CHARACTERIZATION OF THE MUCOSAL AND SYSTEMIC IMMUNE RESPONSES FOLLOWING VIRUS VECTOR-BASED GENE DELIVERY INTO THE COLONIC MUCOSA

By

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ABSTRACT

While adenovirus (Ad) vectors have been shown to elicit potent antigen-specific T cell responses, the kinetics and nature of antigen-specific mucosal and systemic T-cell responses has rarely been examined, especially following mucosal administration of Ad-based vectors. In the present studies, the phenotypic and functional characterization of antigen-specific $CD8^+$ T cell responses following intrarectal (*i.r.*) vaccination with an Ad vector expressing *Gallus gallus* ovalbumin (OVA) was conducted. The frequencies of OVA-specific $CD8^+$ T cells was maximal at 2 weeks post-vaccination in all tissues examined and then declined, demonstrating normal expansion and contraction kinetics. $CD8^+$ T cells induced in the course of immunization exhibited phenotypic characteristics of effector memory T cells including up-regulation of the cell surface molecules CD43, CD44 and a low level of expression of CD127 at both local and systemic sites. While the discordance between the number of tetramer-reactive and cytokine-producing OVA-specific $CD8^+$ T cells was observed, $CD8^+$ T cells appeared to be fully functional *in vivo*. Upon secondary antigen exposure, the $CD8^+$ T cell population expanded dramatically, particularly at the mucosal surfaces. In addition, the $CD8^+$ T cell response generated in the course of *i.r.* priming protected mice from intravaginal (*i.vag.*) vaccinia virus one month after immunization, thus underscoring the importance of inducing a tissue-resident effector memory T cell subset for protection against pathogens at mucosal surfaces. In developing future vaccines for mucosal diseases, the induction of a tissue-resident effector memory T cell subset should be one of the immunization objectives.

Lentiviral vectors represent an attractive mode of genetic vaccination. Most commonly used, vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped lentiviral vectors do not efficiently infect epithelial cells from the apical side, and, therefore, are not suitable as mucosal vaccines. In the present studies, Ebola Zaïre strain glycoprotein (EboZ)-pseudotyped lentiviral vectors, which have been previously used to deliver transgene to the lung epithelium, were delivered *i.r.* and evaluated as a mucosal booster vaccine. Rectal delivery of EboZ-pseudotyped lentiviral vectors expressing β-galactosidase (β-gal) had resulted in low, but detectable levels of β-gal expression 2 weeks after administration. When delivered on its own, EboZ-pseudotyped lentivirus did not prime detectable antigen-specific immune response. However, when delivered *i.r.* 30 days after *i.r.* Adβ-gal immunization, a significant enlargement (boost) of β-gal-specific $CD8^+$ T cell responses, especially in the colonic lamina propria (LP), was observed as compared to the delivery of EboZ-pseudotyped vector encoding different transgenes or VSVG-pseudotyped lentivirus expressing β-gal. When these animals were *i.vag.* challenged with vaccinia virus expressing β-gal, a dramatic expansion of β-gal-specific $CD8^+$ T cells, especially in the vaginal tract, was observed. In addition, this prime and boost strategy protected the mice from *i.vag.* vaccinia virus challenge. Therefore, *i.r.* Ad-based priming followed by *i.r.* EboZ-pseudotyped lentiviral boosting was an effective strategy for eliciting protective mucosal $CD8^+$ T cell responses.

Since mucosal surfaces represent the primary site for entry and transmission of herpes simplex virus type 2 (HSV-2), the utility of *i.r.* priming with Ad vector expressing HSV-1 glycoprotein B (gB) for the induction of antigen-specific mucosal and systemic immune responses following was investigated. This gene-based vaccination via colonic epithelium elicited predominantly gB-specific T helper type 1 (T_H1) response and led to the establishment of protective immunity against a lethal colonic or vaginal HSV-2 challenge. To identify the components of the immune response that were responsible for the antiviral activity in two different acute HSV-2 lethality models, protection studies were conducted in T cell-subset knockout and T cell-depleted animals after *i.r.* immunization. Upon both vaginal and colonic HSV-2 challenges, protection was completely abrogated in mice that were genetically deficient in CD4 molecules and in the major histocompatibility complex class (MHC) II-knockout molecules. In addition, $CD4^+$ cell depletion abolished resistance in vaccinated mice, thus confirming the findings obtained in mice lacking $CD4^+$ cells. On the contrary, $CD8^+$ T cells did not appear to play a role in protection in mucosal HSV-2 challenge. In addition, $NK1.1^+$ cells appeared to contribute to protection from vaginal, but not colonic HSV-2 challenge. Thus, it was concluded that the T-cell responses generated following Ad vector HSV antigen gene delivery to the colonic mucosa was absolutely essential for protection from HSV-2 infection, with $CD4^+$ T cells and NK cells playing major roles in the resistance against vaginally and colonically delivered HSV-2. These differences might reflect altered pathways of protection in colonic versus vaginal tissues and highlight the need for further studies of the spectrum of mucosal immune responses.

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LIST OF ABBREVIATIONS

α-MEM	minimum essential medium α
Adβ-gal	adenovirus vector expressing beta-galactosidase
AdgB	adenovirus vector expressing glycoprotein B
AdOVA	adenovirus vector expressing *Gallus gallus* (chicken) ovalbumin
Ad	adenovirus
Ad5	human Ad serotype 5
AIDS	acquired immunodeficiency syndrome
APC	allophycocyanin
APC-Cy7	allophycocyanin-cyanin 7
β-gal	beta-galactosidase
BALT	bronchial-associated lymphoid tissue
BIV	bovine immunodeficiency virus
BSA	bovine serum albumin
CAEV	caprine arthritis encephalopathy virus
CAR	Coxsackie adenovirus receptor
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CFDA-SE	carboxyfluorescein diacetate, succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
CLA	cutaneous leukocyte antigen

CMIS	common mucosal immune system
CXCL	CXCL-chemokine ligand
CX_3CR1	CX_3C-chemokine receptor 1
CXCR	CXC-chemokine receptor
DC	dendritic cell
DDAO-SE	(1,3-dichloro-9,9-dimethylacridin-2-one)-succinimidyl ester
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E1	early region 1 of adenovirus
E3	early region 3 of adenovirus
Ebo	Ebola virus
EboZ	glycoprotein of Zaïre strain of Ebola virus
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EIAV	equine infectious anemia virus
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked immunospot assay
E-selectin	endothelial-cell selectin
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate

FIV	feline immunodeficiency virus
FoxP3	forkhead box P3
GALT	gut-associated lymphoid tissue
gB	glycoprotein B of herpes simplex virus 1
GI	gastrointestinal
GU	genitourinary
HBSS	Hank's balanced salt solution
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HSV	herpes simplex virus
HSV-2	herpes simplex virus type 2
ICS	intracellular cytokine staining
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILN	iliac lymph node(s)
i.m.	intramuscular(ly)
i.n.	intranasal(ly)
i.p.	intraperitoneal(ly)

i.r.	intrarectal(ly)
i.t.	intratracheal(ly)
i.vag.	intravaginal(ly)
JDV	Jembrana disease virus
LALT	larynx-associated lymphoid tissue
LP	lamina propria
M cells	microfold cells
mRNA	messenger ribonucleic acid
MADCAM1	mucosal addressin cell-adhesion molecule 1
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility
MLN	mesenteric lymph node(s)
MOI	multiplicity of infection
NALT	nasal-associated lymphoid tissue
NK	natural killer cell
NTDL6	(non-toxic, deletion, lung) variant 6
OVA	*Gallus gallus* (chicken) ovalbumin
PBS	phosphate buffered saline
PE	phycoerythrin
PE-Cy5	phycoerythrin-cyanine 5
PE-Cy7	phycoerythrin-cyanine 7
PFU	plaque-forming unit(s)

PP	Peyer's patch(es)
P-selectin	platelet selectin
RNA	ribonucleic acid
s.c.	subcutaneous
SIV	simian immunodeficiency virus
SHIV	simian-human immunodeficiency virus
TCR	T cell receptor
TGF	transforming growth factor
T_H1	T helper type 1
TNF	tumor-necrosis factor
T_{Reg}	regulatory T cells
TU	transducing unit
Vacc	vaccinia virus
VaccOVA	vaccinia virus expressing *Gallus gallus* (chicken) ovalbumin
VG	vaginal tract
VSV	vesicular stomatitis virus
VSVG	glycoprotein G of vesicular stomatitis virus

CHAPTER 1 INTRODUCTION

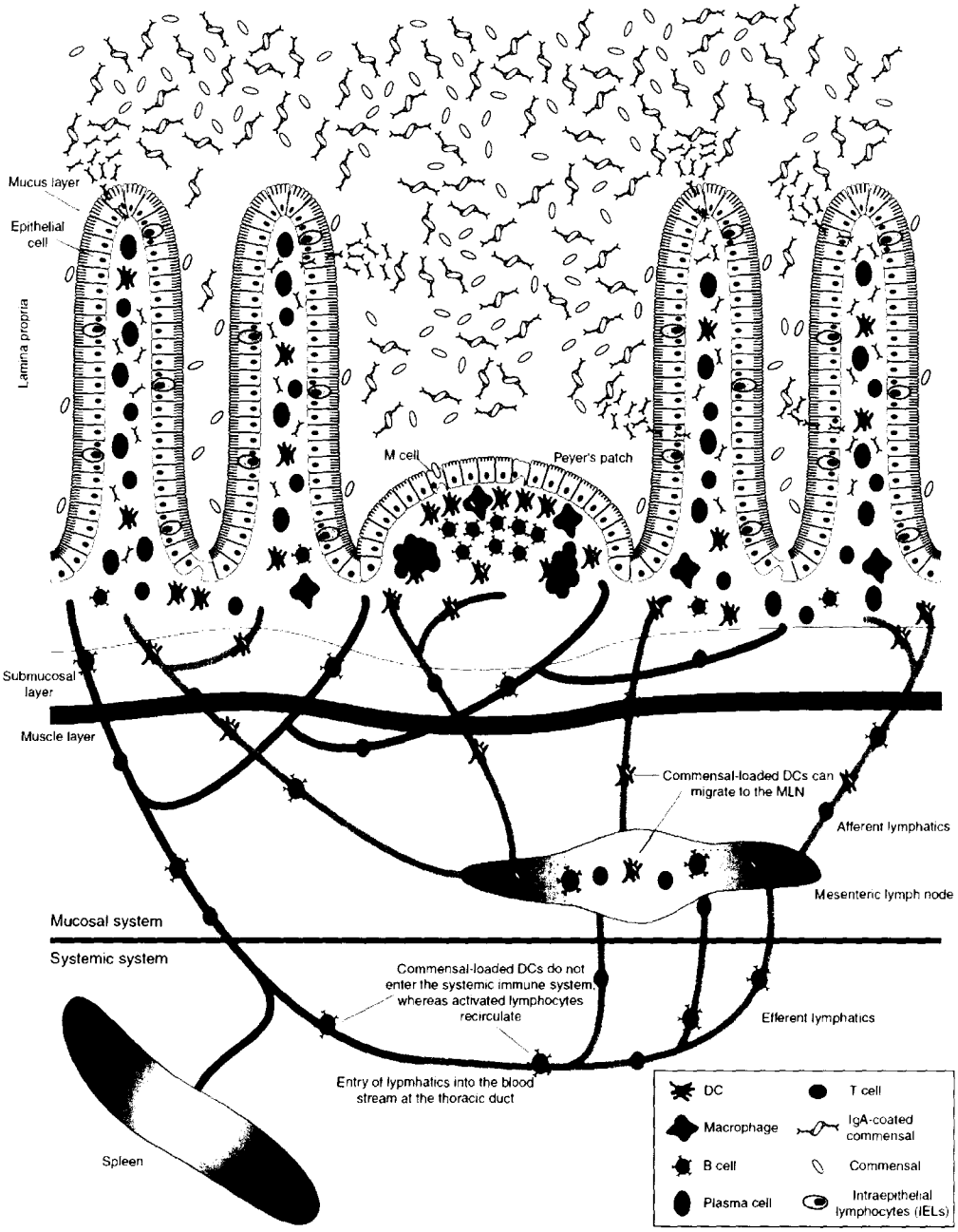
1.1 Mechanisms of mucosal protection

1.1.1 Innate defences at mucosal surfaces

The mucosal surfaces of the respiratory, genito-urinary and gastrointestinal tracts, the ocular surfaces and the lactating mammary glands are constantly in contact with the external environment (Holmgren and Czerkinsky 2005). Therefore, the mucosal immune system has to achieve a delicate balance between the immunological unresponsiveness (tolerance) to innocuous antigens, such as food or beneficial bacteria, and protective immunity to invasion by pathogens and other intruders. This balance is of the utmost importance at the intestinal surface, the largest mucosal site, which presents an area of approximately 100 m^2 and is constantly exposed to dietary and environmental antigens (Artis 2008). In addition, the intestinal mucosal surface plays host to the most densely populated microbial habitat known in biology (Artis 2008). In the gut, commensal organisms, that provide benefit to the host, live predominantly within the intestinal lumen or are associated with the mucus barrier. Thus, the density of mammalian microbiome is separated from a nearly sterile tissue environment within the intestinal villi by a single layer of columnar intestinal epithelial cells (IECs), a distance of only 20 μm (Macpherson, Geuking et al. 2005). Therefore, the mucosal immune system, together with physical defences, plays an important role in separating the host tissues from an environment rich in antigens without causing aberrant inflammation, a function that is essential for host homeostasis.

To achieve this vital separation, the intestinal epithelium exhibits two main structural features including the microvillar extensions and the tight junctions which create a brush border on the apical surface of the epithelium, thus preventing microbial attachment and/or invasion (Fig. 1) (Artis 2008). This physical separation is further reinforced by the presence of the glycocalyx, a heavily glycosylated, mucin-rich layer, produced by goblet cells embedded in the villus and crypt epithelium of both the small and large intestines (Fig. 1). The glycocalyx layer entraps bacteria to be ultimately removed by the flow of the luminal contents. Upon intestinal damage or insult, the production of mucin granules is markedly enhanced and a mucin-rich, semipermeable barrier is maintained to assist in the repair (Artis 2008). In addition, IECs (and other immune cells, such as macrophages, mast cells and neutrophils) produce antimicrobial proteins. These include S100 family proteins, peptidoglycan-recognition proteins, calcium-dependent lectins, iron metabolism proteins and cationic antimicrobial peptides, such as defensins, cathelicidins and calprotectins, many of which have broad activities against Gram-positive and Gram-negative bacteria. In addition to functioning as direct antimicrobial compounds, such as the case with cationic antimicrobial peptides which their amphipathic nature allows for interaction with, and lysis of, bacterial membranes (Artis 2008), these antimicrobial proteins can also function as opsonins, chemokines and modulators of host-cell cytokine production that, in turn, can regulate innate immune responses (Kolls, McCray et al. 2008). Therefore, in addition to digestion and absorption of nutrients, IECs prevent entry of commensal and pathogenic bacteria into the underlying, sterile layer of the intestines.

Figure 1. Intestinal geography of immune responses. Adopted from Macpherson et al. *(Macpherson, Geuking et al. 2005).*


Mucus layer
Epithelial cell
Lamina propria
M cell
Peyer's patch
Submucosal layer
Muscle layer
Commensal-loaded DCs can migrate to the MLN
Afferent lymphatics
Mesenteric lymph node
Mucosal system
Systemic system
Commensal-loaded DCs do not enter the systemic immune system, whereas activated lymphocytes recirculate
Efferent lymphatics
Entry of lypmhatics into the blood stream at the thoracic duct
Spleen
DC
T cell
Macrophage
IgA-coated commensal
B cell
Commensal
Plasma cell
Intraepithelial lymphocytes (IELs)

1.1.2 Cells of the innate immune system

Cells of the innate immune system, which include neutrophils, macrophages, mast cells, eosinophils, basophils and natural killer (NK) cells play important roles in maintaining the immune homeostasis. Phagocytes (neutrophils and macrophages), in particular, are important effectors that regulate microbial density. Following the engulfment of a bacterium, a phagosome is formed into which the reactive oxygen species and hydrolytic enzymes are secreted, and this results in killing of the bacterium (Monack, Mueller et al. 2004). In addition, these cells can also produce a number of the aforementioned antimicrobial peptides. In the presence of microbial colonization or invasion, neutrophils are recruited to the basal side of intestinal epithelial cells in response to CXC-chemokine ligand 8, mainly produced by epithelial cells. These then translocate across the epithelial lining and exert their antimicrobial functions in the gut lumen (Sansonetti 2004). Macrophages also reside in the small and large intestine, undergo local activation in response to microbial stimuli and are able to phagocytose the invading microorganisms by producing various mediators and cytokines (Lee, Starkey et al. 1985; Smythies, Sellers et al. 2005). The cells of the innate system are the first line of defense against many pathogens, although specific pathogens often have evolved numerous strategies to avoid the destruction by innate biocidal mechanisms.

1.1.3 Sensitivity to innate biocidal mechanisms

The way in which the mucosal immune system is adapted to deal with organisms found within the body is largely dependent upon where the organisms reside at the

mucosal surfaces. Commensal bacterial live predominantly within the intestinal lumen or are associated with the mucus layer, whereas most pathogens use a variety of means to attach themselves to the epithelial surface or penetrate it. *Vibrio cholerae* and enteropathogenic *Escherichia coli* colonize epithelial surfaces through close adherence to brush border (Sansonetti 2004). Pathogens can also infect the epithelium or lamina propria, as in the case of rotavirus infections (Sansonetti 2004; Neutra and Kozlowski 2006). Lastly, organisms such as *Salmonella typhi*, *Listeria monocytogenes* and human immunodeficiency virus (HIV) have complex infection patterns that combine both mucosal and systemic modes of invasion (Monack, Mueller et al. 2004; Pamer 2004; Haase 2005). Whereas the commensal flora penetration beyond the epithelial/mucus barrier is effectively controlled first by the innate biocidal mechanisms of phagocytes, pathogens that cross the epithelial lining must avoid or survive phagocytosis to establish productive infections (Macpherson, Geuking et al. 2005). Pathogenic organisms encode a variety of virulence determinants to avoid destruction by macrophages. For example, listeriolysin O secreted by *L. monocytogenes* allows it to escape from the phagosomes by causing destruction of the phagosomal membrane, and type III secretion systems encoded by different pathogenicity islands of *S. typhimurium* induce rapid macrophage death (Monack, Mueller et al. 2004; Pamer 2004). Therefore, while innate immune mechanisms successfully control commensal flora, these are often overcome by pathogens.

1.1.4 Adaptive immune protection at mucosal surfaces

1.1.4.1 Function of IgA

Although predominantly confined to the intestinal lumen, small numbers of commensal bacteria can translocate to Peyer's Patches (PP) and mesenteric lymph nodes (MLN). This allows the efficient priming of the mucosal adaptive immune system without unnecessary systemic priming (Macpherson, Geuking et al. 2005). This microflora-dependent priming results in production of mucosal IgA, in amounts which exceed that of the body's all other immunoglobulins (Artis 2008). IgA appears to be a vital component of defense against an incoming and existing antigenic load, and functions partly as a low affinity biochemical system for containment of microbiota to the intestinal lumen (Macpherson, Geuking et al. 2005). It is hypothesized that the abundance of mucosal IgA, rather than its affinity, is a critical factor for the control of commensal gut flora (Macpherson, Geuking et al. 2005). Therefore, abundance of mucosal IgA together with other mucosal defences restricts the intestinal microbiome to the lumen, such that only local immune responses to the commensal flora are generated.

While low amounts of IgA, mostly in monomeric form, are present in systemic sites, the majority of the body's IgA is found in the form dimers, joined by a small polypeptide termed the "J chain" and covalently bound to a glycoprotein called "secretory component" (Kaetzel 2005). In order to be transported to the intestinal lumen, dimeric IgA is secreted by mucosal plasma cells, bound to the polymeric Ig receptor present on the basolateral surfaces of epithelial cells and is translocated across the cytoplasm of the epithelial cells. During this movement, the ligand-binding portion of polymeric Ig

receptor (secretory component) is cleaved, thereby generating secretory IgA (Kaetzel 2005). Secretory IgA performs a variety of functions which includes immune exclusion of soluble antigens and microorganisms, downmodulation of the expression of proinflammatory bacterial epitopes on commensal bacteria, and maintenance of appropriate bacterial communities. In addition, secretory IgA blocks microbial attachment to epithelial cells, mediates intraepithelial neutralization of incoming pathogens and their products, and facilitates antigen sampling (Macpherson, Geuking et al. 2005). Lastly, secretory IgA can neutralize viruses that entered the epithelial cell cytoplasm by shuttling them back into the lumen through the polymeric Ig receptor or promote bacterial clearance via FcαRI that is present on DCs, neutrophils and other phagocytes (Cerutti and Rescigno 2008).

