrington, LE, Galvan, M, Baum, LG, Altman, JD and Ahmed, R (2000). Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. *J Exp Med* **191**: 1241–1246.
43. Tong, J, Allenspach, EJ, Takahashi, SM, Mody, PD, Park, C, Burkhardt, JK *et al.* (2004). CD43 regulation of T cell activation is not through steric inhibition of T cell-APC interactions but through an intracellular mechanism. *J Exp Med* **199**: 1277–1283.
44. Ng, P, Parks, RJ, Cummings, DT, Evelegh, CM and Graham, FL (2000). An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. *Hum Gene Ther* **11**: 693–699.
45. Yang, TC, Dayball, K, Wan, YH and Bramson, J (2003). Detailed analysis of the $CD8^+$ T-cell response following adenovirus vaccination. *J Virol* **77**: 13407–13411.
46. Bramson, JL, Hitt, M, Gauldie, J and Graham, FL (1997). Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. *Gene Ther* **4**: 1069–1076.
47. Restifo, NP, Bacik, I, Irvine, KR, Yewdell, JW, McCabe, BJ, Anderson, RW *et al.* (1995). Antigen processing *in vivo* and the elicitation of primary CTL responses. *J Immunol* **154**: 4414–4422.
48. Grusby, MJ, Johnson, RS, Papaioannou, VE and Glimcher, LH (1991). Depletion of $CD4^+$ T cells in major histocompatibility complex class II-deficient mice. *Science* **253**: 1417–1420.
49. Hogquist, KA, Jameson, SC, Heath, WR, Howard, JL, Bevan, MJ and Carbone, FR (1994). T cell receptor antagonist peptides induce positive selection. *Cell* **76**: 17–27.
50. Wan, Y, Bramson, J, Pilon, A, Zhu, Q and Gauldie, J (2000). Genetically modified dendritic cells prime autoreactive T cells through a pathway independent of CD40L and interleukin 12: implications for cancer vaccines. *Cancer Res* **60**: 3247–3253.

- Chapter 6 –

Discussion

The research that I conducted during my PhD project has not been directed at creating a vaccine for a specific application. Rather, the aim of my research was to extend our understanding of the T cell response elicited by recombinant human adenovirus type 5 (rHuAd5) in mice. Although this vector has not proven to be extremely effective in humans, the robust immunogenicity of rHuAd5 in mice merits further biological investigation to develop paradigms that can ultimately be extended to humans albeit likely with a different vector platform. In this chapter, I will summarize the knowledge that I have gained regarding protective cellular immunity and discuss areas that require additional investigation to maximize the utility of rAd as a vaccination platform.

What constitutes protective T cell immunity?

The issue of what T cells provide the greatest protective immunity is a highly contested topic. Following the identification of CD62L and CD127 as markers of memory CD8+ T cells with high proliferative capacity, termed "central memory" T cells (T_{cm}), it was suggested that this population represented the most protective CD8+ T cell. However this finding has been questioned following the publication of experimental data that demonstrated that the "age" of the T cells was more important than the expression of CD62L and CD127 (315). Further, Bachmann *et* al demonstrated that the presence of high numbers of T_{eff} correlated with protection against lytic viruses such as vaccinia virus, presumably due to the rapid clearance of infected cells before new viral progenies were produced (145). Also, while both T_{em} and T_{cm} were protective against challenge with *Leishmania major*, the rate at which T_{cm} controlled parasite replication was delayed by at

least 3 weeks presumably because T_{cm} must regain effector function whereas T_{em} can provide immediate effector function (316). Finally, a recent study using a CMV-vectored vaccine demonstrated that T_{em} can provide effective protection against mucosal challenge with SIV; however, this group did not directly compare their results with a vaccination strategy that elicited T_{cm} (317). These results would suggest that either we currently lack the ability to correctly identify T cells by phenotype or, more likely, the optimal protective T cell(s) will be pathogen dependent and determined by the nature of pathogen-host interactions.

An alternate measure of the protective capacity of the T cell response is functionality. As previously described in the introduction, there is considerable evidence to suggest that polyfunctional T cell responses are more protective compared to T cells with less functions. In humans, substantial evidence to support of this theory has been garnered from HIV long-term non-progressor (LTNPs), whose T cells retain the capability to produce IFNγ, TNFα and IL-2 while T cells in progressors lose the capacity to elaborate these cytokines. Loss of cytokine production by T cells has been termed exhaustion and murine models of chronic infection have revealed a hierarchical loss of cytokine production by pathogen-specific CD8+ T cells where loss of IL-2 is followed by loss of TNF-α and, ultimately, loss of IFN-γ (154, 155, 169). It is currently unclear if the functionality of T cells in HIV LTNPs is maintained because they are able to control the infection or if some factor is responsible which permits HIV specific T cells to maintain their functionality and avoid exhaustion. In a murine model of chronic LCMV infection,

exhaustion appears to be promoted by high-antigen loads rather than some virus-associated factor (318). We have observed that the CD8+ T cell population produced by rHuAd5 in mice displays limited polyfunctionality (high IFN-γ but impaired TNF-α and IL-2) which suggested that the CD8+ T cell population elicited by rHuAd5 may be partially exhausted (90, 174). Nevertheless, the CD8+ T cell population produced by rHuAd5 immunization provides robust protection against multiple infectious agent and tumors in murine models. Therefore, although there is evidence that polyfunctionality is an important property of protective T cell immunity, it remains to be determined which functions are most relevant.

In spite of the robust protective immunity produced by rHuAd5 in murine and simian models, a Phase II trial (STEP) investigating the efficacy of a 3 vector vaccine, where each virus expressed 1 of gag, pol or Nef from HIV, failed to provide evidence of protection (309, 310). In fact, patients with pre-existing immunity to HuAd5 appeared to have a higher incidence of infection (309, 310). Why was the rAd vaccine employed in the STEP trail unsuccessful? Although we don't know the exact answer there are numerous possible explanations that could have contributed to it. First, the choice of the HuAd5 serotype was inappropriate since it is highly prevalent within human populations. Pre-existing Ad immunity would have muted responses against the transgenes as well as boost Ad specific responses. This effect could have resulted in T cell responses that were too small to prevent HIV infection. Secondly, the choice of transgenes may have been incorrect or insufficient. The vaccine contained a transgene that expressed gag, nef and

pol which may have been inadequate since STEP trail volunteers elicited only a limited number of epitope specific responses against them. Protection from HIV infection may require a broader response against more epitopes. Third, if studies evaluating functionality are correct for HIV, a vaccine platform that elicits T cells that produce IL-2 in conjunction with IFNγ and/or TNFα may be required. In the STEP trial 88% of responding CD4+ T cells produced IL-2 of which 72% also produced IFNγ or TNFα or both (310). However, very few CD8+ T cells produced IL-2 on their own or in conjunction with IFNγ or TNFα (310). Finally, the route of immunization may be important in positioning cellular immunity appropriately to prevent infection. T cell trafficking research has identified that T cells can be imprinted with specific homing properties depending on the route of infection. Therefore, protection from HIV infection at mucosal surfaces may require a larger number of T cells to be present at the site of infection than parenteral immunization could generate. The STEP vaccine trail has highlighted many questions about the optimal way to employ rAd vectors, and genetic vaccines in general, in humans.

Effect of prolonged antigen presentation on memory CD8+ T cells

Memory CD8+ T cells can be maintained through both antigen-dependent and antigen-independent mechanisms. In the absence of antigen, memory CD8+ T cells are maintained through homeostatic proliferation in response to IL-7 and IL-15 stimulation. By contrast, memory CD8+ T cells generated in the presence of high antigen loads, as in the case of chronic infection with LCMV clone 13, lose the ability to respond to

homeostatic cytokines and become "antigen addicted", where they retain the ability to proliferate in the presence of antigen and die if the antigen is removed (165). Under conditions of lower antigen loads, as in the case of persistent polyoma virus infection, the "memory" population appears to also be antigen-dependent and composed of both "antigen-addicted" cells and recently recruited naive CD8+ T cells (89). Interestingly, following infection with influenza, vesicular stomatitis virus and rAd, acute infectious agents that do not establish persistent infections, prolonged antigen presentation is also observed. Results from chapter 3 have demonstrated that following rAd inoculation, prolonged antigen presentation is required for complete development of CD8+ T cell immunity. Our results suggest that antigen driven proliferation during the first 30 – 60 days following immunization is required to elicit the maximal frequency of memory CD8+ T cells. After day 60, the memory population is no longer dependent upon transgene expression but this does not prove that the population has become antigen-independent. It is entirely possible that a depot of synthesized antigen gets created which provides a source of stimulation in the absence of continued transgene expression.

A number of recent reports have suggested that T cells engaged early in the response differentiate into effector cells whereas those that are engaged later in the response display a less differentiated phenotype and become the memory pool. This model does not appear to explain the maintenance or phenotype of the memory CD8+ T cell population produced by rHuAd5. The late-engaged CD8+ T cells display a less differentiated phenotype which is consistent with the phenotype of T_{cm}. By contrast, the

memory population produced by rHuAd5 is a mixture of T_{eff} and T_{em}. Also, we found that only cells engaged within the first few days of infection actually enter the circulating pool. These "early" engaged cells are dependent upon antigen beyond the expansion phase and exhibit a phenotype consistent with cells that have been persistently exposed to antigen. Unpublished data from our lab generated by my colleague, Jennifer Bassett, revealed that the memory population is actually dependent upon prolonged transgene expression within non-hematopoietic cells located outside the draining lymph nodes. Thus, our combined data suggest that rHuAd5 vectors employ a previously unrecognized mechanism for maintaining an effector-memory CD8+ T cell response.

Another potential result of prolonged CD8+ T cell antigen presentation may be to maintain their presence at the site of infection. In the case of HSV-1 infection, effector CD8+ T cells persist at the trigeminal ganglion where the latent HSV-1 resides; these cells are presumably being repetitively stimulated by HSV-1 genes each time the virus reactivates. Following infection of the respiratory tract with influenza, prolonged antigen presentation can be detected for several weeks yet this persistent antigen presentation does not result in continual activation of naive CD8+ T cells. However, prolonged antigen presentation following influenza infection does result in the continual recruitment of influenza specific CD8+ T cells into the lung lumen. Furthermore, additional studies have found that CD8+ T cells in the lung lumen possess an activated phenotype which may be due to prolonged antigen presentation. A report by Santosuosso *et al*, has found that intranasal immunization with rHuAd5 similarly promoted recruitment of T cells into

the lung lumen and provided robust protection against challenge with mycobacterium in the lung (296). This effect was found to be dependent upon continued antigenic stimulation of the luminal T cells (319). Therefore, the sustained effector-memory population produced by rHuAd5 does have clear value for protection at mucosal surfaces. Further investigation of these properties may also have value for protection against HIV (317), however the route of administration appears to play an important role in efficacy (273).

Effect of prolonged antigen presentation on CD4+ T cells

CD4+ T cells appear to require longer exposure to antigen for complete activation compared to CD8+ T cells. As stated previously, antigen presentation following influenza infection persists for several weeks in the absence of detectable replicating virus (126, 173). Interestingly, in contrast to CD8+ T cells, naive CD4+ T cells continue to be engaged by influenza antigen for up to 4 weeks post infections (126). In this case, late-activated CD4+ T cells formed an important part of the memory pool and possessed a less differentiated phenotype compared to early activated cells (126). Following rAd immunization we do not know what the effects of prolonged antigen presentation are on the CD4+ T cell response. Results from chapter 4, suggest that differences in the magnitude and duration of detectable antigen presentation had a minimal effect on the kinetics and magnitude of the response. However, to achieve a more accurate picture of the effects of prolonged antigen presentation on rAd elicited CD4+ T cells will require a more detailed analysis. Due to similarities in CD8+ T cell antigen presentation between

rAd and influenza, one might predict that prolonged antigen presentation following rHuAd5 immunization may recruit late-comer CD4+ T cells similar to influenza; however, antigen presentation to CD4+ T cells did not appear to persist for a long-time following rHuAd5 immunization. Furthermore, the distribution of the antigen-specific CD4+ T cells was quite different than CD8+ T cells following rHuAd5 immunization. Whereas, high frequencies of antigen-specific CD8+ T cells were measured in the peripheral tissues, the majority of the CD4+ T cells were located within the spleen and lymph nodes. Since we speculate that a non-lymphoid cell is responsible for the persistent stimulation of the CD8+ T cells, it is quite likely that the CD4+ T cells do not receive the same antigenic stimulation since they do not travel to the same locations. Consistent with this hypothesis, we observed that the CD4+ T cell population elicited by rHuAd5 displayed a more conventional kinetic with a peak at day 8 and a marked contraction phase. Thus, it appears that the CD4+ T cell response produced by rHuAd5 is not governed by the same mechanisms that influence the CD8+ T cell response.

Helpless CD8+ T cells; the requirement of CD4+ T cell help

In most infectious models CD4+ T cell help has been shown to be critical for the complete differentiation of memory CD8+ T cells. However, there is considerable controversy regarding the timing and mechanism of CD4+ T cell help. Some studies have found that CD4+ T cells are critical during priming, others have found they are vital for CD8+ T cell maintenance and other models have found CD4+ T cell to be indispensible for recall responses. To fully appreciate CD8+ T cell immunity and

optimize future vaccines, it is important to appreciate the mechanisms by which CD4+ T cells mediate their help.

We have found that CD4+ T cells play an important role in primary CD8+ T cell expansion following immunization with rHuAd5 but they do not appear to be required for CD8+ T cell differentiation or memory maintenance. We did observe impairment in protective immunity by CD8+ T cells but we believe that the reduced protection is simply a reflection of low numbers of memory CD8+ T cells present at the time of challenge. Data from Chapter 5 demonstrated that CD4+ T cells are important in conditioning APCs during the priming phase in order to maximize the magnitude of the CD8+ T cell response, but do not influence the ability of the CD8+ T cells to undergo secondary expansion. Nevertheless, we did observe modest defects in TNFα and granzyme B production in the unhelped CD8+ T cells. When the research was conducted we lacked the ability to assess IL-2 production by CD8+ T cells. If we had measured IL-2 production by unhelped CD8+ T cells, we may have seen further evidence of functional impairment. In other models it has been found that CD4+ T cell help results in epigenetic changes of the IFNγ, IL-2 and T-bet loci. It is currently unknown whether the defects in TNFα and granzyme B production in CD8+ T cells elicited by rHuAd5 are due to differences in epigenetic regulation. Furthermore, since polyfunctionality has recently been shown to be associated with protection from some infectious agents, it may be pertinent to determine if helpless CD8+ T cells generated by rAd immunization can produce Il-2. Understanding the precise interaction between CD4+ and CD8+ T cells will

be essential for optimizing the memory CD8+ T cell response produced by rAd vaccination.

Closing statement

Humans are in a never-ending battle against the infectious agents that surround us. There are numerous microbes for which our immune system is incapable of effectively controlling during a primary infection. The effects of these failures can have devastating consequences resulting in substantial loss of human life. The development of vaccines has saved countless lives and significantly improved our overall quality of life. However, it would appear that current vaccination technologies are unsafe or elicit inadequate immune responses to effectively combat several pathogens. Recombinant adenovirus vectors have shown great promise as vaccine platforms due to their ability to elicit potent adaptive immunity in preclinical studies. However, it is now fully evident that the formulation and deployment of rAd vaccines will require a better understanding of the nature of the T cell responses that they elicit. The research contained within this thesis evaluates both CD8+ T cell and CD4+ T cell responses generated by rAd immunization and will assist in the development of future rAd vaccines.

- Chapter 7 –

References

1. C A Janeway, P. T., M Walport, M Shlomchik. 2001. *Immunobiology 5: The Immune System in Health and Disease*. Garland, New York.
2. Hill, A. V. 2006. Pre-erythrocytic malaria vaccines: towards greater efficacy. *Nat Rev Immunol 6:21.*
3. Watkins, D. I., D. R. Burton, E. G. Kallas, J. P. Moore, and W. C. Koff. 2008. Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. *Nat Med 14:617.*
4. Radosevic, K., A. Rodriguez, A. Lemckert, and J. Goudsmit. 2009. Heterologous prime-boost vaccinations for poverty-related diseases: advantages and future prospects. *Expert Rev Vaccines 8:577.*
5. Robinson, H. L. 2007. HIV/AIDS vaccines: 2007. *Clin Pharmacol Ther 82:686.*
6. Martin, C. 2006. Tuberculosis vaccines: past, present and future. *Curr Opin Pulm Med 12:186.*
7. Relyveld, E. H., B. Bizzini, and R. K. Gupta. 1998. Rational approaches to reduce adverse reactions in man to vaccines containing tetanus and diphtheria toxoids. *Vaccine 16:1016.*
8. Hitchcock, W. P. 2006. Rationale for use of Tdap booster vaccines for adolescent immunization: overview of efficacy, safety, and clinical use. *Clin Pediatr (Phila) 45:785.*
9. Siegrist, C. A. 2007. Mechanisms underlying adverse reactions to vaccines. *J Comp Pathol 137 Suppl 1:S46.*
10. Gundlach, B. R., M. G. Lewis, S. Sopper, T. Schnell, J. Sodroski, C. Stahl-Hennig, and K. Uberla. 2000. Evidence for recombination of live, attenuated immunodeficiency virus vaccine with challenge virus to a more virulent strain. *J Virol 74:3537.*
11. Ruprecht, R. M. 1999. Live attenuated AIDS viruses as vaccines: promise or peril? *Immunol Rev 170:135.*
12. Catelli, E., M. Cecchinato, C. E. Savage, R. C. Jones, and C. J. Naylor. 2006. Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. *Vaccine 24:6476.*
13. Redfield, R. R., D. C. Wright, W. D. James, T. S. Jones, C. Brown, and D. S. Burke. 1987. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *N Engl J Med 316:673.*
14. Cooper, M. D., and M. N. Alder. 2006. The evolution of adaptive immune systems. *Cell 124:815.*
15. Mogensen, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev 22:240.*
16. Carroll, M. C. 2008. Complement and humoral immunity. *Vaccine 26 Suppl 8:I28.*
17. Brandtzaeg, P. 2007. Induction of secretory immunity and memory at mucosal surfaces. *Vaccine 25:5467.*
18. Manz, R. A., A. E. Hauser, F. Hiepe, and A. Radbruch. 2005. Maintenance of serum antibody levels. *Annu Rev Immunol 23:367.*