The generation of mucosal IgA occurs *via* T cell-independent and T cell-dependent pathways. IgA antibodies specific for T cell-dependent antigens derive mostly from B-2 cells which originate in the peritoneal cavity, while those specific for T cell-independent antigens derive from B-1 cells, which originate in the bone-marrow (Cerutti and Rescigno 2008). It appears that B-2 and B-1 cells contribute equally to IgA production, although these have distinct phenotypes and carry different Ig receptor repertoires. It is believed that high affinity IgA emerging from T cell-dependent pathways protects against colonization and invasion by the pathogens while low affinity IgA generated *via* T cell-independent pathways functions in confining commensal bacterial in the intestinal lumen through the immune exclusion. However, this dichotomy is not absolute, such that T cell-independent IgA can provide some degree of protection from the invading pathogens,

while T cell-dependent IgA seems to play an important role in controlling commensal bacteria (Cerutti and Rescigno 2008). Thus, this dichotomy in T cell-independent and T cell-dependent IgA might reflect the differences in the localization and homing properties of B-2 and B-1 cells as well as their activation thresholds and distinct sites for class switch recombination.

1.1.4.2 Function of other immunoglobulins

While IgA predominates in the mucosal secretions, IgG is a predominant isotype in the systemic compartments, such as blood, and peritoneal and lymphatic fluid. However, this isotype is found also in the mucosal secretory fluids including semen, urine and vaginal secretions at low levels (Neutra and Kozlowski 2006). The majority of IgG found in the mucosal compartments is serum-derived and occurs by transudation. The IgG can be directed to intestinal lumen by the polymeric Ig mediated excretory pathway in association with dimeric IgA or *via* the neonatal Fc receptor that is expressed on some intestinal cells even in adults (Yoshida, Claypool et al. 2004; Neutra and Kozlowski 2006).

IgM is the first antibody isotype to be produced by plasma cells and its production is indicative of a recent antigen exposure. Polymeric IgM can be detected at the mucosal surfaces, albeit at a low level. Polymeric IgM can be transcytosed *via* polymeric Ig receptor and can be secreted at the apical surface of epithelial cells as a form of secretory IgM (Rojas and Apodaca 2002; Phalipon and Corthesy 2003). However, the secretory IgM transport is not as efficient as transport of secretory IgA due to its higher molecular

weight (Lamm 1997). In addition, IgM has been known to provide immune compensation in mice lacking IgA such that IgA gene-deleted mice do not develop any immunologically-mediated pathology (Harriman, Bogue et al. 1999).

1.1.4.3 Importance of adaptive immune system in control of commensal flora and pathogens

Adaptive immunity is not essential to tolerate commensal microflora. Antibody-deficient animals and those without B or T cells are able to tolerate their intestinal flora in pathogen-free facilities, although the lack of adaptive immune responses results in increased commensal microbial loads in the mucosa and MLNs of these animals (Macpherson, Geuking et al. 2005). Therefore, compartmentalization of intestinal inductive sites, the commonality of effector cell homing, phagocyte biocidal activity and the broad specificity of anti-commensal IgA responses allows the mucosa beneath the epithelium to remain almost sterile.

In contrast to commensal flora control by immunoglobulins and cellular enzymes, the induction of cell-mediated and humoral adaptive immune responses is vital for effective control and/or clearance of many pathogens. Studies showed a requirement for $CD4^+$ T cells and humoral immunity for the clearance of *S. typhimurium*, while $CD8^+$ T cells provided greater contribution to long-term protective immunity to *L. monocytogenes* (Monack, Mueller et al. 2004; Pamer 2004). The control of early and late-stage simian immunodeficiency virus (SIV) infection in macaques is dependent upon $CD8^+$ T cells (Berzofsky, Ahlers et al. 2001). Therefore, the induction of adaptive mucosal and

systemic immune responses is essential for both control and clearance of many mucosal pathogens.

1.2 The common mucosal immune system

1.2.1 The biology of common mucosal immune system

The common mucosal immune system (CMIS) is a vital network that supports communication between the mucosa-associated lymphoid tissues (MALT) and involves all of the expected immunocompetent cells (B cells, T cells, antigen-presenting cells) to induce potent immune responses to deal effectively with many viruses, bacteria and dietary antigens. Together with physical and innate protective mechanisms, the CMIS is responsible for maintenance of host homeostasis by providing the first line of defense against pathogens (Mestecky 2005).

The MALT can be anatomically subdivided into the gut-associated lymphoid tissue (GALT), the bronchus-associated lymphoid tissue (BALT), the nasal-associated lymphoid tissue (NALT), the larynx-associated lymphoid tissue (LALT), the lactating mammary, lacrimal and salivary glands, and the lymphoid tissue of male and female genital tracts (Mestecky 2005). In addition, these sites, such as GALT and BALT, can be divided also into those that facilitate the generation and the selective localization (operationally defined as homing) of the effectors under homeostatic conditions, plus those that allow the homing and the generation of the effector cells only following the exposure to the pathogens, such as in ocular sites and vaginal tract. The presence of lymphoid tissues at a particular mucosal surface allows the induction of local immune responses at that

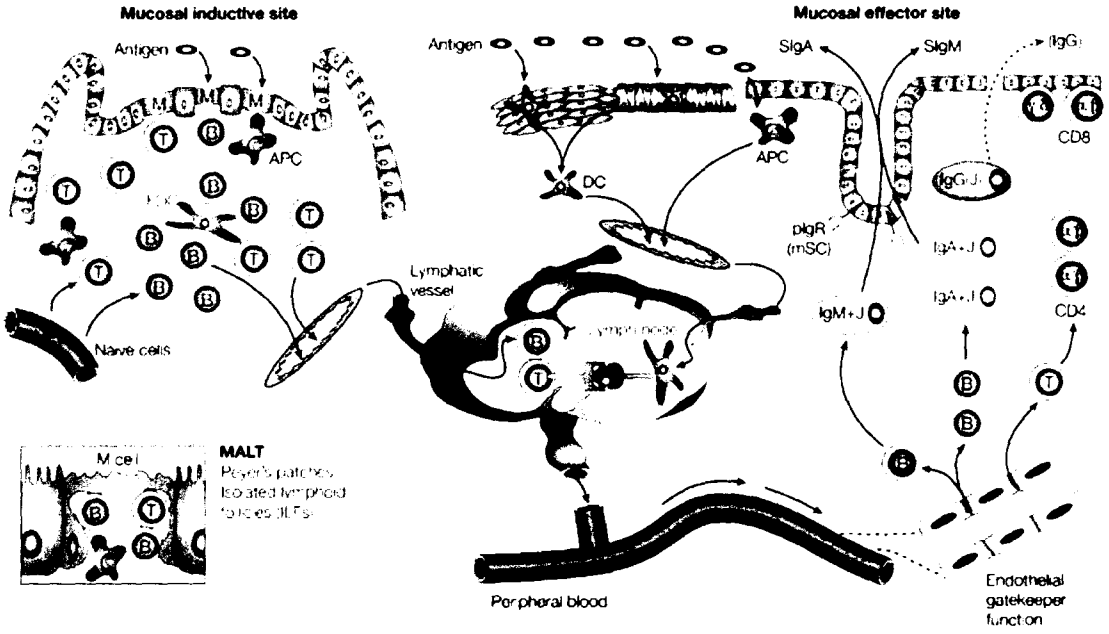
particular tissue site and the specific dissemination of numerous cells from this mucosae which leads to similar or identical immune responses in the systemic compartments of the blood and spleen and at distant mucosal sites. This commonality is a fundamental concept of mucosal immunology and forms the basis for understanding the different immune mechanisms operating at the mucosal surfaces (Zhu, Thomson et al. 2008).

1.2.2 The biology of the GALT

Historically, the GALT was subdivided into the loosely organized effector sites, which consisted of T and B lymphocytes, IgA-secreting plasma cells, and antigen-presenting cells residing in the mucosal epithelium and in the underlying area of the epithelium known as lamina propria, and inductive sites, which are organized lymphoid tissues of PP, MLN and possibly mucosal lymphoid follicles (Fig. 2) (Mestecky 2005). These follicles are structurally similar to PP, which suggests that they might be functionally similar to PP (Mowat 2003). While many of these sites were historically segregated into either effector or inductive sites, many studies indicated that most of these intestinal sites can function either as inductive or effector sites at different stages of immune response. For example, the PP are the location where different intestinal microorganisms and other antigens leaving the intestinal tract encounter immune cells and where critical antigen-specific immune responses are initiated, which is consistent with the PP functioning as inductive sites (Brandtzaeg, Kiyono et al. 2008). These structures are an important source of IgA-producing plasma cells, which demonstrates the important role of PP acting as effector sites (Mowat 2003).

The follicle-associated epithelium that overlies the dome of PP is characterized by microfold (M) cells and the ability to transcytos microorganisms to the subepithelial dome region that is populated by APCs, including a number of dendritic cell (DC) subsets (Artis 2008). Also, an IgA-specific receptor has been identified on the apical surface of M cells that can mediate uptake of luminal IgA into PP and facilitate sampling of luminal immune complexes (Macpherson, Geuking et al. 2005; Neutra and Kozlowski 2006). Not all antigens gain access to M cells, given that size restrictions are defined by the glycocalyx. In addition to these specialized structures, DCs located in the small intestinal lamina propria express tight-junction proteins, such as occludin, claudin 1 and zonula occludens 1, and can protrude the individual dendrites between the adjacent epithelial cells to sample the luminal contents directly (Cerutti and Rescigno 2008), a process that is largely dependent on expression of CX_3C-chemokine receptor 1 (CX_3CR1) on DCs (Artis 2008). In the absence of DCs, bacterial sampling in the intestines is defective, resulting in reduced invasion of enteroinvasive *S. typhimurium* across the epithelium and lack of presentation of *L. monocytogenes* antigens to *Listeria*-specific $CD8^+$ T cells (Jung, Unutmaz et al. 2002; Hapfelmeier, Muller et al. 2008). Lastly, antigens might be actively transported into the underlying region of lamina propria by IgG *via* the neonatal IgG Fc receptor that continues to be present in significant amount on many adult cell types in both rodents and humans, including on IECs (Yoshida, Claypool et al. 2004). Thus, specialized lymphoid structures and immune cells present at the mucosal surfaces are actively participating in antigen sampling to maintain the immune homeostasis.

Figure 2. Depiction of the mucosal immune system. Adopted from Brandtzaeg et al (Brandtzaeg, Kiyono et al. 2008).


Mucosal inductive site
Antigen
APC
Naive cells
Lymphatic vessel
Mucosal effector site
Antigen
SIgA
SIgM
DC
APC
pIgR (mSC)
IgM+J
CD8
CD4
Peripheral blood
Endothelial gatekeeper function
M cell
MALT

1.2.3 The biology of B cell and plasma cell migration and the generation of mucosal IgA

Historically, comparative adoptive transfer studies of cells derived from MLN or peripheral lymph nodes have established that cells from MLN showed a greater propensity to populate mucosal sites, while those from peripheral sites displayed a propensity to localize to their sites of origin. More importantly, the MLN cells localizing in the gut, lungs, MLN and other mucosal sites produced predominantly IgA, while those from peripheral lymph nodes produced mainly IgG (Zhu, Thomson et al. 2008). These studies laid the foundation for the concept of CMIS wherein different mucosal surfaces and mucosal lymphoid sites form an immunological network. Specifically, the homing pattern of IgA-bearing B cells, the precursors of IgA plasma cells, has been elucidated in studies that examined the function and the generation of mucosal IgA response. Studies by Gowans (Gowans and Knight 1964; Pierce and Gowans 1975; Husband and Gowans 1978), Cebra (Craig and Cebra 1971) and their colleagues demonstrated that PP are the early source of IgA precursor cells. Using adoptive transfer experiments based on Ig light chain allotypes, it was demonstrated that cells derived from PP, but not from popliteal lymph nodes, were able to reconstitute lethally-irradiated rabbits with IgA-producing cells. In addition, these studies demonstrated the ability of IgA-producing cell precursors to travel from the circulation to many mucosal sites. It was observed also that immunization with certain antigens at one mucosal site leads to the generation of IgA specific for that antigen not only at that site, but at other distant mucosae. Collectively, these findings suggested the concept of CMIS and found the bias for mucosal, but not systemic, immunization to elicit mucosal immunity.

In the small intestine, the PP are predominant source of IgA-producing precursors. These structures are characterized by features that provide an environment conducive to the activation of B cells that eventually become IgA-producing plasma cells. For example, these structures include germinal centers that promote the interaction between antigen-specific T cells and B cells and are rich in activation-induced cytidine deaminase, the enzyme required for class-switch DNA recombination and somatic hypermutation. PP also produce cytokines, such as transforming growth factor β, interleukin (IL)-4, IL-6 and IL-10, which facilitate the IgA-inducing functions and are needed for the expansion of IgA-expressing B cells and their differentiation to IgA-secreting plasma cells. While PP is a major site of B cell activation, other mucosal sites, such as mucosal lymphoid follicles, the MLN and the lamina propria can function as inductive sites of B cell activation (Mowat 2003; Kiyono and Fukuyama 2004).

Once activated in PP, B cells that have undergone class-switch DNA recombination become IgA^+ effector B cells, including memory B cells and plasmablasts. The IgA^+ plasmablasts upregulate the gut homing receptors, such as $\alpha_4\beta_7$-integrin, CC-chemokine receptor 9 (CCR9) and/or CC-chemokine receptor 10 (CCR10), travel *via* the efferent lymphatics of the PP, through the MLN and *via* the thoracic duct and blood, eventually localizing in the intestinal mucosa and other mucosal sites (Fig. 1). While $\alpha_4\beta_7$-integrin binds mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) on endothelial cells, CCR9 and CCR10 respond to chemokines CC-chemokine ligand-25 (CCL25) and CCL2, respectively, produced by epithelial cells. In the gut lamina propria and at other mucosal sites, IgA^+ plasmablasts terminally differentiate into IgA-secreting plasma cells.

The development of antigen-specific, secretory IgA responses requires 3 to 4 weeks before appreciable IgA antibodies can be detected in the feces, and this might reflect a long period of time needed for activation, proliferation and trafficking of IgA-producing B cell precursors (Cerutti and Rescigno 2008). Importantly, different mucosal lymphoid sites support an environment that is conducive to different stages of immune response, from B cell activation to terminal differentiation into IgA-producing plasma cells. This segregation might prevent the generation of aberrant immune responses to the innocuous antigens and commensal flora (Macpherson, Geuking et al. 2005). In addition, this multi-step pattern that leads to production of IgA might provide the explanation for the importance of mucosal priming in generation of mucosal antibody response.

1.2.4 Intestinal T cell responses

Unlike B cells, the generation of mucosal T cell response does not require as extensive spatial (functional) segregation. For example, it is unknown whether inductive sites, such as PP or MLN are better suited, compared to the lamina propria, to support T cell activation. In addition, there does not appear to be a differentiation step following the return of T cells to the lamina propria. There is evidence that antigen-specific T cells entering the mucosal compartments are armed to a lesser degree than their systemic counterparts, which is argued as important for prevention of aberrant immune responses at the sites with higher antigenic load (Masopust, Vezys et al. 2006). However, the fact that these alterations might occur as a consequence of laboratory methods used for mucosal lymphocyte isolation cannot be ruled out. Also, analogous to mucosal B cell

responses, the recirculation of T cells to mucosal sites appears to be governed by expression of different homing molecules and receptors (Agace 2008).

1.2.4.1 Extralymphoid tropism of effector T-cell subsets

The concept of site-specific homing of memory lymphocytes to different mucosal compartments arose from earlier studies that demonstrated the preferential migration of adoptively transferred lymphocytes to the tissues from which they were isolated. It was later shown that $\alpha_4\beta_7$-integrin and mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) played an important role in mediating the entry of effector $CD4^+$ and $CD8^+$ T cells into the small intestinal mucosa, both under conditions of steady-state (homeostasis) and intestinal inflammation (Agace 2006). In addition, a subset of $\alpha_4\beta_7$-integrin-expressing $CD4^+$ and $CD8^+$ T lymphocytes also selectively expresses CC-chemokine receptor (CCR9), which plays an important role in effector lymphocyte recruitment to the small intestinal epithelium and lamina propria (Agace 2006). This CCR9-mediated homing is achieved by selective expression of its ligand (CC-chemokine ligand 25 (CCL25)) in the small intestinal epithelium. However, these homing molecules are not absolutely required for small intestinal homing since T-cell entry into the intestinal mucosa is not restricted to β_7-expressing $CD8^+$ T lymphocytes and mice deficient in CCR9 have relatively normal numbers of small intestinal T cells (Agace 2006). Similarly, the expression of cutaneous leukocyte antigen (CLA) on a subset of circulating $CD4^+$ lymphocytes is partly responsible for migration of effector T cells to the skin during cutaneous inflammation (Agace 2006). This occurs through the interaction of

CLA with endothelial-cell selectin (E-selectin) and platelet selectin (P-selectin), which are dramatically up-regulated on cutaneous venules and also on the vascular endothelium in several other tissues during inflammation. Also, CCR4 and CCR10 appear to contribute to T-cell homing to the skin, depending on the type of inflammation (Agace 2006). As with intestinal homing ligands, expression of skin-homing ligand alone cannot be regarded as a marker for skin-homing T cells. Together, these results indicate that there is a degree of redundancy in the mechanism of tissue-tropic redistribution of effector T cells.

1.2.4.2 Tissue-tropic effector T-cell generation

The expression of tissue-tropic receptors seems to be governed by the lymph node in which initial activation takes place. As such, T cells activated in the skin-draining lymph nodes aquire the ability to bind E-selectin and P-selectin, while those activated in MLN up-regulate their expression of $\alpha_4\beta_7$-integrin and CCR9 (Agace 2006). Studies, however, indicate that skin-tropic P-selectin ligands are expressed on effector $CD4^+$ T cells following immunization regimens targeting MLN, while transcutaneous priming induces $\alpha_4\beta_7$ expression on effector $CD8^+$ T cells (Belyakov, Hammond et al. 2004).

It is believed that lymph node (and PP) DCs play important roles in the imprinting of the tissue homing potential on effector T cells. Activated mouse T cells cultured in the presence of CD3-specific antibodies (or relevant antigen) and MLN or PP DCs express CCR9 and high levels of $\alpha_4\beta_7$-integrin (Agace 2006). In the MLNs, the ability to drive expression of gut homing molecules was enhanced by enrichment for the $CD103^+$ fraction

of DCs, which are believed to be antigen-presenting cells migrating from the small intestine and not lymph node-resident DCs (Coombes and Powrie 2008). Similarly, co-culture of $CD8^+$ T cells with DCs isolated from skin-draining lymph nodes resulted in increased levels of mRNA for the enzyme responsible for synthesis of ligands for E-selectin and P-selectin in those T cells (Agace 2006). A suggested mechanism by which MLN and PP DCs achieve the compartmentalization of mucosal effector T cells is their ability to generate retinoic acid, a metabolite of vitamin A. The addition of retinoic acid to $CD4^+$ and $CD8^+$ T effector lymphocytes results in induced co-expression of $\alpha_4\beta_7$-integrin and CCR9 and decreased levels of expression of E-selectin and P-selectin ligands (Agace 2006). Splenic DCs are capable also of generating retinoic acid. However, non-intestinal DCs induce the expression of $\alpha_4\beta_7$-integrin, but not CCR9, on responding $CD8^+$ T cells *in vitro* (Agace 2006). In addition, fixed PP DCs failed to induce $\alpha_4\beta_7$-integrin and CCR9 expression on $CD8^+$ T cells, and instead induced a higher expression of E-selectin and P-selectin ligands (Agace 2006). Although this might suggest that CLA induction is a default homing pathway imprinted by DCs, Langerhans cells are superior in inducing CLA expression. Therefore, other, yet unidentified factors produced by skin-localized DCs might be responsible for enhanced effector lymphocyte homing to the skin. In addition to DC-mediated imprinting of intestinal T lymphocyte homing, retinoic acid aids the ability of intestinal DCs to drive the peripheral generation of forkhead box P3 (FoxP3) expressing regulatory T cells (T_{Reg}) and to support generation of gut-homing IgA-secreting B cells (Coombes and Powrie 2008).