19. Shapiro-Shelef, M., and K. Calame. 2005. Regulation of plasma-cell development. *Nat Rev Immunol 5:230.*
20. Crotty, S., P. Felgner, H. Davies, J. Glidewell, L. Villarreal, and R. Ahmed. 2003. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol 171:4969.*
21. Ye, M., and T. Graf. 2007. Early decisions in lymphoid development. *Curr Opin Immunol 19:123.*
22. Maeda, T., T. Merghoub, R. M. Hobbs, L. Dong, M. Maeda, J. Zakrzewski, M. R. van den Brink, A. Zelent, H. Shigematsu, K. Akashi, J. Teruya-Feldstein, G. Cattoretti, and P. P. Pandolfi. 2007. Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF. *Science 316:860.*
23. Bassing, C. H., W. Swat, and F. W. Alt. 2002. The mechanism and regulation of chromosomal V(D)J recombination. *Cell 109 Suppl:S45.*
24. Welsh, R. M., L. K. Selin, and E. Szomolanyi-Tsuda. 2004. Immunological memory to viral infections. *Annu Rev Immunol 22:711.*
25. Pulendran, B., and R. Ahmed. 2006. Translating innate immunity into immunological memory: implications for vaccine development. *Cell 124:849.*
26. Plotkin, S. A. 2008. Vaccines: correlates of vaccine-induced immunity. *Clin Infect Dis 47:401.*
27. Harper, D. M., E. L. Franco, C. M. Wheeler, A. B. Moscicki, B. Romanowski, C. M. Roteli-Martins, D. Jenkins, A. Schuind, S. A. Costa Clemens, and G. Dubin. 2006. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet 367:1247.*
28. Lambert, P. H., M. Liu, and C. A. Siegrist. 2005. Can successful vaccines teach us how to induce efficient protective immune responses? *Nat Med 11:S54.*
29. Galazka, A. M., and S. E. Robertson. 1996. Immunization against diphtheria with special emphasis on immunization of adults. *Vaccine 14:845.*
30. Koutsky, L. A., K. A. Ault, C. M. Wheeler, D. R. Brown, E. Barr, F. B. Alvarez, L. M. Chiacchierini, and K. U. Jansen. 2002. A controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med 347:1645.*
31. Kanra, G., A. Kara, O. Demiralp, M. Contorni, A. K. Hilbert, C. Spyr, and S. Viviani. 2006. Safety and immunogenicity of a new fully liquid DTPw-HepB-Hib combination vaccine in infants. *Hum Vaccin 2:155.*
32. Dupont, J., J. Altclas, A. Lepetic, M. Lombardo, V. Vazquez, C. Salgueira, M. Seigelchifer, N. Arndtz, E. Antunez, K. von Eschen, and Z. Janowicz. 2006. A controlled clinical trial comparing the safety and immunogenicity of a new adjuvanted hepatitis B vaccine with a standard hepatitis B vaccine. *Vaccine 24:7167.*
33. Hammarlund, E., M. W. Lewis, S. G. Hansen, L. I. Strelow, J. A. Nelson, G. J. Sexton, J. M. Hanifin, and M. K. Slifka. 2003. Duration of antiviral immunity after smallpox vaccination. *Nat Med 9:1131.*

34. Jaye, A., A. F. Magnusen, A. D. Sadiq, T. Corrah, and H. C. Whittle. 1998. Ex vivo analysis of cytotoxic T lymphocytes to measles antigens during infection and after vaccination in Gambian children. *J Clin Invest 102:1969.*
35. McElhaney, J. E., D. Xie, W. D. Hager, M. B. Barry, Y. Wang, A. Kleppinger, C. Ewen, K. P. Kane, and R. C. Bleackley. 2006. T cell responses are better correlates of vaccine protection in the elderly. *J Immunol 176:6333.*
36. Hammarlund, E., M. W. Lewis, S. V. Carter, I. Amanna, S. G. Hansen, L. I. Strelow, S. W. Wong, P. Yoshihara, J. M. Hanifin, and M. K. Slifka. 2005. Multiple diagnostic techniques identify previously vaccinated individuals with protective immunity against monkeypox. *Nat Med 11:1005.*
37. Jaye, A., A. F. Magnusen, A. D. Sadiq, T. Corrah, and H. C. Whittle. 1998. Ex Vivo Analysis of Cytotoxic T Lymphocytes to Measles Antigens during Infection and after Vaccination in Gambian Children. *J. Clin. Invest. 102:1969.*
38. Nanan, R., A. Rauch, E. Kampgen, S. Niewiesk, and H. W. Kreth. 2000. A novel sensitive approach for frequency analysis of measles virus-specific memory T-lymphocytes in healthy adults with a childhood history of natural measles. *J Gen Virol 81:1313.*
39. Permar, S. R., S. A. Klumpp, K. G. Mansfield, A. A. Carville, D. A. Gorgone, M. A. Lifton, J. E. Schmitz, K. A. Reimann, F. P. Polack, D. E. Griffin, and N. L. Letvin. 2004. Limited contribution of humoral immunity to the clearance of measles viremia in rhesus monkeys. *J Infect Dis 190:998.*
40. Permar, S. R., S. A. Klumpp, K. G. Mansfield, W. K. Kim, D. A. Gorgone, M. A. Lifton, K. C. Williams, J. E. Schmitz, K. A. Reimann, M. K. Axthelm, F. P. Polack, D. E. Griffin, and N. L. Letvin. 2003. Role of CD8(+) lymphocytes in control and clearance of measles virus infection of rhesus monkeys. *J Virol 77:4396.*
41. Tishon, A., H. Lewicki, A. Andaya, D. McGavern, L. Martin, and M. B. Oldstone. 2006. CD4 T cell control primary measles virus infection of the CNS: regulation is dependent on combined activity with either CD8 T cells or with B cells: CD4, CD8 or B cells alone are ineffective. *Virology 347:234.*
42. Patterson, C. E., D. M. Lawrence, L. A. Echols, and G. F. Rall. 2002. Immune-mediated protection from measles virus-induced central nervous system disease is noncytolytic and gamma interferon dependent. *J Virol 76:4497.*
43. Harrington, L. E., R. Most Rv, J. L. Whitton, and R. Ahmed. 2002. Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. *J Virol 76:3329.*
44. Xu, R., A. J. Johnson, D. Liggitt, and M. J. Bevan. 2004. Cellular and humoral immunity against vaccinia virus infection of mice. *J Immunol 172:6265.*
45. Belyakov, I. M., P. Earl, A. Dzutsev, V. A. Kuznetsov, M. Lemon, L. S. Wyatt, J. T. Snyder, J. D. Ahlers, G. Franchini, B. Moss, and J. A. Berzofsky. 2003. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. *Proc Natl Acad Sci U S A 100:9458.*

46. Fang, M., and L. J. Sigal. 2005. Antibodies and CD8+ T cells are complementary and essential for natural resistance to a highly lethal cytopathic virus. *J Immunol 175:6829.*
47. Belshe, R. B., K. M. Edwards, T. Vesikari, S. V. Black, R. E. Walker, M. Hultquist, G. Kemble, and E. M. Connor. 2007. Live attenuated versus inactivated influenza vaccine in infants and young children. *N Engl J Med 356:685.*
48. Powell, T. J., T. Strutt, J. Reome, J. A. Hollenbaugh, A. D. Roberts, D. L. Woodland, S. L. Swain, and R. W. Dutton. 2007. Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against supralethal challenge with heterosubtypic virus. *J Immunol 178:1030.*
49. Arvin, A. 2005. Aging, immunity, and the varicella-zoster virus. *N Engl J Med 352:2266.*
50. Hata, A., H. Asanuma, M. Rinki, M. Sharp, R. M. Wong, K. Blume, and A. M. Arvin. 2002. Use of an inactivated varicella vaccine in recipients of hematopoietic-cell transplants. *N Engl J Med 347:26.*
51. Levin, M. J., D. Barber, E. Goldblatt, M. Jones, B. LaFleur, C. Chan, D. Stinson, G. O. Zerbe, and A. R. Hayward. 1998. Use of a live attenuated varicella vaccine to boost varicella-specific immune responses in seropositive people 55 years of age and older: duration of booster effect. *J Infect Dis 178 Suppl 1:S109.*
52. Bosselut, R. 2004. CD4/CD8-lineage differentiation in the thymus: from nuclear effectors to membrane signals. *Nat Rev Immunol 4:529.*
53. Florence, W. C., R. K. Bhat, and S. Joyce. 2008. CD1d-restricted glycolipid antigens: presentation principles, recognition logic and functional consequences. *Expert Rev Mol Med 10:e20.*
54. Godfrey, D. I., J. Rossjohn, and J. McCluskey. 2008. The fidelity, occasional promiscuity, and versatility of T cell receptor recognition. *Immunity 28:304.*
55. Purcell, A. W., and T. Elliott. 2008. Molecular machinations of the MHC-I peptide loading complex. *Curr Opin Immunol 20:75.*
56. Busch, R., C. H. Rinderknecht, S. Roh, A. W. Lee, J. J. Harding, T. Burster, T. M. Hornell, and E. D. Mellins. 2005. Achieving stability through editing and chaperoning: regulation of MHC class II peptide binding and expression. *Immunol Rev 207:242.*
57. Ciofani, M., and J. C. Zuniga-Pflucker. 2007. The thymus as an inductive site for T lymphopoiesis. *Annu Rev Cell Dev Biol 23:463.*
58. Weninger, W., M. A. Crowley, N. Manjunath, and U. H. von Andrian. 2001. Migratory properties of naive, effector, and memory CD8(+) T cells. *J Exp Med 194:953.*
59. Kapp, J. A., and R. P. Bucy. 2008. CD8+ suppressor T cells resurrected. *Hum Immunol 69:715.*
60. Shresta, S., C. T. Pham, D. A. Thomas, T. A. Graubert, and T. J. Ley. 1998. How do cytotoxic lymphocytes kill their targets? *Curr Opin Immunol 10:581.*
61. Shen, D. T., J. S. Ma, J. Mather, S. Vukmanovic, and S. Radoja. 2006. Activation of primary T lymphocytes results in lysosome development and polarized granule

exocytosis in CD4+ and CD8+ subsets, whereas expression of lytic molecules confers cytotoxicity to CD8+ T cells. *J Leukoc Biol 80:827.*

62. Ma, J. S., N. Monu, D. T. Shen, I. Mecklenbrauker, N. Radoja, T. F. Haydar, M. Leitges, A. B. Frey, S. Vukmanovic, and S. Radoja. 2007. Protein kinase Cdelta regulates antigen receptor-induced lytic granule polarization in mouse CD8+ CTL. *J Immunol 178:7814.*
63. Pipkin, M. E., and J. Lieberman. 2007. Delivering the kiss of death: progress on understanding how perforin works. *Curr Opin Immunol 19:301.*
64. Salvesen, G. S., and S. J. Riedl. 2008. Caspase mechanisms. *Adv Exp Med Biol 615:13.*
65. Scott, F. L., B. Stec, C. Pop, M. K. Dobaczewska, J. J. Lee, E. Monosov, H. Robinson, G. S. Salvesen, R. Schwarzenbacher, and S. J. Riedl. 2009. The Fas-FADD death domain complex structure unravels signalling by receptor clustering. *Nature 457:1019.*
66. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu Rev Immunol 15:749.*
67. Varfolomeev, E. E., and A. Ashkenazi. 2004. Tumor necrosis factor: an apoptosis JuNKie? *Cell 116:491.*
68. Williams, M. A., A. J. Tyznik, and M. J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature 441:890.*
69. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood 112:1557.*
70. Shevach, E. M. 2006. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity 25:195.*
71. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol 136:2348.*
72. Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley. 1989. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp Med 169:59.*
73. Singh, V. K., S. Mehrotra, and S. S. Agarwal. 1999. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol Res 20:147.*
74. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol 6:1133.*
75. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol 6:1123.*
76. Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol 27:485.*

77. Kirkham, B. W., M. N. Lassere, J. P. Edmonds, K. M. Juhasz, P. A. Bird, C. S. Lee, R. Shnier, and I. J. Portek. 2006. Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). *Arthritis Rheum 54:1122.*
78. Matusevicius, D., P. Kivisakk, B. He, N. Kostulas, V. Ozenci, S. Fredrikson, and H. Link. 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler 5:101.*
79. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol 22:531.*
80. Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science 265:1237.*
81. Jankovic, D., M. C. Kullberg, C. G. Feng, R. S. Goldszmid, C. M. Collazo, M. Wilson, T. A. Wynn, M. Kamanaka, R. A. Flavell, and A. Sher. 2007. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med 204:273.*
82. Brown, D. M., A. M. Dilzer, D. L. Meents, and S. L. Swain. 2006. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. *J Immunol 177:2888.*
83. Brown, D. M., C. Kamperschroer, A. M. Dilzer, D. M. Roberts, and S. L. Swain. 2009. IL-2 and antigen dose differentially regulate perforin- and FasL-mediated cytolytic activity in antigen specific CD4+ T cells. *Cell Immunol 257:69.*
84. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation. *Annu Rev Immunol 25:171.*
85. Butz, E. A., and M. J. Bevan. 1998. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity 8:167.*
86. Whitmire, J. K., N. Benning, and J. L. Whitton. 2006. Precursor frequency, nonlinear proliferation, and functional maturation of virus-specific CD4+ T cells. *J Immunol 176:3028.*
87. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity 8:177.*
88. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity 29:650.*
89. Vezys, V., D. Masopust, C. C. Kemball, D. L. Barber, L. A. O'Mara, C. P. Larsen, T. C. Pearson, R. Ahmed, and A. E. Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J Exp Med 203:2263.*
90. Yang, T. C., J. Millar, T. Groves, N. Grinshtein, R. Parsons, S. Takenaka, Y. Wan, and J. L. Bramson. 2006. The CD8+ T cell population elicited by recombinant adenovirus displays a novel partially exhausted phenotype associated with

prolonged antigen presentation that nonetheless provides long-term immunity. *J Immunol 176:200.*

91. Prlic, M., M. A. Williams, and M. J. Bevan. 2007. Requirements for CD8 T-cell priming, memory generation and maintenance. *Curr Opin Immunol 19:315.*
92. Novak, N., and T. Bieber. 2008. 2. Dendritic cells as regulators of immunity and tolerance. *J Allergy Clin Immunol 121:S370.*
93. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol 2:151.*
94. Villadangos, J. A., and L. Young. 2008. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity 29:352.*
95. Belz, G. T., C. M. Smith, L. Kleinert, P. Reading, A. Brooks, K. Shortman, F. R. Carbone, and W. R. Heath. 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci U S A 101:8670.*
96. Belz, G. T., C. M. Smith, D. Eichner, K. Shortman, G. Karupiah, F. R. Carbone, and W. R. Heath. 2004. Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses. *J Immunol 172:1996.*
97. Smith, C. M., G. T. Belz, N. S. Wilson, J. A. Villadangos, K. Shortman, F. R. Carbone, and W. R. Heath. 2003. Cutting edge: conventional CD8 alpha+ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1. *J Immunol 170:4437.*
98. Probst, H. C., and M. van den Broek. 2005. Priming of CTLs by lymphocytic choriomeningitis virus depends on dendritic cells. *J Immunol 174:3920.*
99. Zammit, D. J., L. S. Cauley, Q. M. Pham, and L. Lefrancois. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity 22:561.*
100. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E. G. Pamer, D. R. Littman, and R. A. Lang. 2002. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity 17:211.*
101. Villadangos, J. A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol 7:543.*
102. Dudziak, D., A. O. Kamphorst, G. F. Heidkamp, V. R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H. W. Lee, C. G. Park, R. M. Steinman, and M. C. Nussenzweig. 2007. Differential antigen processing by dendritic cell subsets in vivo. *Science 315:107.*
103. Belz, G. T., K. Shortman, M. J. Bevan, and W. R. Heath. 2005. CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J Immunol 175:196.*
104. Maroof, A., and P. M. Kaye. 2008. Temporal regulation of interleukin-12p70 (IL-12p70) and IL-12-related cytokines in splenic dendritic cell subsets during Leishmania donovani infection. *Infect Immun 76:239.*
105. Edwards, A. D., S. S. Diebold, E. M. Slack, H. Tomizawa, H. Hemmi, T. Kaisho, S. Akira, and C. Reis e Sousa. 2003. Toll-like receptor expression in murine DC

subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. *Eur J Immunol 33:827.*

106. Fallarino, F., U. Grohmann, C. Vacca, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CD40 ligand and CTLA-4 are reciprocally regulated in the Th1 cell proliferative response sustained by CD8(+) dendritic cells. *J Immunol 169:1182.*
107. Mempel, T. R., S. E. Henrickson, and U. H. Von Andrian. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature 427:154.*
108. Miller, M. J., O. Safrina, I. Parker, and M. D. Cahalan. 2004. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med 200:847.*
109. Zheng, H., B. Jin, S. E. Henrickson, A. S. Perelson, U. H. von Andrian, and A. K. Chakraborty. 2008. How antigen quantity and quality determine T-cell decisions in lymphoid tissue. *Mol Cell Biol 28:4040.*
110. Henrickson, S. E., T. R. Mempel, I. B. Mazo, B. Liu, M. N. Artyomov, H. Zheng, A. Peixoto, M. P. Flynn, B. Senman, T. Junt, H. C. Wong, A. K. Chakraborty, and U. H. von Andrian. 2008. T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat Immunol 9:282.*
111. Blattman, J. N., R. Antia, D. J. Sourdive, X. Wang, S. M. Kaech, K. Murali-Krishna, J. D. Altman, and R. Ahmed. 2002. Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J Exp Med 195:657.*
112. van Stipdonk, M. J., G. Hardenberg, M. S. Bijker, E. E. Lemmens, N. M. Droin, D. R. Green, and S. P. Schoenberger. 2003. Dynamic programming of CD8+ T lymphocyte responses. *Nat Immunol 4:361.*
113. Corbin, G. A., and J. T. Harty. 2004. Duration of infection and antigen display have minimal influence on the kinetics of the CD4+ T cell response to Listeria monocytogenes infection. *J Immunol 173:5679.*
114. Mercado, R., S. Vijh, S. E. Allen, K. Kerksiek, I. M. Pilip, and E. G. Pamer. 2000. Early programming of T cell populations responding to bacterial infection. *J Immunol 165:6833.*
115. Kaech, S. M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol 2:415.*
116. Wong, P., and E. G. Pamer. 2001. Cutting edge: antigen-independent CD8 T cell proliferation. *J Immunol 166:5864.*
117. van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger. 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol 2:423.*
118. Prlic, M., G. Hernandez-Hoyos, and M. J. Bevan. 2006. Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. *J Exp Med 203:2135.*
119. Tewari, K., J. Walent, J. Svaren, R. Zamoyska, and M. Suresh. 2006. Differential requirement for Lck during primary and memory CD8+ T cell responses. *Proc Natl Acad Sci U S A 103:16388.*

120. North, R. J., P. A. Berche, and M. F. Newborg. 1981. Immunologic consequences of antibiotic-induced abridgement of bacterial infection: effect on generation and loss of protective T cells and level of immunologic memory. *J Immunol 127:342.*
121. Williams, M. E., J. J. Densmore, A. W. Pawluczkowycz, P. V. Beum, A. D. Kennedy, M. A. Lindorfer, S. H. Hamil, J. C. Eggleton, and R. P. Taylor. 2006. Thrice-weekly low-dose rituximab decreases CD20 loss via shaving and promotes enhanced targeting in chronic lymphocytic leukemia. *J Immunol 177:7435.*
122. Tseng, K. E., C. Y. Chung, S. H'Ng W, and S. L. Wang. 2009. Early infection termination affects number of CD8+ memory T cells and protective capacities in listeria monocytogenes-infected mice upon rechallenge. *J Immunol 182:4590.*
123. Williams, M. A., and M. J. Bevan. 2004. Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells. *J Immunol 173:6694.*
124. Catron, D. M., L. K. Rusch, J. Hataye, A. A. Itano, and M. K. Jenkins. 2006. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. *J Exp Med 203:1045.*
125. D'Souza, W. N., and S. M. Hedrick. 2006. Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. *J Immunol 177:777.*
126. Jelley-Gibbs, D. M., D. M. Brown, J. P. Dibble, L. Haynes, S. M. Eaton, and S. L. Swain. 2005. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J Exp Med 202:697.*
127. Haring, J. S., V. P. Badovinac, and J. T. Harty. 2006. Inflaming the CD8+ T cell response. *Immunity 25:19.*
128. Harty, J. T., and V. P. Badovinac. 2008. Shaping and reshaping CD8+ T-cell memory. *Nat Rev Immunol 8:107.*
129. Appay, V., D. C. Douek, and D. A. Price. 2008. CD8+ T cell efficacy in vaccination and disease. *Nat Med 14:623.*
130. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708.*
131. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science 291:2413.*
132. Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell, and M. K. Jenkins. 2001. Visualizing the generation of memory CD4 T cells in the whole body. *Nature 410:101.*
133. Bjorkdahl, O., K. A. Barber, S. J. Brett, M. G. Daly, C. Plumpton, N. A. Elshourbagy, J. P. Tite, and L. L. Thomsen. 2003. Characterization of CC-chemokine receptor 7 expression on murine T cells in lymphoid tissues. *Immunology 110:170.*
134. Jabbari, A., and J. T. Harty. 2006. Simultaneous assessment of antigen-stimulated cytokine production and memory subset composition of memory CD8 T cells. *J Immunol Methods 313:161.*