Although homing to the small intestine and skin has received considerable attention, factors responsible for the homing to other mucosal sites have yet to be discovered. As such, neither $\alpha_4\beta_7$ nor CCR9 expression on T cells affects their homing to the large intestine. In addition, while the vitamin A deficiency decreases the number of B and T cells homing to the small intestinal lamina propria, it has no effect on lymphocyte homing patterns to the colon (Moro, Iwata et al. 2008). Similarly, GALT-resident DCs do not appear to induce homing to the large intestine (Moro, Iwata et al. 2008). Recently, $CD62L^{high}$ colonic IELs, in contrast to $CD62L^{low}$ IELs, were shown to express high levels of sphingosine-1-phosphate receptor and their localization and retention within the colonic epithelium were inhibited by a sphingosine-1-phosphate receptor agonist that induces downregulation of sphingosine-1-phosphate receptor (Agace 2006). Therefore, this receptor might play an important role in the recruitment and retention of naïve T cells within colonic epithelium. In addition, the interactions between lamina propria-resident lymphocytes and endothelial vessels in the colonic lamina propria can be partially inhibited by blocking the interaction between CXC-chemokine receptor 4 (CXCR4) and CXC-chemokine ligand 12 (CXCL12) (Agace 2006). However, factors that are responsible for lymphocyte homing to the colon and reasons why homing mechanisms to this compartment are distinct from those important for homing to the small intestine are yet to be determined.

1.3 Cell-mediated immune response and vaccine design

1.3.1 T-cell development and vaccine design

The ability of the immune system to sample the antigens in the mucosal and systemic compartments and to prime systemic and mucosal lymphocytes is essential for well-being of the organism. This same ability is the underlying principle for the success of vaccination, where attenuated or inactivated pathogens or their components are delivered to generate the adaptive immunity to the virulent pathogen. Vaccination has proved to be one of the most effective means for reducing mortality and morbidity caused by infectious agents (Yang, Millar et al. 2007). The ultimate goal of vaccination is to induce a life-long immunological protection upon the encounter. In some situations, protective immunity can be mediated by just one arm of the adaptive immune response, as is the case with many of the vaccines in clinical use today, which depend mostly on neutralizing antibodies (Berzofsky, Ahlers et al. 2001). However, pathogens often evade the immune system by acquiring mutations that result in modification or masking of neutralizing epitopes (Monto and Ohmit 2009). Also, antibodies elicited to one isolate of a pathogen might not protect against other isolates of the same pathogen as a result of molecular differences in epitopes. Therefore, for optimal control of the pathogen, vaccines should engage both the humoral and cell-mediated arms of adaptive immune responses in both systemic and mucosal compartments, such that cytolytic and cytokine-secreting T cells can clear pathogens that have evaded antibody-mediated immunity (Berzofsky, Ahlers et al. 2001; Haase 2005). This would be particularly relevant for pathogens that cause chronic infections, for example HIV, hepatitis C virus and mycobacteria. Given the vital

need for vaccines specific for chronic infections, characterization of immune T-cell responses and mechanisms for the development of effector and memory T cells have received considerable attention. Understanding these cellular immune mechanisms is an important step towards the development of a new generation of vaccines (Seder, Darrah et al. 2008). Since $CD8^+$ T-cell responses have been more extensively characterized *in vivo*, the formation of memory $CD8^+$ T cells, rather than $CD4^+$ T cells, is described below. However, the relevant similarities and differences between $CD8^+$ and $CD4^+$ T-cell responses will also be discussed.

1.3.2 Stages and subsets of adaptive T-cell responses

Antiviral $CD8^+$ T cell responses can be subdivided into three distinct stages (Ahmed and Gray 1996; Kaech, Wherry et al. 2002; Wherry and Ahmed 2004). First, initial exposure to the antigen leads to clonal expansion and the formation of effector $CD8^+$ T cell populations. Following the peak of the $CD8^+$ T cell response at 2-3 weeks after immunization, the effector $CD8^+$ T cell population undergoes contraction, such that only a small percentage (~5-10%) of antigen-specific $CD8^+$ T cells is maintained as a long-lived memory pool. Memory $CD8^+$ T cells play a prominent role in host defense against viruses and intracellular bacteria due to their increased precursor frequency compared to the naïve $CD8^+$ T cell pool, as well as their dramatic proliferation and rapid acquisition of different effector functions, including early inflammatory cytokine production and cytolytic potential (Murali-Krishna, Altman et al. 1998; Cho, Wang et al. 1999; Kaech, Wherry et al. 2002; Byers, Kemball et al. 2003; van Lier, ten Berge et al. 2003; Wherry,

Teichgraber et al. 2003; Wherry and Ahmed 2004). This expansion and contraction of the antigen-specific T lymphocyte pool is observed in both lymphoid and peripheral sites with the similar kinetics that is characteristic of a particular bacterial or viral infection.

According to the current paradigm, memory $CD8^+$ T cells can be segregated into two subsets based on their localization, and phenotypic and functional properties (Sallusto, Lenig et al. 1999; Wherry, Teichgraber et al. 2003; Sallusto, Geginat et al. 2004). The subset designated as central memory T cells (T_{CM}) is characterized phenotypically by the elevated expression of CD62L and CCR7 and these cells are localized mostly in lymphoid organs. Effector memory T cells (T_{EM}), in contrast, express low levels of CD62L and CCR7 and are predominantly associated with peripheral tissues, where it is believed they act as a first line of defense against invading pathogens (Masopust, Vezys et al. 2001). Moreover, the T_{CM} subset is recognized for its enhanced capacity for proliferation and IL-2 production, and appears to be more effective in protecting the host from both systemic and mucosal viral challenge (Wherry, Teichgraber et al. 2003). Thus, over the course of the memory phase, T_{CM} become the predominant subset, reflecting this cell's superior protective capabilities. However, this view has been challenged recently as $CD8^+$ T_{EM} cells with enhanced cytolytic function, but limited proliferative capacity, mediated equal or better protection than $CD8^+$ T_{CM} cells following viral challenge at non-lymphoid sites (Bachmann, Wolint et al. 2005; Seder, Darrah et al. 2008). Therefore, the location and rate at which the T cells are needed might determine the type of response that mediates protection.

Although IL-2 production is one of the hallmarks of the T_{CM} response, depending on the type of infection or difference in the duration of antigen exposure, IL-2 production might be associated with $CD4^+$ T_{CM} or T_{EM}. However, it is rare to find $CD8^+$ T_{EM} cells that produce IL-2. More recent data suggests that not only phenotypic but also the functional attributes of antigen-specific T cell responses need to be considered (Seder, Darrah et al. 2008). While much of the current knowledge regarding the formation of memory $CD8^+$ T cells comes from systemic models of inoculation with lymphocytic choriomeningitis virus (LCMV) or *L. monocytogenes* (Pamer 2004; Tatsis, Fitzgerald et al. 2007), there is little information concerning how memory T cells develop in response to mucosal infections.

1.3.3 Functional characterization of memory T cells by FACS

Whereas early studies suggested that the expression of homing molecules could be used to differentiate functional T-cell subsets, more recent studies suggest that cell-surface phenotype often fails to ascribe functional attributes to the memory T cell phenotypes. The range of T cell functions include the ability to proliferate or induce proliferation by other cells, organize immune responses and carry out effector functions by directly killing target cells through the cytolytic mechanisms or the secretion of cytokines. These functions can be defined as the quality of the T cell response (Seder, Darrah et al. 2008). Methods that define the full characteristics of T cells are crucial for development of both prophylactic and therapeutic vaccines. Currently, multiparameter flow cytometry is one technique that can be used to both quantitatively and qualitatively

define the magnitude, phenotype and functions of T cells that have been induced in the course of immunization. Flow cytometry is a powerful technology that is capable of quantifying individual cell phenotypes independently and simultaneously on a single-cell basis. Currently, quantification of multiple functions of T cells requires at least five-color technology. It allows simultaneous definition of T-cell lineage (CD3, CD4 and CD8), viability, to electronically disselect debris or unwanted cell populations and two or more colors devoted to the phenotype and/or effector functions of interest. Although each approach has limitations, together with MHC class I tetramer technology and intracellular cytokine staining (ICS), these approaches allow extensive analysis of endogenous T-cell responses to immunization or infection (Seder, Darrah et al. 2008). In the present studies, the quantity and quality of $CD4^+$ and $CD8^+$ T cells responses in the context of disease pathogenesis and mucosal vaccine development for viral infections were assessed using these important flow cytometry-based techniques.

Historically, antigen-specific T cells were identified by assays that involved *in vitro* restimulation to generate a measurable response. The use of MHC class I tetramer technology allowed direct visualization of the relevant $CD8^+$ T cells and, consequently, the elucidation of many features of $CD8^+$ T-cell responses in numerous infectious models and human diseases. An MHC class I tetramer is a tetrahedral complex of biotinylated MHC class molecules with the peptide epitope of interest and β_2-microglobulin joined together by a fluorochrome-labeled avidin molecule. Compared to monomeric peptide-MHC complexes that bind poorly to the TCR, tetramers make use of cooperative binding of the TCR and allow efficient detecting of antigen-specific $CD8^+$ T cell populations in

flow cytometric studies (Klenerman, Cerundolo et al. 2002). This technique is extremely useful in defining the kinetics of $CD8^+$ T cell immune responses to numerous epitopes in a variety of models; however, it is limited to a phenotypic characterization and lacks sensitivity when evaluating rare events due to background staining.

ICS is a tool that analyses the ability of T cells to produce cytokines following *in vitro* restimulation with the antigen of interest, such as a protein or a peptide epitope. The technique is based on the ability of certain chemicals to block the usual cytokine secretion pathway, such that intracellular accumulation of the cytokines can be monitored by fluorochrome-labelled antibodies binding to cytokine molecules for detection by flow cytometry. ICS allows for simultaneous measurement of one or more cytokines (Seder, Darrah et al. 2008). However, many laboratories are equipped with flow cytometers that detect up to three different cytokines. Therefore, such staining might not allow for the identification of all relevant T cells.

The frequency of IFN-γ producing T cells has been the most widely used parameter to assess vaccine-induced responses specific for infections that require cellular immunity for protection. In terms of effector function, IFN-γ has been shown to have a role in the clearance of various bacterial, parasitic, viral or fungal infections (Seder, Darrah et al. 2008). Tumor-necrosis factor α (TNF-α) is another cytokine capable of mediating the killing of a variety of intracellular infectious viruses, bacteria and parasites. Furthermore, the production of IFN-γ and TNF-α together leads to enhanced killing of *L. major* and *M. tuberculosis*, as compared to either cytokine alone (Seder, Darrah et al. 2008). The third cytokine in the panel is IL-2. Although IL-2 has little direct effector function, it promotes

the expansion of $CD4^+$ and $CD8^+$ T cells (in an autocrine or paracrine manner), thereby serving to amplify effector T-cell responses (Seder, Darrah et al. 2008). It can also enhance $CD8^+$ T cell memory function and enhance natural killer (NK) cell activity (Seder, Darrah et al. 2008). Therefore, IL-2, TNF-α and IFN-γ provide a set of cytokines that can be used to define vaccine-elicited responses against specific infections that require T cells for protection. The hierarchy of cytokine production from $CD8^+$ T cells has been elucidated in a number of acute infections, which include LCMV, vesicular stomatitis virus (VSV) and influenza virus (Seder, Darrah et al. 2008). In these models, IFN-γ is expressed by the majority of $CD8^+$ T cells at the peak of the immune response. Contained within this population is a fraction that also secretes TNF-α and even smaller fraction that secretes both TNF-α and IL-2. During chronic LCMV infection, persistent levels of antigen result in the loss of effector-cytokine production by $CD8^+$ T cells. Fewer TNF-α- and IL-2-producing $CD8^+$ T cells are detected, with IL-2 being lost first (Fuller, Khanolkar et al. 2004). In addition to cytokines, $CD8^+$ (and some $CD4^+$) T cells mediate cytolytic activity through the release of perforin or granzyme. Measuring these directly or by detecting CD107a as indirect measure of degranulation (Betts, Brenchley et al. 2003) provides further insight into important effector pathways, especially for viral infections. From functional exhaustion studies, it appears that all three cytokine-producing subsets retain the ability to kill virally infected cells.

Evidence that T-cell quality correlates with clinical outcome and/or protection has been generated from a number of both clinical and mouse model studies. Clinical studies of HIV infection have demonstrated that ~40-50% of cytokine-secreting $CD4^+$ T cells in

HIV-infected progressors produced IFN-γ only. In contrast, ~50% of cytokine-secreting $CD4^+$ T cells isolated from long-term non-progressors or individuals recieving anti-retrovirus treatment were either IFN-γ^+TNF-α^+IL-2^+ or IFN-γ^+IL-2^+ (Seder, Darrah et al. 2008). Similarly, long-term non-progressors had significantly higher frequencies of IFN-γ^+TNF-α^+CD107a^+(IL-2^-) $CD8^+$ T cells in both systemic and mucosal sites as compared to progressors (Betts, Brenchley et al. 2003; Critchfield, Lemongello et al. 2007). Studies evaluating different vaccine formulations in the mouse model of *L. major* have demonstrated clearly that generation of IFN-γ^+TNF-α^+IL-2^+ $CD4^+$ T cells correlated with protection against subsequent challenge (Seder, Darrah et al. 2008). Therefore, these results provide compelling evidence that the quality of the T-cell response is a crucial factor in delivering a protective T-cell response.

1.4 Mucosal vaccination

1.4.1 Importance of mucosally-administered vaccines

Many pathogens enter the body *via* mucosa. Some of these infections, such as HIV or *M. tuberculosis*, are causes of global epidemics that kill millions of individuals each year (Haase 2005; Santosuosso, McCormick et al. 2005). Others are ubiquitous human pathogens that occur with high prevalence, such as herpes simplex virus (HSV), which has a major socioeconomic impact and strong association with infections like HIV and *M. tuberculosis* (Santosuosso, McCormick et al. 2005). Effective vaccines for these mucosally-acquired diseases have remained elusive because of the successful adaptation by pathogens to their host and a lack of understanding of immune response correlates of

infection severity and protection. It is believed that protection from mucosally-transmitted pathogens relies on development of mucosal immunity (Berzofsky, Ahlers et al. 2001). As such, the maintenance of antigen-specific T cells is often observed at the sites frequently exploited by the pathogens. For example, the frequencies of lung-resident T cells specific for influenza virus and respiratory syncytial virus were much higher than those observed in the blood of human subjects; however, the frequencies of cytomegalovirus and Epstein-Bar virus were not different between the compartments (de Bree, van Leeuwen et al. 2005). More importantly, mucosal antigen-specific T cell responses are often defined as correlates of protection, as was reported in HIV-1 long-term non-progressors (Sankaran, Guadalupe et al. 2005) and HIV-resistant prostitutes (Berzofsky, Ahlers et al. 2001). Numerous studies have provided evidence that mucosal immunization platforms can elicit superior local immune responses compared to systemic immunization protocols. For example, intranasal (*i.n.*) immunization with recombinant adeno-associated virus encoding the receptor-binding domain of severe acute respiratory syndrome corona virus spike protein induced stronger immune responses when delivered to the lung as compared to intramuscular (*i.m.*) immunization (Du, Zhao et al. 2008). Indeed, mucosal vaccination platforms have been shown to generate superior mucosal immunity both at the site of immunization and distal mucosal sites. For instance, *i.n.* immunization with recombinant vaccinia virus and DNA expressing herpes simplex virus glycoprotein B resulted in increased frequencies of antigen-specific B cells and T cells at distal mucosal sites, such as the vaginal tract, as compared to *i.m.* delivery of the same constructs (Gallichan and Rosenthal 1996). Although numerous studies indicate that

mucosal vaccination is capable of providing protection against many systemic and mucosal pathogens, only a limited number of studies directly evaluated the protective merits of mucosal *vs.* systemic immunization platforms. For example, when the same HIV/SIV peptide vaccine was delivered either intrarectally (*i.r.*) or subcutaneously (*s.c.*), only macaques that received the vaccine mucosally were protected from *i.r.* challenge with a pathogenic strain of SHIV-KU2, whereas *s.c.* immunization resulted in residual viraemia (Kuznetsov, Stepanov et al. 2004). Similarly, in a rectal vaccinia challenge mouse model, only mice that were administered peptide vaccine *i.r.* and not *s.c.* had optimal frequencies of antigen-specific $CD8^+$ T cells present in the colonic mucosa to provide protection against live virus mucosal challenge (Belyakov, Ahlers et al. 1998). Also, recombinant Ad-based immunization delivered mucosally, but not systemically, protected mice from mucosal *M. tuberculosis* challenge (Santosuosso, Zhang et al. 2005). Despite the wealth of evidence about the importance of mucosal immunization, many of the studies evaluating utility of mucosal *vs.* systemic immunizations are plagued by a lack of knowledge of the kinetics of mucosal and systemic immune responses and the phenotypic and functional characteristics of lymphocytes induced by the immunizing agent which can be quite different for the same agent being introduced at different sites. Therefore, boosting or challenge regimens are often chosen arbitrarily and might not be optimal for all modes of administration. In addition, while equivalent doses of the same agent delivered at different sites might differentially seed them with effector cells, titration of immunizing agents (and/or challenge doses) to standardize the number of

effectors present at the time of challenge at the target site might be useful in evaluating the utility of different immunization regimens for systemic or mucosal antigen delivery.

1.4.2 Challenges associated with mucosal vaccination

Despite evidence that the most potent mucosal immune responses are elicited in the course of immunization at the mucosa, clinical vaccine research has relied largely on systemically-delivered vaccines administered either *i.m.* or *s.c.* These injection-based modes of administration deliver known quantities of antigen and the immune responses generated in the process of immunization are measured readily in blood samples (Mitragotri 2005). By contrast, both the understanding of mucosal immunity and development of mucosal vaccines have lagged behind for a number of reasons. Firstly, mucosal vaccines often require labour intensive modes of delivery. Also, the dose of mucosal vaccines that actually reach the immune induction sites in the body cannot be accurately assessed, as the agents administered are facing the same obstacles as those faced by pathogens. As such, vaccines are diluted and transported in mucosal secretions, degraded by proteases, and excluded by epithelial barriers (Berzofsky, Ahlers et al. 2001). Thus, relatively large doses of mucosal vaccines are required. Similarly, infectious agents and antibodies/cytokines in mucosal secretions are difficult to capture and quantify. Lastly, the isolation and characterization of mucosal T cells is mostly an invasive and labour intensive process. As such, only a small number of mucosal vaccines, such as the oral vaccine against poliovirus and the nasal vaccine against influenza virus, have been approved for human use (Neutra and Kozlowski 2006). These

vaccines specifically target the site of induction of immune responses to the relevant pathogen and result in the increased antigen load in the mucosal compartment of interest.

1.4.3 Mucosal immunization with live-vectored vaccines

In common with systemically-delivered vaccines, the ideal mucosal vaccine should be multimeric, it should also adhere to mucosal surfaces and induce potent innate and adaptive immune responses in both systemic and mucosal compartments that are appropriate for the target pathogen. All of these requirements can be fulfilled by live pathogens, which either already make effective mucosal vaccines or are currently under development as potential mucosal vaccines. Indeed, some of the most effective oral vaccines, such as live attenuated poliovirus and *S. typhi*, are derived from pathogens that are successfully adapted to survive in the lumen and invade mucosal tissues (Neutra and Kozlowski 2006). In addition, viscoelastic gel formulations, such as methylcellulose, can be used effectively to enhance antigen uptake and immune responses following viral vector delivery (Sinn, Burnight et al. 2005). In contrast to particulate vaccines, that require a potent adjuvant in order for them to be distinguished from innocuous substances, live attenuated mucosal vaccine vectors possess an armamentarium of molecules that raise alarms in the mucosa and in so doing actively engage the innate and adaptive arms of the immune response (Neutra and Kozlowski 2006). Nevertheless, there are risks associated with some live mucosal vaccine vectors, which include reversion to a virulent phenotype, mild-enteritis following oral administration of viral vectors, and the possibility of retrograde transport to the brain through the olfactory nerve

following *i.n.* adenovirus (Ad)-based gene delivery (Arimoto, Nagata et al. 2002; Lemiale, Kong et al. 2003; Doi, Nibu et al. 2005; Yang, Millar et al. 2007; Damjanovic, Zhang et al. 2008; Ishikawa, Widman et al. 2008). Despite the risks, live-vectored vaccines are attractive vaccine candidates and are actively investigated. Although, non-living vaccines and adjuvants have numerous potential advantages as mucosal vaccines, they will not be discussed in this thesis.

1.5 Adenovirus vector-based immunization

1.5.1 Replication deficient Ad-based vectors

Adenoviral vectors have received considerable attention as possible vaccines. Adenoviruses (Ad) are non-enveloped, double-stranded DNA viruses that are found in many animal species including humans, monkeys, cows, dogs and many others (Yang, Millar et al. 2007). A total of 51 human Ad serotypes are subdivided into six groups (A-H) based on different properties, including hemagglutination, genomic organization and tumorgenicity in rodents. The majority of Ad viruses cause only mild clinical symptoms in humans, such as colds and gastroenteritis. Human Ad serotype 5 (Ad5) viruses are the most studied and allow incorporation of up to 2 kb of exogenous DNA. To generate Ad5 vectors that could accommodate larger inserts such as foreign genes, early (E) regions 1 and 3 were removed, permitting the insertion of up to 8 kb of exogenous DNA (Tatsis and Ertl 2004; Yang, Millar et al. 2007). E1A proteins (encoded by E1) participate in the initiation of virus gene transcription and translation rendering E1-deleted viruses replication-deficient. However, E1-deleted viruses are not fully replication deficient as

some cellular transcription factors may partially compensate for E1 deficiency. E3-region proteins play a role in actively suppressing host immune responses to the vector by numerous mechanisms, such as by inhibition of transport of major histocompatibility complex I molecules to the cell surface and by control of TNFα-mediated cytolysis of infected cells (Feuerbach and Burgert 1993; Beier, Cox et al. 1994; Horwitz, Tufariello et al. 1995; Lee, Abina et al. 1995; Wold, Tollefson et al. 1995). The E1, E3 double-deleted vectors can be amplified easily in culture as E1 proteins are supplied *in trans* by a producing cell line, such as human embryonic kidney (HEK) 293 cells. It is believed that the Ad5 virus enters the cells *via* the Coxsackie adenovirus receptor (CAR), although CAR binding is not the sole means of attachment of Ad viruses since alternate receptors for subgroups B-D have been identified (Tatsis and Ertl 2004). CAR is expressed on a wide variety of cells providing the virus with tropism for a variety of tissues which include liver, kidney, muscle, and epithelial cells. The replication-deficient Ad vectors, lacking both the E1 and E3 regions, are considered attractive vaccine vectors due to their inherent ability to elicit potent immune responses against the transgene product (Babiuk and Tikoo 2000; Tatsis and Ertl 2004; Barouch and Nabel 2005; Yang, Millar et al. 2007). However, efforts to develop Ad vector-based vaccines have been hampered by pre-existing anti-Ad5 immunity elicited by natural infections. A number of studies are underway to overcome these hurdles, for example, using novel delivery vehicles, Ad5 hexon modifications and mucosal administration (Gallichan and Rosenthal 1996; O'Riordan, Lachapelle et al. 1999; Havenga, Lemckert et al. 2002; Sailaja, HogenEsch et

al. 2002; Xiang, Gao et al. 2003; Ophorst, Kostense et al. 2004; Tatsis and Ertl 2004; Barouch and Nabel 2005; Yang, Millar et al. 2007).

1.5.2 Systemic vs. *mucosal immunization with Ad vectors*

Since 1971, adenoviruses of serotypes 4 and 7 have been used as oral vaccines effective against adenovirus infections (Yang, Millar et al. 2007). Ad5 vectors have proven to be highly immunogenic, inducing protective, transgene-specific, humoral and cell-mediated responses in rodents, dogs, and non-human primates, in preclinical studies (Tatsis, Fitzgerald et al. 2007; Yang, Millar et al. 2007). Parenteral Ad-based immunization provides protection from a variety of pathogens, which include, for example, simian-human immunodeficiency virus (SHIV), hepatitis B virus, hepatitis C virus, H5N1 influenza virus and measles. More importantly, their natural tropism for mucosal surfaces due to the abundant expression of CAR on epithelial cells makes Ad5-based vectors excellent candidates for developing mucosal vaccines. Different routes of mucosal delivery of Ad-based vectors were evaluated, which included intranasal (*i.n.*), intratracheal (*i.t.*), intravaginal (*i.vag.*) and intrarectal (*i.r.*) (Gallichan and Rosenthal 1996; Kolb, Margetts et al. 2001; Li, Zhang et al. 2008; Zhu, Thomson et al. 2008). Intranasal administration of Ad5 vectors expressing immunodominant *M. tuberculosis* antigen have proven to be highly effective in the induction of potent mucosal immune responses which protect the mice from *i.t. M. tuberculosis* challenge (Bogers, Bergmeier et al. 2004). Intramuscular (*i.m.*) immunization with the same construct did not protect mice from mucosal challenge with *M. tuberculosis* as it failed to populate the lumen of

the respiratory tract with antigen-specific effector T cells (Santosuosso, McCormick et al. 2007). Also, *i.n.* administration of an Ad5 vector expressing bovine herpes virus glycoprotein D led to induction of protective immunity to intranasally-delivered bovine herpes virus type 1 challenge (Gogev, Vanderheijden et al. 2002). Furthermore, *i.n.* Ad5-based immunization effectively primes distal mucosal sites. Mice primed *i.n.* with Ad5 vectors expressing the HSV glycoprotein B were protected from both long- and short-term *i.vag.* herpes simplex virus type 2 (HSV-2) challenge (Gallichan and Rosenthal 1996). In contrast, intraperitoneal (*i.p.*) delivery of glycoprotein B encoding Ad vectors resulted in short-term protection only.

1.5.3 Rectal Ad-based immunization

The utility of the intrarectal (*i.r.*) delivery of Ad5 vectors has been explored. Adenoviral-based gene transfer *via* the intestinal lumen is impeded severely by several intestinal defense mechanisms including tight junctions, numerous antimicrobial peptides and immunoglobulins, which are part of brush border glycocalyx, and mucus. However, an ethanol enema pretreatment breaches these mucosal defences and allows Ad-based vectors to infect the colonic mucosa (Zhu, Thomson et al. 2008). When carried out successfully, gene delivery to the lumen of the mouse colon, using recombinant adenoviruses, leads to expression of the transgene by colonic epithelial cells. The highest levels of transgene-expression were observed within 48-hours of gene delivery, which is followed by low but detectable levels of gene expression for between 3 and 8 days post-transfer (Wirtz, Galle et al. 1999; Wirtz and Neurath 2003; Vallance, Gunawan et al.

2005; Zhu, Thomson et al. 2008). Unlike *i.m.*, *i.v.* or *i.n.* modes of adenoviral-based gene delivery, where antigen is maintained for a prolonged period of time, the duration of transgene expression in the *i.r.* model appeared to be limited by rapid turnover of the gut epithelium (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006). Intrarectal delivery also resulted in limited transgene expression in the spleen and liver, where the antigen might persist (Wirtz, Galle et al. 1999; Wirtz and Neurath 2003) and in efficient priming of colonic and vaginal mucosal sites, providing protection from both *i.r.* and *i.vag.* HSV-2 challenge (Zhu, Thomson et al. 2008). However, the kinetics and quality of the immune response induced by *i.r.* delivery of Ad5 vectors have not been examined previously.

1.5.4 T cell-mediated of immune response following Ad priming

While Ad vectors have proven efficacious in promoting the development of antigen-specific cellular immunity, limited information is available about the kinetics and nature of antigen-specific mucosal and systemic T-cell responses, especially following mucosal administration of Ad5-based vectors. Ad5 viruses are known to elicit a more predominant $CD8^+$ T cell response, although they also induce $CD4^+$ T cells (less than a tenth of the magnitude of $CD8^+$ T cell response) and an antibody response (Yang, Millar et al. 2007). Until recently, the kinetics and qualitative parameters of Ad-induced $CD8^+$ T cell responses were not defined. However, a number of recent studies have undertaken extensive characterization of systemic and mucosal immune responses following systemic Ad-based immunization (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006; Tatsis, Fitzgerald et al. 2007). Although the kinetics of $CD8^+$ T cell responses to parenteral

immunization varies depending on the mode of delivery, due to the differences in the timing of antigen exposure, immune responses to the transgene peaks around day 14 post-immunization, with the greatest percentage of antigen-specific T cells present at peripheral sites, such as in the lungs and liver. However, the kinetics and phenotype of mucosal and systemic $CD4^+$ and $CD8^+$ T cells following mucosal administration of Ad5-based vectors remains largely undefined. Antigen-specific $CD8^+$ T cells induced in the course of systemic Ad immunizations are capable also of cytolytic activities which are maintained *in vivo* for at least 30 days (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006). Ad-virus immunization is known to induce mainly IFN-γ- and some IFN-γ-TNF-α-producing $CD8^+$ T cells that produce relatively little IL-2. Unlike $CD8^+$ T cell responses, $CD4^+$ T cells induced in the course of immunization appeared to have a normal cytokine profile with 80-90% of $CD4^+$ T cells producing both IFN-γ and TNF-α and 40-50% of $CD4^+$ T cells secreting IL-2 (Yang, Millar et al. 2007). The surface and functional phenotype of $CD8^+$ T cells elicited in the course of Ad5-based immunization is more characteristic of chronic infections, such as polyoma virus or cytomegalovirus, rather than acute infections such as influenza virus or LCMV (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006; Tatsis, Fitzgerald et al. 2007). At least some features of this phenotype might be a consequence of the prolonged antigen exposure and a protracted contraction period associated with some but not all models of Ad immunization.

Overall, an in-depth understanding of T-cell activation, survival and function following mucosal Ad administration is necessary for successful manipulation of the T-

cell compartment and effective application of recombinant Ad5 vectors as mucosal vaccines.

1.6 Utility of lentivirus vector-based immunotherapy

1.6.1 Lentiviruses as gene transfer vectors

Vectors derived from retroviruses, such as oncoretroviruses and lentiviruses, have long been considered to be promising candidates for therapeutic gene transfer. These viruses allow stable integration of a transgene and its propagation in daughter cells. However, there are drawbacks associated with oncoretroviruses, particularly murine leukemia viruses (MLVs), which mainly transduce dividing cells and occasionally cause neoplastic proliferation due to a particular preference for integration upstream of a transcriptional initiation site (Li, Dullmann et al. 2002; Trono 2003). Lentivirus vectors, on the other hand, have shown promise in the transduction of non-proliferating cells types, which include retinal cells, pancreatic islet cells, cells of the central nervous system and hematopoietic cells (Cronin, Zhang et al. 2005). Different lentiviruses have been used as gene transfer vectors. These include vectors derived from human (HIV-1 and HIV-2), simian (SIV), feline (FIV) or bovine (BIV) immunodeficiency viruses, the caprine arthritis encephalopathy virus (CAEV), the equine infectious anemia virus (EIAV), and the Jembrana disease virus (JDV) of bovine origin (Cronin, Zhang et al. 2005). Unlike oncoretroviruses, lentivirus vectors based on HIV-1 integrate within transcriptional units (but not upstream of transcriptional sites) and have never been observed to cause neoplastic growth. In addition, unlike Ad-based immunotherapy, lentivirus vectors do not

induce the type of anti-vector immunity that limits their repeated administration and pre-existing immunity to these vectors is rare. In this thesis, an emphasis on the use of HIV-1 lentiviral vectors will be presented.

1.6.2 Different generations of lentiviral constructs

Although, during the present studies, first generation lentiviral vectors were used, second and third generations of these vectors contain a plethora of safety features that place them amongst the safest viral gene delivery vehicles available currently (Delenda 2004). While an in-depth discussion of the different generations of lentiviral platforms is beyond the focus of this thesis, it is worth mentioning that removal of accessory genes, which are not responsible for viral replication, from first generation lentiviral vectors resulted in what is known as second generation vectors. It appears that removal of accessory genes does not have a negative effect on vector yield. Since accessory genes are crucial for viral pathogenesis, their removal might result in increased immunogenicity of the vectors and alter the transduction efficiency of some cell types. Further optimization of the regulatory elements contained in the lentiviral transfer vector backbone has resulted in so-called third generation lentiviral vectors. Although, third generation vectors display a small reduction in the packaging activity, the safety of these vectors is much improved (Delenda 2004).

1.6.3 Pseudotyping of lentivirus-based vectors

1.6.3.1 Pseudotyping with vesicular stomatitis virus glycoprotein G

Pseudotyping is a process by which enveloped viruses can incorporate heterologous viral glycoproteins into their membranes during budding. In the process, pseudotyped vectors acquire the cellular tropism of the virus from which the glycoprotein was derived. The most commonly used glycoprotein for pseudotyping lentiviral vectors is the vesicular stomatitis virus glycoprotein (VSVG) (Cronin, Zhang et al. 2005). VSVG pseudotyping confers high vector particle stability, such that the resulting vectors are resistant to freeze-thaw cycles and ultracentrifugation. The VSVG pseudotyped vectors are able to grow to relatively high titers *in vitro*. The resulting pseudotype has very broad tropism and can integrate into non-proliferating target cells. A potential drawback for *in vivo* delivery is the fact that VSVG-pseudotyped vectors are rapidly inactivated by human serum. Studies utilizing VSVG pseudotyped lentiviral vector constructs with deletion of some accessory genes have indicated that inserts larger than 5.5 kb of exogenous DNA start to severely impact the yield of the virus, although there appears to be no absolute packaging limit (Delenda 2004). The widespread use of VSVG-pseudotyped lentivirus vectors has made these vectors the standard against which lentiviral vectors pseudotyped with other glycoproteins are evaluated in terms of titer, viral particle stability, toxicity, and host-cell specificity (Cronin, Zhang et al. 2005).

1.6.3.2 Tissue targeting by the means of pseudotyping

Over the course of development of lentiviral vectors for the purposes of targeting gene transfer to specific tissues or cell types, a variety of different glycoproteins have been evaluated. For example, lentiviral vectors pseudotyped with rabies virus RabERA strain glycoprotein target motor neurons, while those pseudotyped with gibbon ape leukemia virus and the cat endogenous retroviral glycoproteins target progenitor and differentiated hematopoetic stem cells (Cronin, Zhang et al. 2005). The devastating mortality associated with cystic fibrosis has prompted development of gene-transfer approaches that particularly target lung mucosa. Initially, mucosal Ad-based gene delivery showed promise, resulting in profound target gene expression in the respiratory mucosa in different rodent models (Boucher 1999; Ferrari, Geddes et al. 2002). As previously discussed, the concerns of pre-existing immunity as well as potent immune response to the vector prevents its re-administration and severely limits the utility of this approach for gene-transfer to the airways in humans. Therapeutic gene delivery to the respiratory mucosa *via* integrating retro- and lentiviral vectors has been attempted as an alternative strategy. In particular, lentiviral vectors pseudotyped with a number of different viral glycoproteins have been evaluated for the purposes of gene transfer to airways. These include lentiviral vectors pseudotyped with VSVG, the envelope glycoprotein of the Zaïre strain of Ebola virus (EboZ), the envelope glycoprotein of Jaagsiekte sheep retrovirus, baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (GP64) and some others (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003; Liu, Halbert et al. 2004; Sinn, Arias et al. 2008). Although commonly used, VSVG-pseudotyped lentiviral

vectors do not efficiently transduce lung epithelial cells. When applied apically, lentiviral vectors pseudotyped with GP64 and Ebola glycoprotein have been shown to be promising tools for *in vitro* and *in vivo* transduction of nasal and respiratory epithelia (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003; Sinn, Arias et al. 2008). For the purposes of this thesis, *i.t.* gene delivery of Ebola pseudotyped-lentiviral vectors will be explored in detail.

1.6.3.3 Pseudotyping with Ebola Zaïre strain glycoprotein

The Ebola virus is an enveloped, non-segmented, negative-sense RNA virus that, together with the closely related Marburg virus, constitutes the filovirus family. Ebola viruses are the causative agents of a hemorrhagic fever disease that has up to 88% mortality rates in humans. The Zaïre strain of Ebola virus expresses a single 140kDa membrane-anchored glycoprotein (EboZ) that is known to exist as a homotrimer and is presumed to be responsible for viral entry into target cells (Wool-Lewis and Bates 1998). EboZ can be packaged with an HIV-based vector (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003). Since EboZ confers specific tropism to lentiviral vectors, its titers, as established by limiting dilution on target cells, are smaller (100-fold) than titers of VSVG pseudotyped vector. However, the reverse transcriptase activity of EboZ-pseudotyped lentiviral vector stocks is comparable to VSVG-pseudotyped lentiviral stocks, thus indicating the presence of similar amounts of virus-like particles in the concentrated preparations. Although the cellular entry of retroviruses pseudotyped with filoviral envelope glycoproteins was initially reported to be dependent on the interaction

with folate receptor α (FRα) (Chan, Empig et al. 2001), viral entry can occur in an FRα-independent manner (Sinn, Hickey et al. 2003). EboZ-pseudotyped lentiviral vectors were specifically evaluated for the purposes of gene-transfer to airway epithelia. Unlike VSVG-pseudotyped lentiviral vectors, those pseudotyped with EboZ efficiently transduce the airway epithelium from the apical surface, both *in vivo* and *in vitro* (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003). Following *i.t.* gene-delivery of EboZ-pseudotyped vectors in a murine model, minimal expression of the transgene can be observed at day 7, while strong expression was achieved by day 28 and still persisted at day 63 (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003). EboZ-pseudotyped lentiviral vectors efficiently transduce cells of the tracheal epithelium, submucosal glands, small airway and alveolar cells. To further increase the titers and improve safety of filovirus-pseudotyped lentiviral vectors, a number of EboZ variants containing internal deletions of the highly variable, mucin-rich region of the glycoprotein were generated and tested (Medina, Kobinger et al. 2003). Pseudotyping with a EboZ variant containing a 217 amino acid deletion in the mucin-rich region (EboZ-non toxic, deletion, lung variant 6 (NTDL6)) led to improved titers of the resulting lentiviral vector that was able to transduce the airway epithelia with efficiency similar to that of unmodified EboZ (Medina, Kobinger et al. 2003). Since *i.t.* administration of lentiviral vectors pseudotyped with filoviral glycoproteins resulted in the efficient transduction of respiratory mucosa, one of the aims of this thesis was to evaluate both EboZ and EboZNTDL6-pseudotyped lentiviral vectors for the purposes of targeting the colonic mucosa.

1.6.4 T cell-mediated immune response to lentivirus vectors

While numerous studies have addressed the longevity of gene expression following different systemic and mucosal modes of administration of lentiviral vectors, information about the kinetics and quality of the immune response is limited. Subcutaneous and *i.v.* delivery of the most widely used VSVG-pseudotyped second or third generation constructs based on HIV-1, were able to induce a cytotoxic $CD8^+$ T cell response that peaked around day 14 post-immunization and then declined by day 60 (Chapatte, Colombetti et al. 2006; Garcia Casado, Janda et al. 2008). At the peak of the immune response, a large fraction of antigen-specific $CD8^+$ T cells in the peripheral blood displayed an activated phenotype, characterized by low expression of CD62L. Antigen-specific $CD8^+$ T cells in the systemic compartment were capable of producing IFN-γ and were able to kill target cells in antigen-specific manner. It remains to be determined whether antigen-specific $CD8^+$ T cells induced by lentiviral immunization are capable also of secreting TNF-α and IL-2. Since only the induction of systemic immune responses was monitored, the kinetics of mucosal immune responses induced by systemic administration of VSVG-pseudotyped vectors remains undefined. It has been shown also that lentiviral vectors induce cytotoxic T cell responses to melanoma antigens that can protect mice from melanoma challenge (Dullaers, Van Meirvenne et al. 2006). Primary responses induced in the course of lentiviral immunization are $CD4^+$-cell dependent (Esslinger, Chapatte et al. 2003). Therefore, lentiviral vectors are potential tools for active immunization. However, in order to fully evaluate the potential of VSVG-pseudotyped lentiviral vectors as systemic vaccines, further characterization of $CD8^+$ T

immune response needs to be carried out. Although, systemically-delivered VSVG-pseudotyped HIV-1 based vectors also induce humoral immune responses, these will not be discussed here. So far, mucosally delivered lentiviral vectors have not been evaluated for the purposes of mucosal vaccination. However, mucosal-delivery of a GP64-pseudotyped feline immunodeficiency virus (FIV)-based vector has been shown to induce a humoral immune response in the circulation and lung upon repeated administration (Sinn, Arias et al. 2008). This response is significantly lower than the humoral immune response induced by *i.n.* Ad-based delivery and does not prevent *i.n.* re-administration of the same lentiviral construct.