135. Galkina, E., K. Tanousis, G. Preece, M. Tolaini, D. Kioussis, O. Florey, D. O. Haskard, T. F. Tedder, and A. Ager. 2003. L-selectin shedding does not regulate constitutive T cell trafficking but controls the migration pathways of antigen-activated T lymphocytes. *J Exp Med 198:1323.*
136. Lefrancois, L., and A. L. Marzo. 2006. The descent of memory T-cell subsets. *Nat Rev Immunol 6:618.*
137. Huster, K. M., V. Busch, M. Schiemann, K. Linkemann, K. M. Kerksiek, H. Wagner, and D. H. Busch. 2004. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A 101:5610.*
138. Schluns, K. S., and L. Lefrancois. 2003. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol 3:269.*
139. Jameson, S. C. 2002. Maintaining the norm: T-cell homeostasis. *Nat Rev Immunol 2:547.*
140. Surh, C. D., O. Boyman, J. F. Purton, and J. Sprent. 2006. Homeostasis of memory T cells. *Immunol Rev 211:154.*
141. Bachmann, M. F., P. Wolint, K. Schwarz, P. Jager, and A. Oxenius. 2005. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol 175:4686.*
142. Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol 4:1191.*
143. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol 4:225.*
144. Lacombe, M. H., M. P. Hardy, J. Rooney, and N. Labrecque. 2005. IL-7 receptor expression levels do not identify CD8+ memory T lymphocyte precursors following peptide immunization. *J Immunol 175:4400.*
145. Bachmann, M. F., P. Wolint, K. Schwarz, and A. Oxenius. 2005. Recall proliferation potential of memory CD8+ T cells and antiviral protection. *J Immunol 175:4677.*
146. Ely, K. H., A. D. Roberts, and D. L. Woodland. 2003. Cutting edge: effector memory CD8+ T cells in the lung airways retain the potential to mediate recall responses. *J Immunol 171:3338.*
147. Roberts, A. D., and D. L. Woodland. 2004. Cutting edge: effector memory CD8+ T cells play a prominent role in recall responses to secondary viral infection in the lung. *J Immunol 172:6533.*
148. Roberts, A. D., K. H. Ely, and D. L. Woodland. 2005. Differential contributions of central and effector memory T cells to recall responses. *J Exp Med 202:123.*
149. Jackson, S. S., J. E. Schmitz, M. J. Kuroda, P. F. McKay, S. M. Sumida, K. L. Martin, F. Yu, M. A. Lifton, D. A. Gorgone, and N. L. Letvin. 2005. Evaluation of CD62L expression as a marker for vaccine-elicited memory cytotoxic T lymphocytes. *Immunology 116:443.*

150. Freitas, A. A., and B. Rocha. 2000. Population biology of lymphocytes: the flight for survival. *Annu Rev Immunol 18:83.*
151. Marzo, A. L., K. D. Klonowski, A. Le Bon, P. Borrow, D. F. Tough, and L. Lefrancois. 2005. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol 6:793.*
152. Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science 272:54.*
153. Masopust, D., S. J. Ha, V. Vezys, and R. Ahmed. 2006. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J Immunol 177:831.*
154. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity 27:670.*
155. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol 77:4911.*
156. Chang, J. T., V. R. Palanivel, I. Kinjyo, F. Schambach, A. M. Intlekofer, A. Banerjee, S. A. Longworth, K. E. Vinup, P. Mrass, J. Oliaro, N. Killeen, J. S. Orange, S. M. Russell, W. Weninger, and S. L. Reiner. 2007. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science 315:1687.*
157. Farber, D. L. 1998. Differential TCR signaling and the generation of memory T cells. *J Immunol 160:535.*
158. Sallusto, F., and A. Lanzavecchia. 2001. Exploring pathways for memory T cell generation. *J Clin Invest 108:805.*
159. Stemberger, C., K. M. Huster, M. Koffler, F. Anderl, M. Schiemann, H. Wagner, and D. H. Busch. 2007. A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. *Immunity 27:985.*
160. Teixeiro, E., M. A. Daniels, S. E. Hamilton, A. G. Schrum, R. Bragado, S. C. Jameson, and E. Palmer. 2009. Different T cell receptor signals determine CD8+ memory versus effector development. *Science 323:502.*
161. Kaech, S. M., and E. J. Wherry. 2007. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity 27:393.*
162. Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin, and S. M. Kaech. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity 27:281.*
163. Gett, A. V., F. Sallusto, A. Lanzavecchia, and J. Geginat. 2003. T cell fitness determined by signal strength. *Nat Immunol 4:355.*
164. Bannard, O., M. Kraman, and D. T. Fearon. 2009. Secondary replicative function of CD8+ T cells that had developed an effector phenotype. *Science 323:505.*

165. Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A 101:16004.*
166. Radziewicz, H., C. C. Ibegbu, M. L. Fernandez, K. A. Workowski, K. Obideen, M. Wehbi, H. L. Hanson, J. P. Steinberg, D. Masopust, E. J. Wherry, J. D. Altman, B. T. Rouse, G. J. Freeman, R. Ahmed, and A. Grakoui. 2007. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol 81:2545.*
167. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature 443:350.*
168. Trautmann, L., L. Janbazian, N. Chomont, E. A. Said, S. Gimmig, B. Bessette, M. R. Boulassel, E. Delwart, H. Sepulveda, R. S. Balderas, J. P. Routy, E. K. Haddad, and R. P. Sekaly. 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med 12:1198.*
169. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med 188:2205.*
170. Usherwood, E. J., D. J. Roy, K. Ward, S. L. Surman, B. M. Dutia, M. A. Blackman, J. P. Stewart, and D. L. Woodland. 2000. Control of gammaherpesvirus latency by latent antigen-specific CD8(+) T cells. *J Exp Med 192:943.*
171. Byers, A. M., C. C. Kemball, J. M. Moser, and A. E. Lukacher. 2003. Cutting edge: rapid in vivo CTL activity by polyoma virus-specific effector and memory CD8+ T cells. *J Immunol 171:17.*
172. Polic, B., H. Hengel, A. Krmpotic, J. Trgovcich, I. Pavic, P. Luccaronin, S. Jonjic, and U. H. Koszinowski. 1998. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. *J Exp Med 188:1047.*
173. Zammit, D. J., D. L. Turner, K. D. Klonowski, L. Lefrancois, and L. S. Cauley. 2006. Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity 24:439.*
174. Tatsis, N., J. C. Fitzgerald, A. Reyes-Sandoval, K. C. Harris-McCoy, S. E. Hensley, D. Zhou, S. W. Lin, A. Bian, Z. Q. Xiang, A. Iparraguirre, C. Lopez-Camacho, E. J. Wherry, and H. C. Ertl. 2007. Adenoviral vectors persist in vivo and maintain activated CD8+ T cells: implications for their use as vaccines. *Blood 110:1916.*
175. Turner, D. L., L. S. Cauley, K. M. Khanna, and L. Lefrancois. 2007. Persistent antigen presentation after acute vesicular stomatitis virus infection. *J Virol 81:2039.*

176. Khanolkar, A., V. P. Badovinac, and J. T. Harty. 2007. CD8 T cell memory development: CD4 T cell help is appreciated. *Immunol Res 39:94.*
177. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature 421:852.*
178. Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science 300:337.*
179. Sun, J. C., M. A. Williams, and M. J. Bevan. 2004. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol 5:927.*
180. Sun, J. C., and M. J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science 300:339.*
181. Grakoui, A., N. H. Shoukry, D. J. Woollard, J. H. Han, H. L. Hanson, J. Ghrayeb, K. K. Murthy, C. M. Rice, and C. M. Walker. 2003. HCV persistence and immune evasion in the absence of memory T cell help. *Science 302:659.*
182. Agnellini, P., M. Wiesel, K. Schwarz, P. Wolint, M. F. Bachmann, and A. Oxenius. 2008. Kinetic and mechanistic requirements for helping CD8 T cells. *J Immunol 180:1517.*
183. Novy, P., M. Quigley, X. Huang, and Y. Yang. 2007. CD4 T cells are required for CD8 T cell survival during both primary and memory recall responses. *J Immunol 179:8243.*
184. Marzo, A. L., V. Vezys, K. D. Klonowski, S. J. Lee, G. Muralimohan, M. Moore, D. F. Tough, and L. Lefrancois. 2004. Fully functional memory CD8 T cells in the absence of CD4 T cells. *J Immunol 173:969.*
185. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature 393:474.*
186. Belz, G. T., D. Wodarz, G. Diaz, M. A. Nowak, and P. C. Doherty. 2002. Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. *J Virol 76:12388.*
187. Bennett, S. R., F. R. Carbone, F. Karamalis, J. F. Miller, and W. R. Heath. 1997. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J Exp Med 186:65.*
188. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature 393:480.*
189. Wang, J. C., and A. M. Livingstone. 2003. Cutting edge: CD4+ T cell help can be essential for primary CD8+ T cell responses in vivo. *J Immunol 171:6339.*
190. Hernandez, M. G., L. Shen, and K. L. Rock. 2007. CD40-CD40 ligand interaction between dendritic cells and CD8+ T cells is needed to stimulate maximal T cell responses in the absence of CD4+ T cell help. *J Immunol 178:2844.*
191. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478.*

192. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science 297:2060.*
193. Lee, B. O., L. Hartson, and T. D. Randall. 2003. CD40-deficient, influenza-specific CD8 memory T cells develop and function normally in a CD40-sufficient environment. *J Exp Med 198:1759.*
194. Riberdy, J. M., J. P. Christensen, K. Branum, and P. C. Doherty. 2000. Diminished primary and secondary influenza virus-specific CD8(+) T-cell responses in CD4-depleted Ig(-/-) mice. *J Virol 74:9762.*
195. Shedlock, D. J., J. K. Whitmire, J. Tan, A. S. MacDonald, R. Ahmed, and H. Shen. 2003. Role of CD4 T cell help and costimulation in CD8 T cell responses during Listeria monocytogenes infection. *J Immunol 170:2053.*
196. Hamilton, S. E., A. R. Tvinnereim, and J. T. Harty. 2001. Listeria monocytogenes infection overcomes the requirement for CD40 ligand in exogenous antigen presentation to CD8(+) T cells. *J Immunol 167:5603.*
197. Sun, J. C., and M. J. Bevan. 2004. Cutting edge: long-lived CD8 memory and protective immunity in the absence of CD40 expression on CD8 T cells. *J Immunol 172:3385.*
198. Whitmire, J. K., R. A. Flavell, I. S. Grewal, C. P. Larsen, T. C. Pearson, and R. Ahmed. 1999. CD40-CD40 ligand costimulation is required for generating antiviral CD4 T cell responses but is dispensable for CD8 T cell responses. *J Immunol 163:3194.*
199. Hertz, C. J., S. M. Kiertscher, P. J. Godowski, D. A. Bouis, M. V. Norgard, M. D. Roth, and R. L. Modlin. 2001. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J Immunol 166:2444.*
200. Cho, H. J., K. Takabayashi, P. M. Cheng, M. D. Nguyen, M. Corr, S. Tuck, and E. Raz. 2000. Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat Biotechnol 18:509.*
201. Le Bon, A., N. Etchart, C. Rossmann, M. Ashton, S. Hou, D. Gewert, P. Borrow, and D. F. Tough. 2003. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat Immunol 4:1009.*
202. Mintern, J. D., G. M. Davey, G. T. Belz, F. R. Carbone, and W. R. Heath. 2002. Cutting edge: precursor frequency affects the helper dependence of cytotoxic T cells. *J Immunol 168:977.*
203. Badovinac, V. P., K. A. Messingham, T. S. Griffith, and J. T. Harty. 2006. TRAIL deficiency delays, but does not prevent, erosion in the quality of "helpless" memory CD8 T cells. *J Immunol 177:999.*
204. Fuse, S., C. Y. Tsai, M. J. Molloy, S. R. Allie, W. Zhang, H. Yagita, and E. J. Usherwood. 2009. Recall responses by helpless memory CD8+ T cells are restricted by the up-regulation of PD-1. *J Immunol 182:4244.*
205. Intlekofer, A. M., N. Takemoto, C. Kao, A. Banerjee, F. Schambach, J. K. Northrop, H. Shen, E. J. Wherry, and S. L. Reiner. 2007. Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J Exp Med 204:2015.*

206. Janssen, E. M., N. M. Droin, E. E. Lemmens, M. J. Pinkoski, S. J. Bensinger, B. D. Ehst, T. S. Griffith, D. R. Green, and S. P. Schoenberger. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature 434:88.*
207. Slifka, M. K., and J. L. Whitton. 2000. Activated and memory CD8+ T cells can be distinguished by their cytokine profiles and phenotypic markers. *J Immunol 164:208.*
208. Khanolkar, A., M. J. Fuller, and A. J. Zajac. 2004. CD4 T cell-dependent CD8 T cell maturation. *J Immunol 172:2834.*
209. Northrop, J. K., R. M. Thomas, A. D. Wells, and H. Shen. 2006. Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells. *J Immunol 177:1062.*
210. Sacks, J. A., and M. J. Bevan. 2008. TRAIL deficiency does not rescue impaired CD8+ T cell memory generated in the absence of CD4+ T cell help. *J Immunol 180:4570.*
211. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature 439:682.*
212. Belz, G. T., N. S. Wilson, C. M. Smith, A. M. Mount, F. R. Carbone, and W. R. Heath. 2006. Bone marrow-derived cells expand memory CD8+ T cells in response to viral infections of the lung and skin. *Eur J Immunol 36:327.*
213. Lauterbach, H., E. I. Zuniga, P. Truong, M. B. Oldstone, and D. B. McGavern. 2006. Adoptive immunotherapy induces CNS dendritic cell recruitment and antigen presentation during clearance of a persistent viral infection. *J Exp Med 203:1963.*
214. Wong, P., and E. G. Pamer. 2003. CD8 T cell responses to infectious pathogens. *Annu Rev Immunol 21:29.*
215. Heller, K. N., C. Gurer, and C. Munz. 2006. Virus-specific CD4+ T cells: ready for direct attack. *J Exp Med 203:805.*
216. Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med 203:2865.*
217. Jellison, E. R., S. K. Kim, and R. M. Welsh. 2005. Cutting edge: MHC class II-restricted killing in vivo during viral infection. *J Immunol 174:614.*
218. Demachi-Okamura, A., Y. Ito, Y. Akatsuka, K. Tsujimura, Y. Morishima, T. Takahashi, and K. Kuzushima. 2008. Epstein-Barr virus nuclear antigen 1-specific CD4+ T cells directly kill Epstein-Barr virus-carrying natural killer and T cells. *Cancer Sci 99:1633.*
219. Woodland, D. L., and R. W. Dutton. 2003. Heterogeneity of CD4(+) and CD8(+) T cells. *Curr Opin Immunol 15:336.*
220. Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M.

Roederer, and R. A. Koup. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood 107:4781.*

221. Darrah, P. A., D. T. Patel, P. M. De Luca, R. W. Lindsay, D. F. Davey, B. J. Flynn, S. T. Hoff, P. Andersen, S. G. Reed, S. L. Morris, M. Roederer, and R. A. Seder. 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med 13:843.*
222. Precopio, M. L., M. R. Betts, J. Parrino, D. A. Price, E. Gostick, D. R. Ambrozak, T. E. Asher, D. C. Douek, A. Harari, G. Pantaleo, R. Bailer, B. S. Graham, M. Roederer, and R. A. Koup. 2007. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med 204:1405.*
223. Wille-Reece, U., B. J. Flynn, K. Lore, R. A. Koup, R. M. Kedl, J. J. Mattapallil, W. R. Weiss, M. Roederer, and R. A. Seder. 2005. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proc Natl Acad Sci U S A 102:15190.*
224. Kannanganat, S., C. Ibegbu, L. Chennareddi, H. L. Robinson, and R. R. Amara. 2007. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *J Virol 81:8468.*
225. Tilton, J. C., M. R. Luskin, A. J. Johnson, M. Manion, C. W. Hallahan, J. A. Metcalf, M. McLaughlin, R. T. Davey, Jr., and M. Connors. 2007. Changes in paracrine interleukin-2 requirement, CCR7 expression, frequency, and cytokine secretion of human immunodeficiency virus-specific CD4+ T cells are a consequence of antigen load. *J Virol 81:2713.*
226. Chavez-Galan, L., M. C. Arenas-Del Angel, E. Zenteno, R. Chavez, and R. Lascurain. 2009. Cell death mechanisms induced by cytotoxic lymphocytes. *Cell Mol Immunol 6:15.*
227. Alcami, A., and U. H. Koszinowski. 2000. Viral mechanisms of immune evasion. *Immunol Today 21:447.*
228. Corbin, G. A., and J. T. Harty. 2005. T cells undergo rapid ON/OFF but not ON/OFF/ON cycling of cytokine production in response to antigen. *J Immunol 174:718.*
229. Slifka, M. K., F. Rodriguez, and J. L. Whitton. 1999. Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells. *Nature 401:76.*
230. Slifka, M. K., and J. L. Whitton. 2000. Antigen-specific regulation of T cell-mediated cytokine production. *Immunity 12:451.*
231. Bogdan, C., H. Moll, W. Solbach, and M. Rollinghoff. 1990. Tumor necrosis factor-alpha in combination with interferon-gamma, but not with interleukin 4 activates murine macrophages for elimination of Leishmania major amastigotes. *Eur J Immunol 20:1131.*
232. Liew, F. Y., Y. Li, and S. Millott. 1990. Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide. *J Immunol 145:4306.*

233. Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom. 1992. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med 175:1111.*
234. D'Souza, W. N., and L. Lefrancois. 2003. IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. *J Immunol 171:5727.*
235. Boyman, O., M. Kovar, M. P. Rubinstein, C. D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science 311:1924.*
236. Belz, G. T., W. Xie, and P. C. Doherty. 2001. Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. *J Immunol 166:4627.*
237. Roman, E., E. Miller, A. Harmsen, J. Wiley, U. H. Von Andrian, G. Huston, and S. L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J Exp Med 196:957.*
238. Freeman, M. M., and H. K. Ziegler. 2005. Simultaneous Th1-type cytokine expression is a signature of peritoneal CD4+ lymphocytes responding to infection with Listeria monocytogenes. *J Immunol 175:394.*
239. Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol 75:11983.*
240. Gea-Banacloche, J. C., S. A. Migueles, L. Martino, W. L. Shupert, A. C. McNeil, M. S. Sabbaghian, L. Ehler, C. Prussin, R. Stevens, L. Lambert, J. Altman, C. W. Hallahan, J. C. de Quiros, and M. Connors. 2000. Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. *J Immunol 165:1082.*
241. Schellens, I. M., J. A. Borghans, C. A. Jansen, I. M. De Cuyper, R. B. Geskus, D. van Baarle, and F. Miedema. 2008. Abundance of early functional HIV-specific CD8+ T cells does not predict AIDS-free survival time. *PLoS One 3:e2745.*
242. Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol 3:1061.*
243. Freeman, G. J., E. J. Wherry, R. Ahmed, and A. H. Sharpe. 2006. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J Exp Med 203:2223.*
244. Ciuffreda, D., D. Comte, M. Cavassini, E. Giostra, L. Buhler, M. Perruchoud, M. H. Heim, M. Battegay, D. Genne, B. Mulhaupt, R. Malinverni, C. Oneta, E. Bernasconi, M. Monnat, A. Cerny, C. Chuard, J. Borovicka, G. Mentha, M. Pascual, J. J. Gonvers, G. Pantaleo, and V. Dutoit. 2008. Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication. *Eur J Immunol 38:2665.*