1.7 Prime-boost approach

1.7.1 Different prime-boost strategies

Ideally, a single dose of vaccine should confer robust, life-long immunity. This is especially relevant in countries with limited access to health services. In practice, very few vaccines have achieved this goal and most vaccines are administered repeatedly and in combination to increase their immunogenicity. This approach is generically referred to as "prime-boosting". While booster immunization with the same vaccine (homologous boosting) often results in enhancement of the humoral immune response, it can be inefficient at enhancing cellular immune responses, since prior immunity to the vaccine immunogen(s) effectively limits its antigen presentation and the generation of appropriate signals (McShane and Hill 2005). This is relevant for viruses, such as vaccinia and adenovirus, which are known to induce antibody and T cell-mediated responses that are

capable of neutralizing viral infection and, thus, limit the efficacy of re-administering the same virus. To circumvent this problem, a strategy known as heterologous prime-boost, can be employed (McShane and Hill 2005). It involves administering different vaccines that encode the same antigens at various time points by the same or alternative routes. The utility of this approach was first described, when influenza virus-induced $CD8^+$ T cells responses were effectively boosted by vaccinia virus and protected mice from malaria virus challenge (Woodland 2004). The hallmark of this approach was the resultant synergy between two vaccines, such that sequential administration of different immunizing agents achieved levels of immunity that were superior to those induced by multiple administrations of the same vaccine. Over the years, the protective efficacy of prime-boost vaccination strategies has been demonstrated for variety of pathogens, including *M. tuberculosis*, HIV, malaria, *L. monocytogenes*, Ebola virus and others (Woodland 2004; McShane and Hill 2005). Heterologous prime-boost vaccination regimens often combine different vaccines, such as naked DNA, subunit and live, viral vectored vaccines. Numerous combinations of viral vectors have been used also in heterologous prime-boost studies, of which poxvirus- and Ad-based vectors are most commonly used. One advantage of these large viruses is that they can be engineered to carry not only one or more antigens, but also a panel of co-stimulatory molecules to improve the immunogenicity of the vaccination.

1.7.2 Mechanism of action of heterologous prime-boost

The exact mechanisms by which heterologous prime-boost immunization strategies induce such potent immune responses, as compared to homologous prime-boosting with the same vector, are not fully elucidated. Although possible reasons for the success of certain prime-boost regimens include the nature of the immunizing agents as well as the ability of the booster immunization to preferentially expand the T cell population(s) specific for the particular immunodominant epitope(s), primed during the initial immunization (Woodland 2004). In some instances, not only is the maginitude of the immune response dramatically enhanced upon secondary immunization, but also the avidity of the T cell response is improved. For example, in a system where DNA immunization was followed by recombinant poxvirus boost, the resulting antigen-specific T cells were able to eliminate target cells pulsed with 10-100-fold less immunogenic peptide than T cells from mice immunized with either vector alone (Gherardi and Esteban 2005). In addition, many of the viral vectors are highly immunogenic and composed of many antigens which results in the expansion of immune responses to the virus but not the transgene of interest (Robert-Guroff 2007). Therefore, the use of one vector to prime and different viral vector to boost should focus the expansion of the cell-mediated immune response on the target antigen rather than on native viral proteins. Furthermore, both recombinant pox-viruses and recombinant Ad viruses induce non-specific, co-stimulated responses that expand pre-existing memory T cells due to potent adjuvant properties and bystander activation (Robert-Guroff 2007). The optimal combination of antigens, dose, order and timing in which they should be used to prime and subsequently

boost the immune response remains a subject of intense discussion. Although largely dependent on the immunizing agent, it appears that modestly immunogenic vaccines should be delivered first, followed by the boost with a highly immunogenic immunizing agent (Woodland 2004). The timing of the booster immunization delivery should be determined strongly by the kinetics of contraction of the primary immune response (Woodland 2004). As such, booster vaccines can be delivered sooner if the primary immunization has fast contraction kinetics. This contraction might be dictated by many factors including antigen maintenance. For example, prolonged contraction following *i.m.* delivery of Ad- and adeno-associated virus-based vectors, especially when high doses of these vectors were used, was reported to be strongly dependent on presence of antigen (Yang, Millar et al. 2006; Lin, Hensley et al. 2007; Tatsis, Fitzgerald et al. 2007). Therefore, the primary immune response could not be effectively boosted while the antigen remained present and the primary T-cell responses did not contract (Lin, Hensley et al. 2007; Tatsis, Fitzgerald et al. 2007). In studies using an Ad-based prime and orthopoxvirus modified vaccinia virus Ankara strain (MVA) boost, protection from blood-stage malaria virus could only be achieved if MVA boost was delivered 8 weeks following the primary intradermal immunization, but not when delivered 2 weeks after immunization (Woodland 2004). Lastly, it appears that upon re-exposure to the antigen, memory T cells are driven towards effector-like properties and show preferential accumulation in non-lymphoid tissues, even following sequential exposure to the antigen systemically (Masopust, Vezys et al. 2001; Tatsis, Fitzgerald et al. 2007). It is not yet

know if this phenomenon has positive or negative effects on protective immune responses.

1.7.3 Ad in heterologous prime-boost targeting mucosal surfaces

Systemic prime-boost protocols utilizing Ad-based vectors induce potent cellular and antibody-mediated immune responses and protect mice and non-human primates from systemic challenge with simian-human immunodeficiency virus 89.6P and malaria (Shiver, Fu et al. 2002; Santra, Seaman et al. 2005; Seaman, Santra et al. 2005; Wille-Reece, Flynn et al. 2006). However, for mucosal pathogens, such as HIV and *M. tuberculosis*, it might be important to induce a mucosal immune response at the point of pathogen entry. The natural tropism of Ad-based vectors for epithelial cells make these viruses attractive candidates as vectors for vaccines, and they are extensively used in prime-boost regimens to specifically target induction of local mucosal immune responses. For example, these vectors, when administered *i.n.*, can efficiently boost the local immune responses elicited by parenterally administered *Bacillus-Calmette-Guerin* and provide improved protection from *i.t. M. tuberculosis* challenge (Santosuosso, McCormick et al. 2006). Ad-based *i.n.* boosting, administered after a DNA vaccine prime, elicits immune responses and protection against airborne Venezuelan equine encephalitis virus (Perkins, O'Brien et al. 2006). Intravaginal delivery of *L. monocytogenes* encoding an HIV epitope followed by Ad-based immunization targeting the vaginal mucosa has been shown to lead to induction of strong cellular immunity, especially in vaginal tissue and provided robust protection from *i.vag.* vaccinia challenge

for as long as five months (Li, Zhang et al. 2008). Although studies examining a direct comparison of the systemic and mucosal application of Ad-vectors are rare, there is evidence to indicate that the protective efficacy of mucosal Ad administration is strongly dependent on its ability to recruit antigen-specific T cells in the mucosal compartment. For example, in the study by Li *et al.* (Li, Zhang et al. 2008), it was shown that while *i.m.* Ad boosting resulted in increased systemic frequencies of antigen-specific lymphocytes, *i.vag.* Ad delivery failed to induce high frequencies of vagina-resident antigen-specific T cells. Also, unlike *i.n.* delivered Ad, *i.m.* administration of Ad-vector encoding antigen 85 of *M. tuberculosis* did not induce protection from *i.t.* challenge (Santosuosso, Zhang et al. 2005). However, *i.n.* delivery of non-specific inflammatory agonists, such as Ad vector not encoding any antigens or CpG oligonucleotides, which are known to have immunostimulatory effect, results in recruitment of antigen-specific $CD8^+$ T cells in the airway lumen and short-term immune protection, which then wanes once loss of antigen-specific $CD8^+$ T cells from the lumen occurs. Lastly, other mechanisms might contribute to the potency of Ad-based vectors used in prime-boost regimens. For example, mucosal prior to systemic application of Ad boosting in DNA-primed macaques resulted in greater breadth and strength of SIV-specific systemic T cell responses and exhibited lower vector-specific immune responses (Schulte, Suh et al. 2009). Therefore, different recombinant virus-based prime-boost regimens delivered mucosally can prove to be effective immunization strategies against mucosal pathogens.

Although never previously used together in prime-boost protocols, this thesis examined the pairing of filovirus-pseudotyped lentiviral and Ad-based vectors to assess

the potency of these live vectors in inducing cellular immune responses in the large intestine and evaluated the resultant protective efficacy against *i.vag.* vaccinia virus challenge in mice.

1.8 Vaccinia virus as a tool in immunologic studies

Vaccinia virus is a prototype member of the family Poxviridae that consists of enveloped viruses with double-stranded DNA genomes of 130-300 kb in length. Vaccinia virus is a complex virus having more than 190 open reading frames which allows for the insertion of large foreign DNA fragments (over 25 kb). The high immunogenicity of vaccinia infection, which can produce long-lasting, neutralizing antibodies and cytotoxic T cells, was a crucial factor in the success of vaccinia virus immunizations for the eradication of smallpox (Gherardi and Esteban 2005). Therefore, vaccinia strains and related poxviruses are extensively used for immunotherapy in different animal models and in humans. The efficacy of poxvirus vectors, as inducers of immune responses against a variety of antigens, has been clearly demonstrated. Single, sequential and combination vaccine treatments with replication competent poxviruses elicit protection against a broad range of infectious diseases (Gherardi and Esteban 2005). Although vaccinia virus provides a very effective means of priming and/or boosting the immune responses, it also provides a valuable model for evaluating the potency of the immunization. More importantly, examining a variety of routes of delivery of vaccinia virus has led to the development of both systemic and mucosal challenge models, which include *i.p.*, *i.v.*, *i.n.*, *i.vag.* or *i.r.* Following *i.p.*, *i.vag.* or *i.r.* delivery of vaccinia virus to

naïve/mock immunized mice, the virus can be detected in the ovaries (and other tissues, such as oviducts, spleen and colon) 5-7 days post-inoculation using a plaque assay for live virus or polymerase chain reaction to detect vaccinia virus DNA. Protection in both systemic and mucosal models of vaccinia virus challenge is mediated by antigen-specific cytotoxic $CD8^+$ T cells (Belyakov, Derby et al. 1998; Jiang, Patrick et al. 2005). Therefore, vaccination regimens that result in viral clearance or low titers of vaccinia virus detected in the target tissue can be evaluated as having effectively primed the $CD8^+$ T cell arm of the cellular immune response. Importantly, this live vaccinia virus challenge model provides a functional determination of the efficacy of the immunization scheme in inducing antigen-specific $CD8^+$ T cells. While *i.r.* and *i.vag.* routes of delivery of vaccinia virus were characterized initially as mucosal models, the ability of the virus to target and replicate in non-mucosal compartments, such as the ovaries, precludes their evaluation as strictly mucosal models. While the virus first encounters tissue-resident $CD8^+$ T cells, which might eliminate the virus, ultimately the ability of the virus to access other systemic organs excludes the possibility of determining whether the virus was cleared in the mucosal or systemic compartments. Therefore, this thesis examined *i.vag.* delivery system of vaccinia virus encoding different model antigens to determine the protective efficacy of Ad-based immunization and prime-boost approaches. One of the hallmarks of the *i.vag.* vaccinia challenge model is an age-dependent loss of susceptibility of mice to vaccinia virus infection. To circumvent this problem, the mice were pre-treated with long-term progestin, Depo-Provera®, to sustain them in prolonged diestrus and render them susceptible to *i.vag.* infection with vaccinia (Jiang, Patrick et al. 2005).

1. 9 Model antigens

1.9.1 Gallus gallus *(chicken) ovalbumin*

To investigate the induction of antigen-specific $CD8^+$ T cell immune responses and to evaluate the potency of *i.r.* Ad vaccination, Ad- and vaccinia vectors were engineered to encode chicken ovalbumin (OVA). OVA was chosen as a model antigen, as it has been used in many earlier studies in C57BL/6 mice and the immunologic properties of the molecule are well documented, including the specific H-$2K^b$-restricted immunodominant $CD8^+$ T cell epitope, SIINFEKL ($OVA_{257-264}$) . This immunodominant peptide can be used directly for ICS, pulsing of target cells or can be incorporated into MHC class I tetramers to fully characterize the magnitude and the quality of the $CD8^+$ T cell response induced by *i.r.* AdOVA immunization. In addition, the availability of SIINFEKL T-cell receptor transgenic OT-1 mice on a C57BL/6 background (henceforth referred to as OT-1 mice) provided an important immunological tool for *in vitro* and *in vivo* proliferation studies.

1.9.2 β-Galactosidase

In order to assess the comparative ability of filovirus pseudotyped lentiviral vectors to boost immune responses induced by Ad immunization, Ad-, lentivirus-, and vaccinia-based vectors were engineered to encode *E .coli* β-galactosidase (β-gal). β-gal is a commonly used reporter molecule that allows visualization of virally infected cells in culture and in animal tissues. This enzyme cleaves the organic compound 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) provided exogenously, yielding galactose

and 5-bromo-3-chloro-3-hydroxyindole, which is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble, blue product. Although gene expression studies using β-gal are not as sensitive as those utilizing *Photinus pyralis* (firefly) luciferase as reporter gene, these studies do not require special equipment for detection of bioluminescence and are not time-sensitive. More importantly, the immunologic properties of β-gal in C57BL/6 mice, including H-2K^b-restricted immunodominant CD8$^+$ T cell peptides DAPIYTNV (β-gal$_{96-103}$) and ICPMYARV (β-gal$_{497-504}$), are well-documented. Therefore, viral titration, *in vivo* gene expression and characterization of CD8$^+$ T-cell response studies can be carried out with the same model antigen.

1.10 Herpes simplex virus type 2 (HSV-2) infection

1.10.1 HSV-2 pathogenesis and epidemiology

The plethora of vaccination strategies that have been developed in recent years is only exceeded by the range of pathogens against which they have been evaluated. While many of these have proved to be useful immunologic tools for evaluating the responses induced by different immunization strategies, these strategies were ultimately designed with clinically relevant infections in mind, such as HSV-2 for which no effective vaccine exists (Koelle and Corey 2008). Vaccines against numerous pathogens have been investigated for many years and a variety of approaches have been developed. These approaches are often first tested in animal models, such as the one previously described that relies on evaluating immune responses to model antigens and utilizing experimental live virus challenge. These are extremely useful immunologic tools for evaluation of the

immune responses induced by different immunization strategies; however, these strategies were ultimately designed with clinically relevant infections in mind, such as HSV-2 for which no effective vaccine currently exists.

HSV-2 is a double-stranded DNA virus with a large, complex genome and is a member of the *Herpesviridae* family. The virion consists of a DNA-containing core, a capsid which surrounds the core, an unstructured tegument surrounding the capsid, and an outer envelope in which 11 different glycoproteins are embedded (Fields, Knipe et al. 2007). Interactions between HSV glycoproteins and cellular receptors results in initial binding and viral entry. Following the entry, viral DNA is delivered to the nucleus, where viral transcription, DNA replication, encapsidation, and egress take place. Viral genes, expressed sequentially, are responsible for mediating these processes. During productive infection as many as 80 viral proteins are expressed. While 47 of these proteins are dispensable for the ability of the virus to replicate *in vitro*, many of them are required for replication in animals or for modifying the host response to the virus (Fields, Knipe et al. 2007).

HSV-2 is an extremely prevalent sexually-transmitted disease that primarily infects the genitourinary tract and surrounding areas. HSV-2 first enters mucosal surfaces by infecting local epithelial cells (Kimura, Koya et al. 2007). Following the infection, HSV induces cells to fuse and form multinucleated giant cells. Cell lysis causes fluid to accumulate between the epidermis and dermal layer of the skin containing cellular debris, inflammatory cells and progeny virions. Rupture of the fluid-filled vesicles results in formation of shallow ulcers. Following the spread in the mucosal sites, HSV-2 enters

sensory neurons where further replication and the establishment of latency can occur. The virus starts being shed about 1 week after entering the body and continues to be shed for about 1-2 weeks (Koelle and Corey 2008). The virus persists for the life due to latency in the infected individual and periodically reactivates, resulting in viral shedding, which may occur in either the presence or absence of clinical symptoms. However, compared to asymptomatic individuals, virus titers from individual vaginal lesions are 100-1000-fold higher and the efficiency of transmission during symptomatic phases is increased (Koelle and Corey 2008). Also, among immunocompetent, HSV-2 seropositive persons, 95% of persons shed HSV-2 25% of the time. This high frequency of reactivation is a major factor in transmission to others.

Although recurrent mucocutaneous infections associated with HSV are painful and socially concerning, medically-serious complications, such as encephalitis, hepatitis and, pneumonia are rare (Koelle and Corey 2008). However, primary HSV infections can be devastating in newborns and in immunosupressed hosts. HSV-2 has estimated seroprevalence rates of 10-30% (Looker, Garnett et al. 2008). Seropositivity increases with age and is higher among females than males. In many countries of sub-Saharan Africa, the prevalence reached a maximum of 70% among women and around 55% among men (Looker, Garnett et al. 2008). In addition, genital herpes is associated with an increased risk of HIV acquisition, HIV transmission and may account for 40-60% of new HIV infections in populations with high HSV-2 prevalence.

1.11.2 HSV-2 infection mouse model

The mouse is an important experimental animal model for studies of immunity to HSV-2 infection. McDermott *et al.,* demonstrated that wild-type HSV-2 *i.vag.* infection of 6-8 week old BALB/c mice resulted in severe neurological disease that killed most mice in 8–14 days (Zhu, Thomson et al. 2008). However, this model was not suitable for long-term studies of immunity because only 10% of mice were susceptible to HSV-2 by 16 weeks of age and C57BL/6 mice are naturally resistant to HSV-2 infection. However, several studies have shown that the age-related and natural resistance of mice to *i.vag.* HSV-2 infection can be overcome by pretreatment with progesterone or the long-acting progestin, Depo-Provera® (Tatsis, Fitzgerald et al. 2007). The progesterone-dominated vaginal epithelium consists of a layer of columnar mucous cells overlying several layers of smaller basal cells. This epithelium is relatively thin and is permeable to a variety of intraluminally-administered proteins. Thus, progestin-induced susceptibility to *i.vag.* HSV-2 infection could result from the increased permeability of the epithelial layer or be influenced by other factors, such as possible changes in virus receptors on the epithelial cells or immune modulation.

A small number of studies have evaluated the ability of HSV-2 to infect the rectal mucosa. As observed in models of *i.r.* Ad infection, mice with intact rectal mucosa are resistant to HSV-2 infection and successful *i.r.* HSV-2 infections require epithelial destruction and/or abrasion of rectal mucosa by agents, such as nonoxynol-9 (Phillips and Zacharopoulos 1998; Zeitlin, Hoen et al. 2001) or an aluminum oxide-coated cylinder. Pretreatment of the rectal mucosa with aqueous ethanol enema, in the same was as

described for *i.r.* Ad delivery, allows HSV-2 to successfully gain entry to the colonic mucosa.

Mice infected *i.vag.* or *i.r.* with HSV-2 display similar symptoms to those experienced by humans. HSV-2 infection in unmanipulated mice results in gross inflammation and lesions in the skin in close proximity to infected site. Once the mucosal defences are breached, HSV-2 enters the dorsal root ganglia and leads to hind limb paralysis. Immunization with relevant HSV antigen can protect the mice from mucosal HSV-2 infection resulting in diminished or complete abrogation of symptoms. Thus, the severity of the herpes virus infection is a sensitive indication of the potency of the induced anti-viral immunity at the mucosal sites (Tatsis, Fitzgerald et al. 2007). Therefore, one of the aims of the research presented in this thesis was to further evaluate the protective efficacy of Ad-based immunization in mucosal HSV-2 challenge models. More importantly, unlike live virus challenge with vaccinia virus, these models provided a mucosally-contained challenge that can evaluate the potency of local immune responses generated during Ad-based priming. Although a mouse model of HSV-2 infection has many attractive features, it does not mimic the relapsing disease pattern of HSV-2 infection in humans presumably due to an inability of the virus to re-activate from murine neurons.

Another HSV-2 mouse model developed has relied on the *i.vag.* delivery of HSV-2 virus containing partial deletion of thymidine kinase gene following the pre-treatment with Depo-Provera® (Zhu, Thomson et al. 2008). This virus does not cause the lethality associated with wild-type HSV-2 virus. More importantly, immunization with this virus leads to protection from wild-type HSV-2 infection and is considered to be the standard

by which murine immunization against HSV-2 is judged (Tatsis, Fitzgerald et al. 2007). However, this model was not utilized in the present studies due to the poor immunogenicity of HSV-2-based vaccines containing extensive deletion of the same gene in humans (Meignier and Roizman 1985; Stanberry, Cunningham et al. 2000).

1.10.3 Glycoproteins as target antigens for vaccine design

To test ability of the *i.r.* Ad-based immunization to induce immunity to HSV-2 infection, HSV-1 viral glycoprotein was expressed in the viral backbone. Viral glycoproteins represent very attractive targets for immunization because they induce strong host immune responses. For example, it has been shown that 70-90% of all T-cells generated in response to HSV-1 infection were directed against a single epitope, the immunodominant peptide of glycoprotein B (gB) (Wallace, Keating et al. 1999). gB is the most highly conserved glycoprotein encoded by herpes viruses (Eberle, Tanamachi et al. 1997). It is required for infectivity and functions to allow penetration of cells by promoting fusion of the virion and plasma membranes. It has been proposed that gB participates in a multisubunit protein complex that controls fusion after initial interactions with cell-surface receptors. According to recent findings, gB functions together with other viral proteins to form a hydrophobic fusion pore (Subramanian and Geraghty 2007).

1.10.4 Correlates of protection

Antibody responses to HSV-2 are broad and react with envelope glycoproteins as well as tegument and capsid proteins. Cervical HSV-specific antibodies (mainly IgGs) can

neutralize virus *in vitro*. Also, passive transfer of monoclonal IgGs can protect mice from systemic HSV-2 challenge. However, immunization of mice deficient in $Ig\mu^{-/-}$ and $IgA^{-/-}$ still leads to protection from *i.vag.* HSV-2 infection, albeit with slower viral clearance kinetics at early time points after challenge (Tatsis, Fitzgerald et al. 2007; Tengvall, O'Hagan et al. 2008). Although the $Ig\mu^{-/-}$ strain of mice does not contain a complete immunoglobulin gene deletion as it retains minimal levels of T-cell independent IgA, the results of protection studies collectively suggest that antibodies do not play a major role in protecting the mice from HSV-2 challenge. However, vaccine formulations that generated antibody levels equivalent to those observed in humans with natural HSV-2 infections have not been effective in clinical trials. In addition, no correlation has been observed between elevated or deficient antibody levels in individual patients and severity of HSV infection. A similar observation has been made in our laboratory, where levels of antibodies induced by *i.r.* Ad immunization in individual mice did not correlate with levels of protection from *i.vag.* HSV-2 challenge (unpublished observation). Therefore, it appears that while antibodies are involved in reducing viral infection at early points post-challenge, their presence is not necessary for viral clearance.

Following immunization, the immune mechanisms responsible for clearing HSV infections are predominantly mediated by T cells (Rouse and Gierynska 2001). Most studies emphasize that protection and clearance of virus from the infected site can be accomplished by either $CD4^+$ or $CD8^+$ T cells, depending on the mouse strain, infection model and virus dose (Milligan, Dudley-McClain et al. 2004) . Some studies have implicated HSV-specific $CD4^+$ T cells as a subset responsible for conferring immunity to

vaginal viral replication (Tengvall, O'Hagan et al. 2008; Gill and Ashkar 2009). In addition, most reports suggest that control of viral reactivation within the nervous system might be mediated by $CD8^+$ T cells (Liu, Halbert et al. 2004; Knickelbein, Khanna et al. 2008; Koelle and Corey 2008). Clinical data indicate that $CD8^+$ T lymphocytes persist in the site of recurrent HSV-2 genital lesions and the kinetics of viral clearance from lesions correlates with the infiltration of $CD8^+$ T cells (Koelle and Corey 2008). It appears that T cell-mediated viral resolution in mucosal HSV-2 models is, at least in part, dependent on the ability of these cells to make IFN-γ. This cytokine, unlike TNF-α, is crucial for HSV-2 viral clearance and animals depleted of IFN-γ at the time of challenge do not effectively resolve HSV-2 infection (Milligan and Bernstein 1997). Thus, questions remain concerning the function of different lymphocyte subsets in immune protection from HSV infection, particularly regarding protection against challenge at mucosal surfaces.

The innate immune mechanisms provide early defense against many viral pathogens. In the genital tract, NK cells are involved in the immune response against HSV-2. Studies by Ashkar *et al.,* have identified NK cells as important players in mediating protection from *i.vag.* HSV-2 infection early post-challenge (Kaushic, Ashkar et al. 2003). Similar to T cell-mediated protection, the ability of NK cells to control *i.vag.* HSV-2 infection might rely on the release of IFN-γ (Torseth and Merigan 1986; Milligan and Bernstein 1997). Also, studies in humans have demonstrated NK cell accumulation at the site of HSV-2 infection (Koelle and Corey 2008). Defects in NK cell function have been shown to be associated with increased HSV-2 viral titers. Although the association is not direct, several studies have found that patients with severe HSV-1 or HSV-2

infections were deficient in NK cells (Biron, Byron et al. 1989; Jawahar, Moody et al. 1996; Dalloul, Oksenhendler et al. 2004).

Establishing the role of innate and adaptive immune responses and defining correlates of protection from HSV-2 infection in different murine models provides valuable information to direct the development of treatment options and vaccine design. While cell-mediated immunity appears crucial for resolution of HSV-2 infections, human correlates of protection remain to be defined.

1.11 Hypothesis and objectives

Priming and presenting antigen at a specific mucosal tissue will lead to induction of local and systemic immune responses and the development of beneficial and effective protective immunity against subsequent pathogen challenge at the mucosal site.

Although, much work has been done to advance an understanding of mucosal immunity and to develop mucosal vaccines, many questions remain unanswered. In the course of the present studies, the utility of adenovirus (Ad) vectors as potential mucosal vaccines was evaluated, and mucosal and systemic T cell responses induced by colonic administration of Ad-based vectors were characterized (*Sections 3.1-3.8*). The present study aimed to improve our understanding of the kinetics of $CD8^+$ T cell responses, as well as their phenotypic and functional characteristics, occuring at the intestinal mucosa and spleen following *i.r.* Ad-based immunization and to evaluate whether the *i.r.* immunization with this vector can protect from mucosal viral challenge. Within this aim,

the ability of *i.r.* Ad-based immunization to prime mucosal and systemic immune responses was compared to systemic routes of Ad immunization.

The experiments described in the second part of these studies (*Sections 3.9-3.12*), the utility of VSVG- and Zaïre strain of Ebola virus-pseudotyped lentiviral vectors for the *in vivo* transduction of the colonic epithelium was evaluated. In addition, the lentivirus-based vectors were also evaluated as potential mucosal vaccines and whether the *i.r.* delivery of these vectors either before or after *i.r.* immunization with Ad-based vectors can boost immune responses induced by either vector alone or lead to enhanced protection from mucosal viral challenge.

Since mucosal surfaces represent the primary site for entry and transmission of HSV-2, the induction of protective immune responses following *i.r.* priming with Ad vector expressing HSV-1 glycoprotein B was evaluated. This Ad-based *i.r.* vaccination has been shown to be effective in establishing protective immunity against a lethal colonic and *i.vag.* HSV-2 virus challenge delivered 2-3 weeks after initial immunization, since no prior knowledge of the kinetics of the immune responses following *i.r.* immunization existed (Zhu, Thomson et al. 2008). The experiments in the present studies (*Sections 3.13-3.16*) evaluated whether this protection can be observed at later times, when primary immune responses to Ad-based vectors subside. More importantly, the correlates of protection were not established previously. Therefore, the present studies were aimed at determining cell subsets that play major roles in resistance against *i.vag.* and *i.r.* delivered HSV-2.

CHAPTER 2 MATERIALS AND METHODS

2.1 Animals

Six to 8-week-old C57Bl/6 female mice were purchased from Charles River Laboratories International Inc. (Wilmington, MA). OT-1, B6.129S2-Cd4tm1Mak/J, B6.129S2-Cd8atm1Mak/J and MHC class II gene-deleted female mice were bred in the Central Animal Facility, McMaster University. B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (CD45.1^{+}) congenic female mice were purchased from Taconic Farms Inc. (Germantown, NY). All animals were housed under specific pathogen-free conditions. Animal handling was carried out in biological safety cabinets. All animal experiments were approved by the Animal Ethics Research Board of McMaster University and conducted in accordance with the regulations of the Canadian Council on Animal Care.

2.2 Cell lines

293 cells (ATCC CRL-1573), 293T cells (ATCC CRL-11268) and CV-1 cells (ATCC CRL-10478) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Inc., Carlsbad, CA) supplemented with 10% v/v fetal bovine serum (FBS) (Invitrogen Inc.), 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen Inc.) and 0.01M N-(2-hydroxyethyl)-piperazine-2"-(2-ethanesulfonic acid) (HEPES), pH 7.2, in a 100% humidified incubator at 37°C. African Green monkey kidney (Vero) cells (ATCC CCL-81) were grown in Minimum Essential Medium α (α-MEM) (Invitrogen Inc.) supplemented with 5% v/v FBS (Invitrogen Inc.), 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Inc.), pH 7.2, in a

5% CO_2/100% humidified incubator at 37°C. 293 cells were used to propagate replication-deficient adenovirus heterologous expression vectors (Ad). 293T cells were used for the generation of lentiviruses pseudotyped with different glycoproteins. CV-1 cells were used for titration of recombinant vaccinia viruses. Vero cells were used for propagation and titration of herpes simplex virus type 2 (HSV-2) strain 333, a gift from Dr. K.L. Rosenthal (McMaster University).

2.3 Viruses

AdOVA (Ad5delE1MCMV-OVA.1) and AdgB (Ad5E3SV40gB) were E1/E3-deleted, replication-deficient Ad heterologous expression vectors encoding full length *Gallus gallus* (chicken) ovalbumin (OVA) (Zhu, Thomson et al. 2008) and glycoprotein B of HSV-1 (Hutchinson, Graham et al. 1993), respectively. Adβ-gal (Ad5E1mCMVCA35LacZ) encoded *E. coli* β-galactosidase (β-gal) (Zhu, Thomson et al. 2008). Addl70 was an E1/E3-deleted virus that does not contain or express a transgene (Addison, CL ,(Zhu, Thomson et al. 2008). VaccOVA was a recombinant vaccinia virus (Vacc) expressing full length OVA. Vaccβ-gal was a recombinant vaccinia virus expressing full length β-gal. VaccOVA and Vaccβ-gal were a gift from Dr. J.W. Yewdell (National Institutes of Health, USA).

Ad vectors were propagated in 293 cells and purified by a CsCl gradient as described previously (Celis 1998). All virus stocks were aliquoted and stored at -70°C until used. HSV-2 strain 333 was propagated in Vero cells as previously described (Zhu, Thomson et al. 2008). Briefly, Vero cells were infected with HSV-2 at a multiplicity of infection

(MOI) of 0.1 in a minimal volume of Vero cell culture medium without FBS at 37°C for 1-2 h. Vero cell culture medium containing 5% FBS was added to the culture and the infected cells were harvested 24-48 h later. To release the virus, the cells were centrifuged (400 × g; 10 min, 4°C) and disrupted by six rounds of sonication for 15 seconds at 25% output power using a Sonicator/Ultrasonic processor (Misonix, Inc., Farmingdale, NY). After centrifugation (400 × g; 10 min, 4°C), the supernatant containing the virus was aliquoted and stored at -70°C until required.

For pseudotyped lentivirus production, the helper packaging construct pCMVΔR8.2 encoding for the human immunodeficiency virus (HIV) helper function, the transfer vector pHR'*LacZ* encoding for β-gal or pHR'*EGFP* encoding for enhanced green fluorescent protein (EGFP) from *Aequorea victoria*, and plasmids encoding for the various viral envelope proteins were used for triple transfection. Plasmids encoding the following viral envelopes were used to generate the pseudotyped viruses: pLTRMVG (encoding for the Rhabdoviridae vesicular stomatitis virus (VSV) G protein envelope), pCB6-Ebo-GP (encoding for the filovirus Ebola Zaire envelope glycoprotein), and pCB6-Ebo-GP-NTDL6 (encoding for the filovirus Ebola Zaire (EboZ) envelope glycoprotein containing 217 amino acid deletion from the variable, mucin-rich GP1 region located downstream from the EboZ binding domain). All plasmids were a kind gift form Dr. J.M. Wilson (University of Pennsylvania). The HIV vector was produced by a triple transfection method using CalPhos kit (Clontech Laboratories, Inc., Takara Bio, Madison, WI) with a 2:4:1 molar ratio of packaging, transfer, and envelope plasmids, respectively. The calcium phosphate transfection procedure was carried out in accordance with the

manufacturer's instructions. Briefly, 160 μg of an endotoxin-free DNA mixture was applied to each 150 mm plate (Thermo Fisher Scientific, Inc., Rochester, NY) of 293T cells grown to 75% confluency. At 48 h after transfection, DMEM medium was added to each plate for 16 h before collection of virus. The medium, containing virus-like particles, was filtered through a 0.45 μm membrane filter (Millipore Inc., Billerica, MA). The cell-free supernatant was concentrated by ultracentrifugation at 141,000 × *g* for 2 h at 4°C using a SW32Ti rotor (Beckman Coulter, Inc., Fullerton, CA) and stored at -70 °C until used (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003).

All virus stocks were diluted in PBS, pH 7.4, and used for both mucosal and systemic inoculations. AdOVA was used for mucosal and parenteral immunizations to characterize the kinetics of the anti-transgene $CD8^+$ T cell response. Vaginal administration of VaccOVA was used in a live virus challenge model to evaluate the protective potential of parental and colonic Ad-based immunization. AdgB was used to evaluate the mechanisms of protection elicited by rectal immunization from both rectal and vaginal HSV-2 challenge. To evaluate the colonic prime and boost strategy, Adβ-gal, EboZβ-gal, EboZEGFP, EboZNTDL6β-gal and VSVGβ-gal viruses were used. Vaginal administration of Vacc β-gal was used in a live virus challenge model to evaluate the protective potential of colonic Ad-based immunization and EboZ-based boost regimen.

2.4 Delivery of viral vectors

2.4.1 Intrarectal delivery of viruses

The protocol for virus-mediated gene delivery to the colonic mucosa was developed previously and leads to substantial foreign gene expression in colonic epithelial cells (Wirtz, Galle et al. 1999; Vallance, Gunawan et al. 2005; Zhu, Thomson et al. 2008). Briefly, C57Bl/6 mice were anaesthetized using Isoflurane® (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, Abbot Laboratories, Ltd., Montreal, QC), given an intrarectal (*i.r.*) enema of 150 µl of aqueous 45 % ethanol solution. To ensure adequate distribution of ethanol throughout the colon, the mice were held in a vertical position for 30 seconds after the instillation. The mice were then kept under anaesthetic for 40 minutes. The colons were flushed with 5 ml of PBS. The mice were allowed to recover for 30 minutes and anaesthetized again. Ad virus vectors were injected, slowly, in a total volume of 100 µl, in PBS, using a 1 ml pipettor. To prevent leakage, the anal opening was temporarily sealed with one drop of Dermabond® adhesive (Johnson & Johnson Inc., Guelph, ON) and the mice were maintained in the anaesthetic machine for another 50 min. At two hours following immunization, the Dermabond® was removed. This colonic delivery technique was used also for the delivery other viruses, such as HSV-2 and differentially pseudotyped lentiviral vectors (Zhu, Thomson et al. 2008).

2.4.2 Intravaginal delivery of viruses

For intravaginal (*i.vag.*) challenge, the mice were pretreated with 3.0 mg of progesterone (Depo-Provera®; Upjohn, Don Mills, ON), subcutaneously, to render them

diestrous and, thereby, increasing their susceptibility to HSV-2 infection (Tatsis, Fitzgerald et al. 2007). Five to seven days later, when the lining of the vaginal epithelium had become thinner, the mice were challenged by *i.vag.* inoculation with recombinant vaccinia virus or HSV-2 in 10 µl of PBS.

2.4.3 Subcutaneous and intramuscular administration of viruses

Mice were injected subcutaneously (*s.c.*) with 200 µl of the Ad-based vector in PBS by inserting a 28-gauge needle between the skin and muscle of the left flank. For intramuscular (*i.m.*) immunization, the virus was diluted in 100 µl of sterile PBS and injected *i.m.* in both rear thighs of each mouse using a 28-gauge needle.

2.5 Clinical pathology scores of HSV-2 infected mice

Rectal and vaginal HSV-2 infection was assessed by monitoring the genital pathology daily and scored on a five point scale: 0, no apparent infection; 1, a slight redness of external vagina and rectum; 2, a redness and swelling of external vagina and rectum; 3, an extreme redness and swelling of external vagina/rectum and hair loss from the surrounding tissues; 4, the appearance of genital ulcerations with severe redness and hair loss from the genital and surrounding tissues; and 5, the appearance of severe genital ulcerations extending to the surrounding tissue and sometimes hind leg paralysis. Animals that developed severe lesions (i.e., a score of 5) were deemed to have reached the pathological endpoint and were euthanized (Zhu, Thomson et al. 2008).

2.6 Sample collection and preparation

2.6.1 Collection of vaginal washings

The vaginal secretions of *i.vag.* challenged mice were collected by pipetting 30 µl of PBS into and out of the vagina six to eight times. This procedure was repeated thrice. This vaginal wash was collected and stored at -70°C until viral titers were determined by virus plaque assays using monolayers of Vero cells as described previously (Zhu, Thomson et al. 2008); (*Section 2.3*).

2.6.2 Collection of tissues

Mice were sacrificed by cervical dislocation and their abdominal cavities were opened. The lungs and spleens were asceptically resected first. Iliac lymph nodes (ILN) were identified on both sides of the abdominal aorta above its bifurcation into the iliac arteries and were aseptically resected. The large intestines, vaginal tracts and ovaries were all aseptically removed. All tissues were placed into tubes containing DMEM without supplements and stored on ice until further processing.

2.6.3 Preparation of T cell populations

Single cell suspensions of spleens or lymph nodes were prepared by gently compressing the tissues between sterile glass slides. The cell suspensions were centrifuged at 400 × *g* for 5 min at 4°C. Erythrocytes were lysed by treating the spleen cells with 2 ml of M-lyse buffer (Mouse Erythrocyte Lysing Kit; R&D Systems, Minneapolis, MN) for 13 min. Following incubation, PBS was added to the splenic cell

suspension and this was centrifuged at 400 × *g* for 5 min at 4°C. Cell pellets were resuspended in PBS at 2×10^7 cells per ml for use in different assays. All subsequent ones were kept at 4°C until further use.

Lamina propria (LP) and intraepithelial lymphocytes (IEL) were isolated, as described previously, with modifications (Belyakov, Moss et al. 1999). Briefly, the lumens of the resected large intestines of individual mice (cecum to rectum) were flushed with PBS. The organs were divided into 5 mm lengths and washed three times in HEPES-buffered, Ca^{2+}, Mg^{2+}-containing Hanks' Balanced Salt Solution (HBSS) (Invitrogen Inc.), pH 7.2,. Intestinal pieces were shaken at 37°C for 20 minutes in 5 mM ethylene diamine tetraacetic acid (EDTA) in HEPES-buffered, Ca^{2+}, Mg^{2+}-free HBSS (Invitrogen Inc.), pH 7.2. This procedure was repeated three times to create a crude cell suspension.

The crude cell suspension which contained IEL was collected and centrifuged 400 × *g* for 5 min at 4°C. The resulting pellet was resuspended in 40% Percoll® solution and centrifuged through a 40%/70% Percoll® gradient (Pharmacia Inc., Uppsala, Sweden) for 30 minutes at 600 × *g* at room temperature. The cells at the interface of the gradient were collected. PBS was added to these cells and the resulting cell suspension was centrifuged at 600 × *g* for 15 min at room temperature. The cell pellet was resuspended in PBS at 2×10^7 cells per ml for use in different assays.

For LP cell isolation, the intestinal tissue was digested in HEPES-buffered Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen Inc.) supplemented with 300 U/ml collagenase type VIII (Sigma-Aldrich, Inc., St. Louis, MO), 10% v/v FBS, 2 mM L-glutamine and 100 μg/ml of gentamycin (Invitrogen Inc.), pH 7.2. The resulting crude

cell suspension was washed by centrifugation in 5% v/v FBS in PBS and centrifuged through a 40%/70% Percoll® gradient (Pharmacia Inc.). The cells at the interface of the gradient were collected. PBS was added to the cells and the resulting cell suspension was centrifuged at 600 × *g* for 15 min at room temperature. Cell pellets were resuspended in PBS at 2×10^7 cells per ml for use in different assays.

For vaginal tract (VG) cell isolation, the entire genital tract (from the cervical/uterine bifurcation distally) was removed and cut into 5 mm pieces, subjected to collagenase type VIII (Sigma-Aldrich) digestion (300 U/ml) in RPMI 1640 supplemented with 10% v/v FBS, 2 mM L-glutamine and 100 μg/ml of gentamycin (Invitrogen Inc.), pH 7.2, and subsequently processed using the procedure described for LP cell isolation.

For lung cell isolation, the lungs and the heart were dissected, and perfused with 10 ml HBSS through the right ventricle to remove erythrocytes. The lungs were separated from the heart, divided into small pieces and digested for 1 h at 37°C in type VIII collagenase-supplemented RPMI 1640 (10% v/v FBS, 2 mM L-glutamine and 100 μg/ml of gentamycin (Invitrogen Inc.), pH 7.2) with continuous agitation. The lung pieces were then passed through a 100-μm pore size cell strainer (Becton Dickinson, Inc., San Diego, CA). Following the addition of PBS, the resultant cell suspension was centrifuged at 400 × *g* for 5 min at 4°C. Cell pellets were resuspended in PBS at 2×10^7 cells per ml for use in different assays.