245. Harari, A., S. Petitpierre, F. Vallelian, and G. Pantaleo. 2004. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood 103:966.*
246. Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J Exp Med 198:1909.*
247. Boaz, M. J., A. Waters, S. Murad, P. J. Easterbrook, and A. Vyakarnam. 2002. Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. *J Immunol 169:6376.*
248. Aagaard, C., J. Dietrich, M. Doherty, and P. Andersen. 2009. TB vaccines: current status and future perspectives. *Immunol Cell Biol 87:279.*
249. Flynn, N. M., D. N. Forthal, C. D. Harro, F. N. Judson, K. H. Mayer, and M. F. Para. 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis 191:654.*
250. Gilbert, P. B., M. L. Peterson, D. Follmann, M. G. Hudgens, D. P. Francis, M. Gurwith, W. L. Heyward, D. V. Jobes, V. Popovic, S. G. Self, F. Sinangil, D. Burke, and P. W. Berman. 2005. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. *J Infect Dis 191:666.*
251. Bojang, K. A., P. J. Milligan, M. Pinder, L. Vigneron, A. Alloueche, K. E. Kester, W. R. Ballou, D. J. Conway, W. H. Reece, P. Gothard, L. Yamuah, M. Delchambre, G. Voss, B. M. Greenwood, A. Hill, K. P. McAdam, N. Tornieporth, J. D. Cohen, and T. Doherty. 2001. Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. *Lancet 358:1927.*
252. Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw. 2003. Antibody neutralization and escape by HIV-1. *Nature 422:307.*
253. Pantophlet, R., and D. R. Burton. 2006. GP120: target for neutralizing HIV-1 antibodies. *Annu Rev Immunol 24:739.*
254. Baruch, D. I., B. Gamain, J. W. Barnwell, J. S. Sullivan, A. Stowers, G. G. Galland, L. H. Miller, and W. E. Collins. 2002. Immunization of Aotus monkeys with a functional domain of the Plasmodium falciparum variant antigen induces protection against a lethal parasite line. *Proc Natl Acad Sci U S A 99:3860.*
255. Bull, P. C., B. S. Lowe, M. Kortok, C. S. Molyneux, C. I. Newbold, and K. Marsh. 1998. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat Med 4:358.*
256. Scherf, A., J. J. Lopez-Rubio, and L. Riviere. 2008. Antigenic variation in Plasmodium falciparum. *Annu Rev Microbiol 62:445.*

257. Doherty, P. C., S. J. Turner, R. G. Webby, and P. G. Thomas. 2006. Influenza and the challenge for immunology. *Nat Immunol 7:449.*
258. Naniche, D., M. Garenne, C. Rae, M. Manchester, R. Buchta, S. K. Brodine, and M. B. Oldstone. 2004. Decrease in measles virus-specific CD4 T cell memory in vaccinated subjects. *J Infect Dis 190:1387.*
259. Yang, T. C., J. B. Millar, N. Grinshtein, J. Bassett, J. Finn, and J. L. Bramson. 2007. T-cell immunity generated by recombinant adenovirus vaccines. *Expert Rev Vaccines 6:347.*
260. Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott, and T. G. Ward. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med 84:570.*
261. Hilleman, M. R., and J. H. Werner. 1954. Recovery of new agent from patients with acute respiratory illness. *Proc Soc Exp Biol Med 85:183.*
262. Berk, A. J. 2007. 63 Adenoviridae: The virus and their replication. In *Fields Virology*. Wolters kluwer/Lippincott Williams & Wilkins, p. 2355.
263. Russell, W. C. 2009. Adenoviruses: update on structure and function. *J Gen Virol 90:1.*
264. Bangari, D. S., and S. K. Mittal. 2006. Development of nonhuman adenoviruses as vaccine vectors. *Vaccine 24:849.*
265. Ghosh-Choudhury, G., Y. Haj-Ahmad, and F. L. Graham. 1987. Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *Embo J 6:1733.*
266. Ng, P., R. J. Parks, D. T. Cummings, C. M. Evelegh, and F. L. Graham. 2000. An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. *Hum Gene Ther 11:693.*
267. Russell, W. C. 2000. Update on adenovirus and its vectors. *J Gen Virol 81:2573.*
268. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol 36:59.*
269. Fallaux, F. J., A. Bout, I. van der Velde, D. J. van den Wollenberg, K. M. Hehir, J. Keegan, C. Auger, S. J. Cramer, H. van Ormondt, A. J. van der Eb, D. Valerio, and R. C. Hoeben. 1998. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther 9:1909.*
270. Yang, Y., Q. Li, H. C. Ertl, and J. M. Wilson. 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol 69:2004.*
271. Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gonczol, and J. M. Wilson. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A 91:4407.*
272. Hoelscher, M. A., S. Garg, D. S. Bangari, J. A. Belser, X. Lu, I. Stephenson, R. A. Bright, J. M. Katz, S. K. Mittal, and S. Sambhara. 2006. Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. *Lancet 367:475.*

273. Wang, J., L. Thorson, R. W. Stokes, M. Santosuosso, K. Huygen, A. Zganiacz, M. Hitt, and Z. Xing. 2004. Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol 173:6357.*
274. Folgori, A., S. Capone, L. Ruggeri, A. Meola, E. Sporeno, B. B. Ercole, M. Pezzanera, R. Tafi, M. Arcuri, E. Fattori, A. Lahm, A. Luzzago, A. Vitelli, S. Colloca, R. Cortese, and A. Nicosia. 2006. A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. *Nat Med 12:190.*
275. Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Y. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel. 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature 424:681.*
276. Sullivan, N. J., A. Sanchez, P. E. Rollin, Z. Y. Yang, and G. J. Nabel. 2000. Development of a preventive vaccine for Ebola virus infection in primates. *Nature 408:605.*
277. Thorner, A. R., A. A. Lemckert, J. Goudsmit, D. M. Lynch, B. A. Ewald, M. Denholtz, M. J. Havenga, and D. H. Barouch. 2006. Immunogenicity of heterologous recombinant adenovirus prime-boost vaccine regimens is enhanced by circumventing vector cross-reactivity. *J Virol 80:12009.*
278. Sumida, S. M., D. M. Truitt, A. A. Lemckert, R. Vogels, J. H. Custers, M. M. Addo, S. Lockman, T. Peter, F. W. Peyerl, M. G. Kishko, S. S. Jackson, D. A. Gorgone, M. A. Lifton, M. Essex, B. D. Walker, J. Goudsmit, M. J. Havenga, and D. H. Barouch. 2005. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol 174:7179.*
279. Roberts, D. M., A. Nanda, M. J. Havenga, P. Abbink, D. M. Lynch, B. A. Ewald, J. Liu, A. R. Thorner, P. E. Swanson, D. A. Gorgone, M. A. Lifton, A. A. Lemckert, L. Holterman, B. Chen, A. Dilraj, A. Carville, K. G. Mansfield, J. Goudsmit, and D. H. Barouch. 2006. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature 441:239.*
280. O'Riordan, C. R., A. Lachapelle, C. Delgado, V. Parkes, S. C. Wadsworth, A. E. Smith, and G. E. Francis. 1999. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum Gene Ther 10:1349.*
281. Fisher, K. D., Y. Stallwood, N. K. Green, K. Ulbrich, V. Mautner, and L. W. Seymour. 2001. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther 8:341.*
282. Shiver, J. W., and E. A. Emini. 2004. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu Rev Med 55:355.*
283. Capone, S., A. Meola, B. B. Ercole, A. Vitelli, M. Pezzanera, L. Ruggeri, M. E. Davies, R. Tafi, C. Santini, A. Luzzago, T. M. Fu, A. Bett, S. Colloca, R. Cortese, A. Nicosia, and A. Folgori. 2006. A novel adenovirus type 6 (Ad6)-based hepatitis

C virus vector that overcomes preexisting anti-ad5 immunity and induces potent and broad cellular immune responses in rhesus macaques. *J Virol 80:1688.*