2.7 Flow cytometry

2.7.1 General flow cytometry procedures

Prior to various staining procedures, the cells suspensions from different tissue origins were filtered through a 40-μm pore size cell strainer (Becton Dickinson, Inc.). For antibody and tetramer staining, the cells were resuspended in PBS supplemented with FACS buffer (0.5% w/v bovine serum albumin; Sigma-Aldrich, Inc.) and aliquoted into round-bottomed, 96-well plates at 2×10^6 cells/well (Becton Dickinson, Inc.). The plates were centrifuged at $400 \times g$ for 5 min at 4°C and the FACS buffer was discarded. Non-specific antibody binding was blocked by first incubating the cell suspensions with 50 μl of purified anti-mouse CD16/CD32 (clone 2.4G2, FcBlock; Becton Dickinson, Inc.), diluted 1:100 in FACS buffer, for 20 min at 4°C. Antibodies and tetramers conjugated to the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin conjugated cyanine dye 5 (PE-Cy5), phycoerythrin conjugated cyanine dye 7 (PE-Cy7) and allophycocyanin (APC) were used to detect different cell surface markers and intracellular cytokines by flow cytometric analyses. To determine the optimal binding concentration of antibodies and tetramers used in the experiments, these reagents were titrated by doubling dilutions from 1:25 to 1:400 in FACS buffer. The optimal dilution was chosen as that greater than the dilution which provided the maximum separation of positive and negative cell populations as judged by flow cytometric fluorescence analyses. All the titrations were conducted on the splenic cell suspensions, except for anti-TCRβ-APC, anti-TCRγδ-PE, anti-CD103-biotin and anti-CD8β-FITC, which were titrated using IEL cell populations. All reactions were conducted in a 50 μl final volume

of FACS buffer and washed using 150 µl of FACS buffer by centrifuging at 400 × *g* for 5 min at 4°C, unless stated otherwise. Cell pellets were resuspended in 200 µl of FACS buffer or fixed in the same volume of 1% w/v paraformaldehyde in PBS. All events were acquired using a LSRII flow cytometer (Becton Dickinson, Inc.) and analyzed using FlowJo flow cytometric software (Tree Star, Inc., Ashland, OR).

2.7.2 Flow cytometry reagents

The following antibodies were used: anti-CD8α-PE-Cy7 (clone 53-6.7, 1:100), anti-CD43-PE (clone 1B11, 1:50), anti-CD44-PE-Cy5 (clone IM7, 1:400), anti-CD44-FITC (1:200), anti-CD107a-FITC (clone 1D4B, 1:100), anti-IFN-γ-APC (clone XMG1.2, 1:100), anti-TNF-α-PE (clone MP6-XT22, 1:100), anti-IL-2-FITC (clone JES6-5H4, 1:100), anti-CD4-PE-Cy5 (clone RM4-5, 1:100), anti-CD8α-APC (clone 53-6.7, 1:100), anti-CD3-PE-Cy7 (clone 145-2C11, 1:100), anti-TCRβ-APC (clone H57-597, 1:100), anti-TCRγδ-PE (clone GL-3, 1:200), anti-CD103-biotin (clone M-290, 1:200) (all obtained from Becton Dickinson, Inc.), anti-CD127-FITC (clone A7R34, 1:25), anti-CD8β-FITC (clone CT-CD8β, 1:100) and anti-CD45.2-PE (clone 104, 1:200) (eBiosciences, Inc., San Diego, CA)). Anti-granzyme B-PE (clone GB12, 1:25) was purchased from Invitrogen, Inc. Streptavidin-conjugated PE-Cy7 (1:400) was purchased from Becton Dickinson, Inc. MHC class I fluorescently-labelled K^b/SIINFEKL (APC), K^b/ICPMYARV (PE), K^b/DAPIYTNV (PE) and K^b/SSIEFARL (PE) tetramers were obtained from the Molecular Biology Core at the Trudeau Institute, Inc. (Saranac Lake, NY).

2.7.3 Tetramer staining

To determine the magnitude of the anti-OVA immune response following colonic Ad virus immunization, splenic and mucosal cells were reacted with fluorescently-labelled K^b/SIINFEKL tetramers for 1 h at 25°C in FACS buffer. The cells were washed by the addition of the FACS buffer and centrifugation at 400 × *g* for 5 min at 4°C. Subsequently, anti-CD8α-PE-Cy7, anti-CD44-PE-Cy5, and anti-CD43-PE and anti-CD127-FITC mAbs were added and incubated for 30 min at 4°C. Following the addition of FACS buffer, the cells were washed by centrifugation at 400 × *g* for 5 min at 4°C. The cells were fixed in 200 µl PBS containing 1% w/v paraformaldehyde (Sigma-Aldrich, Inc.) for further analyses.

To assess the efficiency of AdgB priming, the leukocytes were reacted with K^b/SSIEFARL tetramers and anti-CD8α-PE-Cy7 and anti-CD44-FITC antibodies using the procedure identical to that described for analyses of anti-OVA $CD8^+$ T cell immune responses. The presence of β-gal-specific $CD8^+$ T cells was determined by co-staining isolated splenic and mucosal leukocytes with K^b/ICPMYARV and K^b/DAPIYTNV tetramers together with anti-CD8α-PE-Cy7, anti-CD43-PE and anti-CD44-FITC antibodies using the procedure identical to the one used for SIINFEKL-specific T cells. Flow cytometric analyses were performed using a LSRII flow cytometer (Becton Dickinson, Inc.). Data were analyzed with FlowJo software (Tree Star, Inc.).

2.7.4 Intracellular cytokine staining

Peptide stocks of SIINFEKL, SSIEFARL, ICPMYARV, and DAPIYTNV were prepared in distilled water and stored at -20°C until used. Various tissue cell populations were cultured for 5-6 h at 37°C in the presence or absence of 1 μg/ml of SIINFEKL (the OVA immunodominant class-I epitope $OVA_{257-264}$), SSIEFARL (the HSV-1 gB immunodominant class-I epitope $gB_{498-505}$), ICPMYARV (the β-gal immunodominant class-I epitope β-$gal_{497-504}$) and DAPIYTNV (β-gal the immunodominant class-I epitope β-gal_{96-103}) peptides, all synthesized by Dalton Chemical Laboratories, Toronto, ON. The peptides were prepared in 200 μl of RPMI 1640 supplemented with 10% v/v FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen Inc.), pH 7.2, containing 4 μl/ml of Brefeldin A-containing GolgiPlug™ (Becton Dickinson, Inc.), to halt protein secretion. For analyses of cytokine production by $CD4^+$ T lymphocytes, the cells were cultured for 12 h with 10 μg/ml of gB glycoprotein. Brefeldin A was added for the final 6 h of culture. The cells were washed by the addition of FACS buffer and centrifuged at 400 × *g* for 5 min at 4°C. The cells were assessed for the presence of cell surface antigens by incubation with anti-CD4-PE-Cy5 or anti-CD8α-PE-Cy7 antibodies for 30 min at 4°C. Following the addition of FACS buffer, the cells were washed by centrifugation at 400 × *g* for 5 min at 4°C. The cells were fixed in 100 μl of Cytofix/Cytoperm Buffer (Becton Dickinson, Inc.) for 20 minutes at 4°C and washed twice with Perm/Wash solution (Becton Dickinson, Inc.) by centrifugation at 400 × *g* for 5 min at 4°C. The permeabilized cells were incubated with anti-IFN-γ-APC, anti-TNF-α-PE and anti-IL-2-FITC for 30 minutes at 4°C, washed twice by the addition of

Perm/Wash solution (Becton Dickinson, Inc.), at 400 × *g* for 5 min at 4°C. The cells were resuspended in 200 µl of 1% w/v paraformaldehyde in PBS.

2.7.5 Degranulation assay

Cells were restimulated with SIINFEKL for 5 h at 37°C in the presence of Monensin (to halt cytokine secretion) and anti-CD107a-FITC antibodies (both from Becton Dickinson, Inc.), before assessment for CD8α, intracellular IFN-γ and intracellular TNF-α, using a procedure identical to the that used for intracellular cytokine staining, as described by Betts *et al.* (Betts, Brenchley et al. 2003).

2.7.6 Granzyme B detection

Cells were restimulated with SIINFEKL for 5 h at 37°C in the presence of Monensin (to halt cytokine secretion) and anti-CD107a-FITC antibodies (both from Becton Dickinson, Inc.). Following the incubation with FcBlock, the cells were incubated for 1 h at room temperature with fluorescent anti-Granzyme B antibodies (Becton Dickinson, Inc.), before assessment for CD8α, intracellular IFN-γ using a procedure identical to that used for intracellular cytokine staining.

2.7.7 Adoptive transfer and proliferation of OT-1 cells

Carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) was purchased from Invitrogen Inc. CFDA-SE was kept as a 0.5 mM (100×) stock in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Inc.) and stored at -20°C. Single-cell suspensions from the

lymph nodes of OT-I mice were prepared as described in *Section 2.6.3* and $CD8^+$ cells were purified using a mouse $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec, Inc., Auburn, CA). $CD8^+$ OT-I cells were incubated in 5 μM CFDA-SE for 5 minutes at 25°C. The cells were washed thrice by centrifugation at 400 × *g* for 5 min at 4°C following the addition of FACS buffer. One million OT-1 lymph node cells, resuspended in 200 μl of PBS, were injected *via* a tail vein into each congenic recipient. After 72 h, the recipients were given AdOVA, *i.r.*, as described in *Section 2.4.1.* Four and seven days later, lymphocytes from the spleens, the iliac lymph nodes, the LP and the IEL populations were isolated as described in *Section 2.6.3.* Adoptively transferred cells were identified as such populations by co-staining for CD8α, the congenic marker CD45.2 and K^b/SIINFEKL tetramers. Proliferation was analyzed on a LSR II flow cytometer (Becton Dickinson, Inc.) by the dilution of CFSE fluorescence.

2.7.8 In vivo *cytotoxic assay*

Cytotoxic activity was assessed by the method described by Hermans *et al.* (Hermans, Silk et al. 2004), with modifications. Briefly, splenic single cell suspensions were prepared as described in *Section 2.6.3.* Syngeneic splenocyte populations were labelled with different dye combinations: a control population was labelled with 2.5μM CFSE, a population of cells labelled with 0.05 μM CFSE, a populations labelled with 5 μM (1,3-dichloro-9,9-dimethylacridin-2-one)-succinimidyl ester (DDAO-SE ; Invitrogen, Inc.), a population labelled with 5 μM DDAO-SE and 2.5μM CFSE, and a population labelled with 5 μM DDAO-SE and 0.05μM CFSE. The cells were incubated with the various

reagents for 5 min at 25°C, followed by addition of FACS buffer and centrifugation at 400 × *g* for 5 min at 4°C. Cell pellets were resuspended in RPMI 1640 supplemented with 10% v/v FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Inc.), pH 7.2, and the four populations were exposed to varying concentrations of SIINFEKL peptide (from 10 μM to 0.01 μM in single log increments) by incubating the cells for 1 hr in 100% humidified incubator at 37°C. The control population was untreated. Following the addition of FACS buffer and centrifugation at 400 × *g* for 5 min at 4°C, the 3×10^6 cells from each population were combined in 200 μl of PBS and injected *via* a tail vein into mice immunized with AdOVA 14 days earlier. Twelve hours later, the spleens of injected mice were resected, and splenic cells suspensions were prepared as described in *Section 2.6.3.* The cytotoxicity was evaluated by disappearance of peptide-containing target cells as compared to cells not exposed to the SIINFEKL peptide by flow cytometric analyses.

2.8 Virus titration

2.8.1 Vaccinia virus titration

To determine the viral burden following *i.vag.* VaccOVA or Vaccβ-gal inoculation, mice were sacrificed at day 6 post-infection. The ovaries were harvested and homogenized in 1ml of 1mM Tris buffer (pH 9.0). Viral titers were determined by plaque-forming assays using CV-1 cell monolayers, as previously described (Belyakov, Moss et al. 1999). Briefly, serial 50-fold dilutions of virus in DMEM supplemented with 5% v/v fetal bovine serum (FBS) (Invitrogen Inc.), 50 μg/ml penicillin, 50 μg/ml

streptomycin and 2 mM L-glutamine (Invitrogen Inc.), pH 7.2, were conducted in 12-well plates (Becton Dickinson, Inc.) containing a 90% confluent monolayer of CV-1 cells and incubated at 37°C for 48 h. The medium was removed and the plaques were counterstained with 0.1% w/v crystal violet and enumerated at each dilution. The virus titer was expressed as PFU/ml of homogenate.

2.8.2 HSV-2 virus titration

For titration of HSV-2, vaginal washes were collected at days 1, 2 and 3 post-infection. To determine virus replication in the vaginal tract, virus titers were determined in doubling dilutions of vaginal washes. Briefly, serial 10-fold dilutions of virus in α-MEM 5% v/v fetal bovine serum (FBS) (Invitrogen Inc.), 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Inc.), pH 7.2, were conducted in 24-well plates (Becton Dickinson, Inc.) containing a 90% confluent monolayer of Vero cells and incubated at 37°C for 48 h. The medium was removed and the plaques were counterstained with 5% w/v crystal violet and enumerated at each dilution. The virus titer was expressed as PFU/ml of vaginal wash.

2.8.3 Lentiviral titration

Concentrated virus stocks were thawed and limiting dilutions (50- to 5×10^5-fold) of the viral stocks were then performed on 75% confluent 293T cell monolayers grown in 24-well plates (Becton Dickinson, Inc.). Thirty six hours later, the virus-containing medium was removed and the cells were fixed in 0.05% v/v glutaraldehyde solution in

PBS for 10 min. The monolayers were washed twice in PBS and then assessed for β-galactosidase activity by adding X-Gal solution (3.5×10^{-2} M potassium ferricyanide [$K_3Fe(CN)_6$], 3.5×10^{-2} M potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$], 1×10^{-4} M magnesium chloride ($MgCl_2$), and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal], all prepared in PBS). Virus titers were determined indirectly by microscopic examination of the cells for the presence of β-galactosidase-containing blue cells. Enumeration of β-galactosidase-containing cells demonstrated 3-5 $\times 10^7$ transducing units (TU)/ml for EboZ, 5×10^7 to 1×10^8 TU/ml for EboZNTDL6 and 3 to 5 $\times 10^9$ TU/ml for VSVG pseudotyped lentiviral vectors (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003).

2.9 NK cell and T lymphocyte depletion

$CD4^+$ and $CD8^+$ T cells were depleted at -3, -1, and 1 days before and after immunization by intraperitoneal (*i.p.*) injection of 500 μg anti-CD4 (clone GK1.5; rat IgG2b) and anti-CD8 (clone GK2.43; rat IgG) antibodies in 500 μl of PBS. $CD4^+$ and $CD8^+$ T cells were depleted 6 times at -3, -1, 1, 3, 5, 7 days before and after HSV-2 instillation by *i.p.* injection of 500 μg anti-CD4 and anti-CD8 antibodies in 500 μl of PBS. NK cells were depleted 6 times at -3, -1, 1, 3, 5, 7 days before and after HSV-2 inoculation by *i.p.* injection of 500 μg anti-NK1.1 (clone PK136; mouse IgG2a) in 500 μl of PBS. Mice injected *i.p.* with 500 μg of irrelevant rat IgG or mouse IgG2a in 500 μl of PBS were used as controls. These protocols depleted $CD4^+$, $CD8^+$ and $NK1.1^+$ cells, and this depletion was sustained for at least 10 days as confirmed by FACS analyses of the

single cell suspensions isolated from spleens, iliac lymph nodes, colons and vaginal tracts resected from the antibody-treated mice.

2.10 β-galactosidase gene expression

At different times following EboZ and VSVG pseudotyped lentiviral vector administration, the animals were sacrificed and their lungs inflated with Optimal Cutting Temperature (O.C.T.) Compound (Sakura Finetek, Inc., Torrance, CA)/PBS (combined 1:1, 500 µl per lung) and frozen sections were prepared. For intestinal preparations, colons, from cecum to rectum, were resected and flushed with PBS. The colon was then opened longitudinally, wound "Swiss roll" fashion, mucosa side outwards, and frozen in OCT. Cryosections (10 µm) of lungs and colons were prepared, placed on microscope slides and fixed in 0.05% v/v glutaraldehyde in PBS for 10 min. The slides were rinsed twice with PBS and then assessed for β-galactosidase activity by incubation with X-Gal solution (3.5×10^{-2} M potassium ferricyanide [$K_3Fe(CN)_6$], 3.5×10^{-2} M potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$], 1×10^{-4} M magnesium chloride ($MgCl_2$), and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal] all prepared in PBS) overnight. Images of X-gal-stained tissues where captured using a Leica microscope (Leica Microsystems, Inc., Richmond Hill, ON).

2.11 Quantitative real-time polymerase chain reactions.

Colons were resected asceptically and homogenized in 5 ml of Trizol (Invitrogen, Inc.). RNA was extracted according to the manufacturer's instructions. RNA integrity

and concentration were determined with a microfluidic gel analysis (Agilent 2100, Agilent Technologies, Inc., Santa Clara, CA). RNA (1μg) was DNase-treated and reverse-transcribed using a standard protocol (Invitrogen, Inc.). A quantitative real-time polymerase chain reaction (RT-PCR) for HSV-2 glycoprotein D (gD) to detect HSV-2 infection (to distinguish gB RNA introduced by AdgB immunization) and 18S ribosomal RNA was performed using a 7500 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). Negative control samples (no template or no reverse transcriptase) were included concurrently. The results were normalized to 18S ribosomal RNA. HSV-2 gD and 18S gene-specific primers (HSV gDF, 5'-GTGCCCCTACAACAAGTCGTG-3', HSV gDR, 5'-GCATCAGGAATCCCAGGTTA TC-3', 18SF, 5'-GCCGCTAGAGGTG AAATTCTTG-3', 18SR, 5'-CATTCTTGGCAA ATCCT TTCG-3') (Mobix, Department of Biology, McMaster University, Hamilton, ON) and a fluorescence-labelled probe (HSV gD, 5' Fam-TACTATGACAGCTTTAGCGC-Tamra 3', 18S, 5'-Vic-ACCGGCG CAAGACGGACCAG-Tamra-3') (Applied Biosystems, Inc.) were used to detect HSV-2 mRNA.

2.12 Statistical Analyses

Quantitative data were expressed as the mean ± standard error of the mean (SEM) for at least n = 3. Data shown were representative of at least two separate experiments that yielded similar results. The statistical significance of the flow cytometry data was determined by an unpaired, two-tailed Student's *t* test. Statistical differences of the viral

titers were determined by unpaired, two-tailed, log-transformed Student's t test. Values from these tests of $p < 0.05$ were considered statistically significant.