284. Gao, W., P. D. Robbins, and A. Gambotto. 2003. Human adenovirus type 35: nucleotide sequence and vector development. *Gene Ther 10:1941.*
285. Stone, D., S. Ni, Z. Y. Li, A. Gaggar, N. DiPaolo, Q. Feng, V. Sandig, and A. Lieber. 2005. Development and assessment of human adenovirus type 11 as a gene transfer vector. *J Virol 79:5090.*
286. Rich, D. P., L. A. Couture, L. M. Cardoza, V. M. Guiggio, D. Armentano, P. C. Espino, K. Hehir, M. J. Welsh, A. E. Smith, and R. J. Gregory. 1993. Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis. *Hum Gene Ther 4:461.*
287. Nan, X., B. Peng, T. W. Hahn, E. Richardson, A. Lizonova, I. Kovesdi, and M. Robert-Guroff. 2003. Development of an Ad7 cosmid system and generation of an Ad7deltaE1deltaE3HIV(MN) env/rev recombinant virus. *Gene Ther 10:326.*
288. Tatsis, N., L. Tesema, E. R. Robinson, W. Giles-Davis, K. McCoy, G. P. Gao, J. M. Wilson, and H. C. Ertl. 2006. Chimpanzee-origin adenovirus vectors as vaccine carriers. *Gene Ther 13:421.*
289. Michou, A. I., H. Lehrmann, M. Saltik, and M. Cotten. 1999. Mutational analysis of the avian adenovirus CELO, which provides a basis for gene delivery vectors. *J Virol 73:1399.*
290. Hofmann, C., P. Loser, G. Cichon, W. Arnold, G. W. Both, and M. Strauss. 1999. Ovine adenovirus vectors overcome preexisting humoral immunity against human adenoviruses in vivo. *J Virol 73:6930.*
291. Kremer, E. J., S. Boutin, M. Chillon, and O. Danos. 2000. Canine adenovirus vectors: an alternative for adenovirus-mediated gene transfer. *J Virol 74:505.*
292. Reddy, P. S., N. Idamakanti, L. A. Babiuk, M. Mehtali, and S. K. Tikoo. 1999. Porcine adenovirus-3 as a helper-dependent expression vector. *J Gen Virol 80 (Pt 11):2909.*
293. Reddy, P. S., N. Idamakanti, B. H. Hyun, S. K. Tikoo, and L. A. Babiuk. 1999. Development of porcine adenovirus-3 as an expression vector. *J Gen Virol 80 (Pt 3):563.*
294. Reddy, P. S., N. Idamakanti, Y. Chen, T. Whale, L. A. Babiuk, M. Mehtali, and S. K. Tikoo. 1999. Replication-defective bovine adenovirus type 3 as an expression vector. *J Virol 73:9137.*
295. Sumida, S. M., D. M. Truitt, M. G. Kishko, J. C. Arthur, S. S. Jackson, D. A. Gorgone, M. A. Lifton, W. Koudstaal, M. G. Pau, S. Kostense, M. J. Havenga, J. Goudsmit, N. L. Letvin, and D. H. Barouch. 2004. Neutralizing antibodies and CD8+ T lymphocytes both contribute to immunity to adenovirus serotype 5 vaccine vectors. *J Virol 78:2666.*
296. Santosuosso, M., X. Zhang, S. McCormick, J. Wang, M. Hitt, and Z. Xing. 2005. Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. *J Immunol 174:7986.*

297. Magalhaes, I., D. R. Sizemore, R. K. Ahmed, S. Mueller, L. Wehlin, C. Scanga, F. Weichold, G. Schirru, M. G. Pau, J. Goudsmit, S. Kuhlmann-Berenzon, M. Spangberg, J. Andersson, H. Gaines, R. Thorstensson, Y. A. Skeiky, J. Sadoff, and M. Maeurer. 2008. rBCG induces strong antigen-specific T cell responses in rhesus macaques in a prime-boost setting with an adenovirus 35 tuberculosis vaccine vector. *PLoS One 3:e3790.*
298. Lo, C. Y., Z. Wu, J. A. Misplon, G. E. Price, C. Pappas, W. P. Kong, T. M. Tumpey, and S. L. Epstein. 2008. Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: cold-adapted vaccine versus DNA prime-adenovirus boost strategies. *Vaccine 26:2062.*
299. Roy, S., Y. Zhi, G. P. Kobinger, J. Figueredo, R. Calcedo, J. R. Miller, H. Feldmann, and J. M. Wilson. 2006. Generation of an adenoviral vaccine vector based on simian adenovirus 21. *J Gen Virol 87:2477.*
300. Patel, A., Y. Zhang, M. Croyle, K. Tran, M. Gray, J. Strong, H. Feldmann, J. M. Wilson, and G. P. Kobinger. 2007. Mucosal delivery of adenovirus-based vaccine protects against Ebola virus infection in mice. *J Infect Dis 196 Suppl 2:S413.*
301. Wang, D., M. Hevey, L. Y. Juompan, C. M. Trubey, N. U. Raja, S. B. Deitz, J. Woraratanadharm, M. Luo, H. Yu, B. M. Swain, K. M. Moore, and J. Y. Dong. 2006. Complex adenovirus-vectored vaccine protects guinea pigs from three strains of Marburg virus challenges. *Virology 353:324.*
302. Swenson, D. L., D. Wang, M. Luo, K. L. Warfield, J. Woraratanadharm, D. H. Holman, J. Y. Dong, and W. D. Pratt. 2008. Vaccine to confer to nonhuman primates complete protection against multistrain Ebola and Marburg virus infections. *Clin Vaccine Immunol 15:460.*
303. Casimiro, D. R., L. Chen, T. M. Fu, R. K. Evans, M. J. Caulfield, M. E. Davies, A. Tang, M. Chen, L. Huang, V. Harris, D. C. Freed, K. A. Wilson, S. Dubey, D. M. Zhu, D. Nawrocki, H. Mach, R. Troutman, L. Isopi, D. Williams, W. Hurni, Z. Xu, J. G. Smith, S. Wang, X. Liu, L. Guan, R. Long, W. Trigona, G. J. Heidecker, H. C. Perry, N. Persaud, T. J. Toner, Q. Su, X. Liang, R. Youil, M. Chastain, A. J. Bett, D. B. Volkin, E. A. Emini, and J. W. Shiver. 2003. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. *J Virol 77:6305.*
304. Casimiro, D. R., A. J. Bett, T. M. Fu, M. E. Davies, A. Tang, K. A. Wilson, M. Chen, R. Long, T. McKelvey, M. Chastain, S. Gurunathan, J. Tartaglia, E. A. Emini, and J. Shiver. 2004. Heterologous human immunodeficiency virus type 1 priming-boosting immunization strategies involving replication-defective adenovirus and poxvirus vaccine vectors. *J Virol 78:11434.*
305. Casimiro, D. R., A. Tang, L. Chen, T. M. Fu, R. K. Evans, M. E. Davies, D. C. Freed, W. Hurni, J. M. Aste-Amezaga, L. Guan, R. Long, L. Huang, V. Harris, D. K. Nawrocki, H. Mach, R. D. Troutman, L. A. Isopi, K. K. Murthy, K. Rice, K. A. Wilson, D. B. Volkin, E. A. Emini, and J. W. Shiver. 2003. Vaccine-induced immunity in baboons by using DNA and replication-incompetent adenovirus type

5 vectors expressing a human immunodeficiency virus type 1 gag gene. *J Virol 77:7663.*

306. Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans, Z. Q. Zhang, A. J. Simon, W. L. Trigona, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature 415:331.*
307. Letvin, N. L., J. R. Mascola, Y. Sun, D. A. Gorgone, A. P. Buzby, L. Xu, Z. Y. Yang, B. Chakrabarti, S. S. Rao, J. E. Schmitz, D. C. Montefiori, B. R. Barker, F. L. Bookstein, and G. J. Nabel. 2006. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science 312:1530.*
308. Catanzaro, A. T., R. A. Koup, M. Roederer, R. T. Bailer, M. E. Enama, Z. Moodie, L. Gu, J. E. Martin, L. Novik, B. K. Chakrabarti, B. T. Butman, J. G. Gall, C. R. King, C. A. Andrews, R. Sheets, P. L. Gomez, J. R. Mascola, G. J. Nabel, and B. S. Graham. 2006. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. *J Infect Dis 194:1638.*
309. Buchbinder, S. P., D. V. Mehrotra, A. Duerr, D. W. Fitzgerald, R. Mogg, D. Li, P. B. Gilbert, J. R. Lama, M. Marmor, C. Del Rio, M. J. McElrath, D. R. Casimiro, K. M. Gottesdiener, J. A. Chodakewitz, L. Corey, and M. N. Robertson. 2008. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet 372:1881.*
310. McElrath, M. J., S. C. De Rosa, Z. Moodie, S. Dubey, L. Kierstead, H. Janes, O. D. Defawe, D. K. Carter, J. Hural, R. Akondy, S. P. Buchbinder, M. N. Robertson, D. V. Mehrotra, S. G. Self, L. Corey, J. W. Shiver, and D. R. Casimiro. 2008. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet 372:1894.*
311. Girard, M. P., and G. P. Bansal. 2008. HIV/AIDS vaccines: a need for new concepts? *Int Rev Immunol 27:447.*
312. Malkevitch, N., D. Rohne, J. Pinczewski, K. Aldrich, V. S. Kalyanaraman, N. L. Letvin, and M. Robert-Guroff. 2004. Evaluation of combination DNA/replication-competent Ad-SIV recombinant immunization regimens in rhesus macaques. *AIDS Res Hum Retroviruses 20:235.*
313. Malkevitch, N., L. J. Patterson, K. Aldrich, E. Richardson, W. G. Alvord, and M. Robert-Guroff. 2003. A replication competent adenovirus 5 host range mutant-simian immunodeficiency virus (SIV) recombinant priming/subunit protein boosting vaccine regimen induces broad, persistent SIV-specific cellular

immunity to dominant and subdominant epitopes in Mamu-A*01 rhesus macaques. *J Immunol 170:4281.*

314. Liu, J., K. L. O'Brien, D. M. Lynch, N. L. Simmons, A. La Porte, A. M. Riggs, P. Abbink, R. T. Coffey, L. E. Grandpre, M. S. Seaman, G. Landucci, D. N. Forthal, D. C. Montefiori, A. Carville, K. G. Mansfield, M. J. Havenga, M. G. Pau, J. Goudsmit, and D. H. Barouch. 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature 457:87.*
315. Jabbari, A., and J. T. Harty. 2006. Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype. *J Exp Med 203:919.*
316. Zaph, C., J. Uzonna, S. M. Beverley, and P. Scott. 2004. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. *Nat Med 10:1104.*
317. Hansen, S. G., C. Vieville, N. Whizin, L. Coyne-Johnson, D. C. Siess, D. D. Drummond, A. W. Legasse, M. K. Axthelm, K. Oswald, C. M. Trubey, M. Piatak, Jr., J. D. Lifson, J. A. Nelson, M. A. Jarvis, and L. J. Picker. 2009. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med 15:293.*
318. Mueller, S. N., and R. Ahmed. 2009. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A 106:8623.*
319. Santosuosso, M., S. McCormick, E. Roediger, X. Zhang, A. Zganiacz, B. D. Lichty, and Z. Xing. 2007. Mucosal luminal manipulation of T cell geography switches on protective efficacy by otherwise ineffective parenteral genetic immunization. *J Immunol 178:2387.*

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