CHAPTER 3 RESULTS

3.1 Kinetics of expansion and/or recruitment of in vivo *generated effectors*

Since previous studies (Yang, Dayball et al. 2003) showed that mouse $CD8^+$ T cells became activated within 3 days following systemic Ad inoculation, studies were conducted to determine whether a similar $CD8^+$ T cell activation occurred following mucosal inoculation with the heterologous expression vector AdOVA. To determine this, a hybrid mouse model was used in which a known population of OVA-reactive $CD8^+$ T cells was present. To create this model, $CD45.1^+$ congenic mice were engrafted by adoptive transfer with CFSE-labelled, $CD45.2^+$ $CD8^+$ T cells prepared from the systemic lymph nodes of OT-I mice. Since OT-1 T cells bear a transgenic T cell receptor (TCR) specific for the OVA peptide epitope SIINFEKL, in this model, presentation of OVA to such cells induces T cell activation and proliferation only in the OT-I T cell population (Hogquist, Jameson et al. 1994). Thus, engrafted mice were inoculated intrarectally (*i.r.*) 72 h after OT-I T cell transfer with AdOVA or the irrelevant adenovirus Add170. Cell suspensions of their spleens, iliac lymph nodes (ILNs), colons (intraepithelial and lamina propria lymphocytes; IEL and LP, respectively) were prepared 4 and 7 days later. $CD45.2^+$ OT-I $CD8^+$T cells in these preparations were detected by fluorescence flow cytometry. Fig. 3A shows that on both days, compared to animals given Add170, in those infected with AdOVA the majority of the donor cells, 9.2 % and 17.2 %, respectively, were found in the colonic LP although on day 7 increased percentages were found also in the spleen and IEL preparations. Very few OT-I T cells were detectable in the ILN cell preparations of both AdOVA and Add170-immunized mice, thus indicating that antigen-

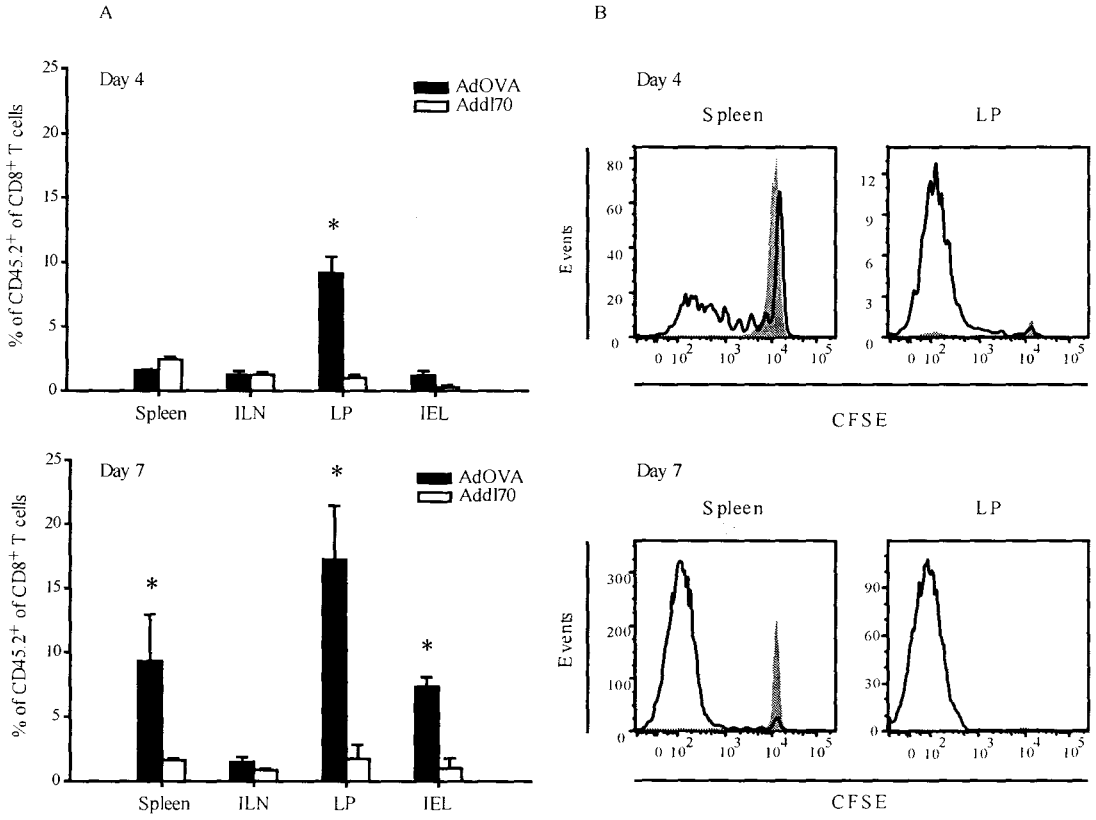
specific T cells were not being recruited to and/or expanded at this site. These results indicated that, initially, OVA specific OT-I $CD8^+$ T cells were found principally at the mucosal site displaying OVA by virtue of colonic AdOVA infection and, subsequently, at distant systemic locations.

Since mitotic division of CFSE-labelled cells decreases their fluorescence intensity by approximately one-half, successive cell divisions of the OT-I transgenic T cells could be detected following exposure to OVA (De Boer, Ganusov et al. 2006). Therefore, *in vivo* OT-I cell division in different tissue sites was examined by observing the decrease of CFSE fluorescence using flow cytometry. The decline in cytoplasmic CFSE fluorescence intensity was observed in all cell preparations on days 4 and 7, thereby confirming the ability of the method to detect the dividing cells (Fig. 3B and data not shown). The percentage of proliferating cells was determined by the summation of percentages of cells that had undergone one or more rounds of cell division. At day 4 post-immunization, 41 % and 64% of donor cells recovered from the spleens and ILNs, respectively, were proliferating cells, and only 10% of these had undergone six or more cell divisions (Fig. 3B and data not shown). At this time post-immunization, greater than 90% of OT-I T cells found at the mucosal sites were proliferating cells, and >60% of these had divided six or more times (Fig. 3B and data not shown). By 7 days post-priming, greater than 95% of all donor cells in all organ sites examined had lost detectable CFSE (Fig. 3B and data not shown), thus indicating that they had undergone more than six rounds of division (De Boer, Ganusov et al. 2006). These data showed that mucosal AdOVA immunization activated transgenic OT-I cells and elicited their expansion in and/or recruitment to both

systemic and colonic compartments. The presence of T cells that had undergone six or more cell divisions in the colonic compartments is consistent with the antigen-driven migration of activated effector T cells into non-lymphoid tissues, such as the colonic LP, following Ad vector infection.

Figure 3. Kinetics of tissue accumulation and proliferation of OT-I cells in AdOVA immunized mice. To follow the proliferation of naïve $CD8^+$ T lymphocytes *in vivo* after intrarectal (*i.r.*) immunization, 1×10^6 naïve CFSE-labeled $CD8^+$ T cells from the systemic lymph nodes of OT-I mice were engrafted into $CD45.1^+$ congenic mice ($B6.SJL\text{-}Ptprc^aPep3^b/BoyJ$). Engrafted mice were primed with AdOVA or Adl170 *via* the colonic mucosa 72 hours later. Four and 7 days after inoculation, cells from the spleens, ILNs, IEL and LP populations were isolated. In panel *A*, the histogram represents the percentage of donor $CD45.2^+$ $CD8^+$ T cells as detected by flow cytometry. The results represent the mean ± SEM of 4 mice per group. Student's t test was used to compare the significance of data between AdOVA- and Adl70-immunized mice and significant values were represented by an asterisk ($P < 0.05$). In panel B, 4 and 7 days post-immunization, $CD45.2^+$ $CD8^+$ transgenic T cells in the spleen, ILN, IEL and LP compartments shown in panel A were analyzed for cell division by detecting intracellular fluorescence of CFSE using flow cytometry. The shaded areas correspond to total $CD8^+$ T cells and the open areas correspond to tetramer-reactive T cells. The results represent one of two independent experiments.

Figure 3


A
B
Day 4
AdOVA
Add170
% of CD45.2+ of CD8+ T cells
Spleen
ILN
LP
IEL
Day 7
Day 4
Spleen
LP
Events
CFSE
Day 7
Spleen
LP
Events
CFSE

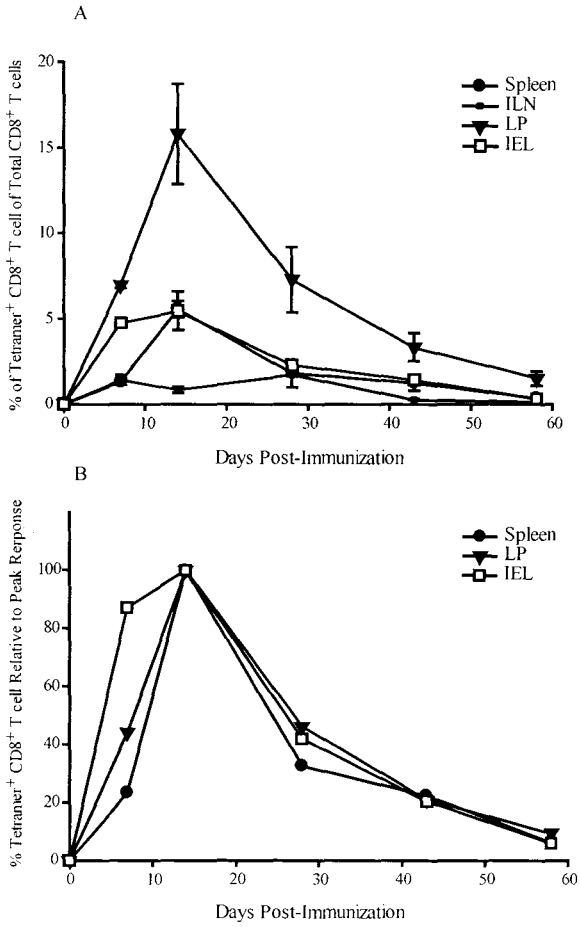
3.2 Tissue-specific kinetics of SIINFEKL-specific CD8$^+$ T cell responses to intrarectal Ad immunization

Although the engraftment mouse model utilizing transgenic TCR T cells provided a tool for examining the *in vivo* activation of T cells in systemic and mucosal sites following mucosal immunization, it has deficiencies including the inability to measure *in situ* mucosal activation of CD8$^+$ T cells and the possibility that engrafted transgenic TCR T cells, when used in high initial naïve T cell precursor frequencies, might undergo a different programming pathway of memory differentiation as compared to the endogenous T cell population, thereby resulting in different activation kinetics (Masopust, Vezys et al. 2001). To surmount these difficulties, fluorescent tetrameric MHC class I-SIINFEKL peptide complexes (operationally termed tetramers) were used to identify antigen-specific T cells by flow cytometric analysis *in situ*. Therefore, to characterize mucosal and systemic immune responses in unengrafted mice that received colonic immunization with AdOVA, K^b/SIINFEKL tetramers were utilized to quantify endogenous OVA-specific CD8$^+$ T cell responses in cells recovered from the splenic, ILN, colonic IEL, and colonic LP lymphocyte populations. Tetramer binding to OVA-specific CD8$^+$ T cells was analyzed on days 7 through 58 post-immunization. The response kinetics revealed distinct expansion and contraction phases of the relevant T cell populations as determined by the enumeration of tetramer-positive cells (Fig. 4A). In absolute terms, the percentage of tetramer-binding cells (the CD8$^+$ T cell response to SIINFEKL) was maximal in the spleen, colonic LP and IEL populations at 14 days post *i.r.* immunization and declined by day 58 (Fig. 4A). The highest percentage, 15.8% of

SIINFEKL-specific $CD8^+$ T cells, was detected in the LP cell population at day 14 post-immunization. No antigen-specific lymphocytes were found in the ILN. The greatest number of OVA-specific T cells in the LP population was consistent with the presence of antigen at the colonic mucosa. Therefore, expansion and contraction of the SIINFEKL-specific T cell populations occurred at the mucosal and systemic sites previously identified by transgenic TCR T cell transfer experiments, with the LP compartment being the largest reservoir of antigen-specific T cells. To more clearly examine the expansion and contraction phases of immune response to rectal Ad infection, the percentage of tetramer-positive T cells at a given time was related to the maximal percentage of tetramer-binding T cells at each respective tissue. Eight weeks following *i.r.* priming, the frequency of tetramer-binding T cells waned to 6-10% of the maximal cell number (Fig. 4B). In all tissue sites where SIINFEKL-specific T cells were detected, a contraction phase that was characteristic of acute viral infection occurred. Typically, acute viral or bacterial infections result in clonal expansion of antigen-specific T cells, the majority of which (90-95%) are eliminated over the weeks that follow antigen clearance. Only a small, long-lived memory pool survives, which represents 5-10% of the T cell numbers at the height of expansion. Therefore, rectal Ad-based immunization resulted in normal kinetics of expansion and contraction of antigen-specific $CD8^+$ T cells.

Figure 4. Kinetics of antigen-specific T cell responses to $OVA_{257-264}$ (SIINFEKL) in mice immunized intrarectally with AdOVA. At various times after intrarectal (*i.r.*) AdOVA immunization, splenic, ILN, IEL and LP T cells were isolated and reacted with fluorescent H2-K^b $OVA_{257-264}$ tetramers. Tetramer-binding cells were enumerated by flow cytometry. Figures in panel A represent the percentage (mean ± SEM) of tetramer-binding $CD8^+$ T cells from 3-5 animals per group. Figures in panel B indicate the percentage of tetramer-binding $CD8^+$ T cells in the splenic, LP and IEL populations depicted in panel A relative to the maximal response of the respective lymphocyte population. Due to low tetramer binding in ILN, data for this tissue is not shown in panel B. The results are representative of two independent experiments.

Figure 4


A
% of Tetramer+ CD8+ T cell of Total CD8+ T cells
Spleen
ILN
LP
IEL
Days Post-Immunization
B
% Tetramer+ CD8+ T cell Relative to Peak Reponse
Spleen
LP
IEL
Days Post-Immunization

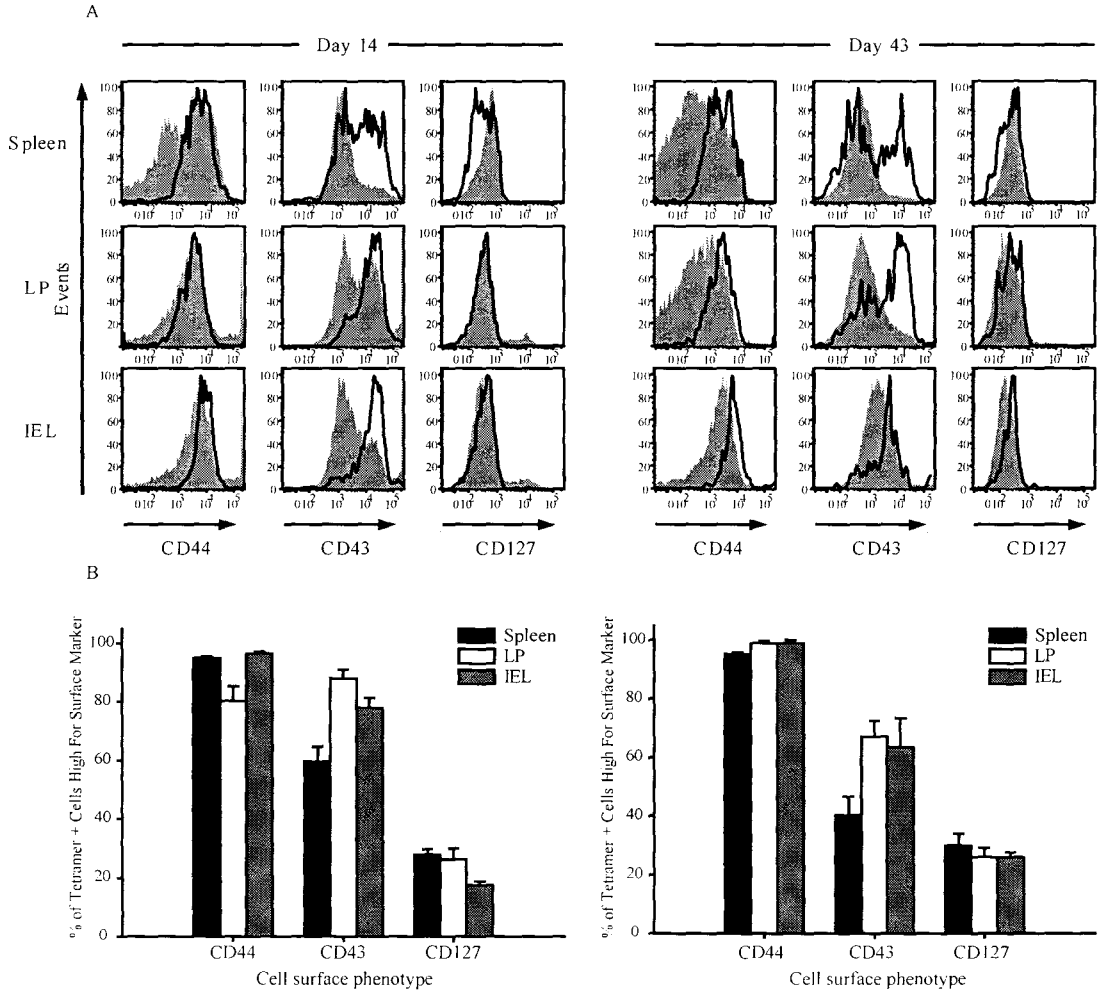
3.3 Phenotypic characterization of Ad-induced SIINFEKL-specific CD8$^+$ T cells

Phenotypic analysis is an important tool in the characterization of immune response to the immunization because it can help to identify many features of a single T cell including, but not limited to, its antigenic experience, activation status, homing potential, and ability to respond to chemokine stimulation (Seder, Darrah et al. 2008). In conjunction with a functional assessment, the pattern of expression of surface markers, such as CD62L, CD43 and CD127, can help to subdivide antigen-specific T cells into effector memory (T_{EM}) and central memory (T_{CM}) T cells based upon their localization and proliferation capacity (Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004). The results from previous phenotypic and functional studies of CD8$^+$ T cell responses following systemic administration of Ad5-based vectors suggested that these vectors induced predominantly effector CD8$^+$ T cells. These antigen-experienced effector CD8$^+$ T cells were characterized by increased activation, the inability to respond to IL-7 and the capability of homing to non-lymphoid effector sites, such as the intestinal LP. Therefore, to further analyze the CD8$^+$ T cell populations induced by *i.r.* administration of an Ad vector, the expression of CD43, CD44 and CD127 on K^b/SIINFEKL-binding CD8$^+$ T cells was evaluated by flow cytometric analyses at 14 and 43 days post-priming with AdOVA. The tetramer-binding CD8$^+$ T cells isolated from the splenic, LP and IEL populations highly expressed the memory T cell marker, CD44, for the entire eight weeks examined (Fig. 5 and data not shown). Antigen-specific CD8$^+$ T cells examined up to 58 days post-immunization did not express CD127, a marker believed to identify a population of effector CD8$^+$ T cells that differentiate into persistent memory T cells

(Wherry, Teichgraber et al. 2003). When the immune response was maximal, isoform 1B11 of CD43, which is expressed on $CD8^+$ T cells following antigen activation, was up-regulated on SIINFEKL-specific $CD8^+$ T cells after *i.r.* AdOVA inoculation. Five weeks after any measurable AdOVA-induced antigen expression ceased to occur, the pattern of expression of CD43 by the antigen-specific cell population was re-examined. While the tetramer-binding $CD8^+$ T cell population in the spleen showed decreased CD43 expression by day 43 post-priming, CD43 expression was still sustained in 60-70% of antigen-specific cells in the colonic IEL and LP populations. Taken together, these results demonstrated that rectal Ad-based immunization elicited antigen-specific $CD8^+$ T cells that displayed an effector phenotype during the expansion and contraction phases of the immune response. These data also indicated that the phenotype of antigen-specific $CD8^+$ T cells induced in the course of rectal immunization was consistent with that observed following systemic administration of Ad viruses (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006; Tatsis, Fitzgerald et al. 2007).

Figure 5. Phenotypic characterization of OVA-specific CD8$^+$ T cells elicited after colonic AdOVA priming in mice. Fourteen and 43 days following intrarectal (*i.r.*) AdOVA immunization, SIINFEKL-specific splenic, LP and IEL CD8α T cells were detected using tetramer and co-reacted with fluorescently-labelled antibodies specific for CD43, CD44 and CD127. The cells were enumerated by flow cytometry. In panel *A*, representative data from one mouse for each tissue are shown. The shaded areas correspond to total CD8$^+$ T cells and the open areas correspond to tetramer-reactive T cells. Tetramer-binding populations were classified as high and low fluorescence with respect to the above cell surface phenotype. The percentages of CD8$^+$ T cells with a high level of expression of CD43, CD44 and CD127 are shown as mean ± SEM of 3-5 mice *per* group and time point. The results are representative of two independent experiments.

Figure 5


A
Day 14
Day 43
Spleen
LP
IEL
Events
CD44
CD43
CD127
B
% of Tetramer + Cells High For Surface Marker
Spleen
LP
IEL
Cell surface phenotype

3.4 Flow cytometric analysis of colonic CD8$^+$ IEL following intrarectal Ad immunization

Although small intestinal IEL have been extensively studied, colonic IEL have received far less attention despite considerable differences in both the function and the luminal environment of the different regions of the intestine. Phenotypically, a markedly lower proportion of CD8$^+$ cells is present within the large intestinal epithelium compared to that of small intestine (Little, Bell et al. 2005; Hapfelmeier, Muller et al. 2008). Similar to the small intestine, the CD8$^+$ IEL population of the colon consists primarily of TCR$\alpha\beta^+$ CD8$\alpha\alpha^+$ T cells, TCR$\gamma\delta^+$ CD8$\alpha\alpha^+$ T cells and TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ T cells. While thymus-independent TCR$\gamma\delta^+$ CD8$\alpha\alpha^+$ predominate in the small intestine, they are much less abundant in the large intestine. Different parasitic, bacterial or viral infections cause changes in the phenotypic composition of IEL in both small and large intestinal compartments (Little, Bell et al. 2005; Hapfelmeier, Muller et al. 2008). In general, the number of small intestinal TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ T cells increases with age and upon microbial colonization, whereas the relative frequencies of TCR$\alpha\beta^+$ CD8$\alpha\alpha^+$ and TCR$\gamma\delta^+$ CD8$\alpha\alpha^+$ T cells decrease under these conditions. Also, the relative frequencies of small intestinal TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ IEL increased during acute infection with LCMV, and this subset mediated the principal virus-specific cytotoxic activity of IELs (Hapfelmeier, Muller et al. 2008). When adoptively transferred, such cells protected naïve, LCMV-infected recipient mice from productive virus infection. Large intestinal CD8α^+ IEL have been reported to increase upon parasitic infection with *Trichuris muris* (Little, Bell et al. 2005). However, colonic IELs have much less cytolytic activity *in vitro* compared to small intestinal IELs.

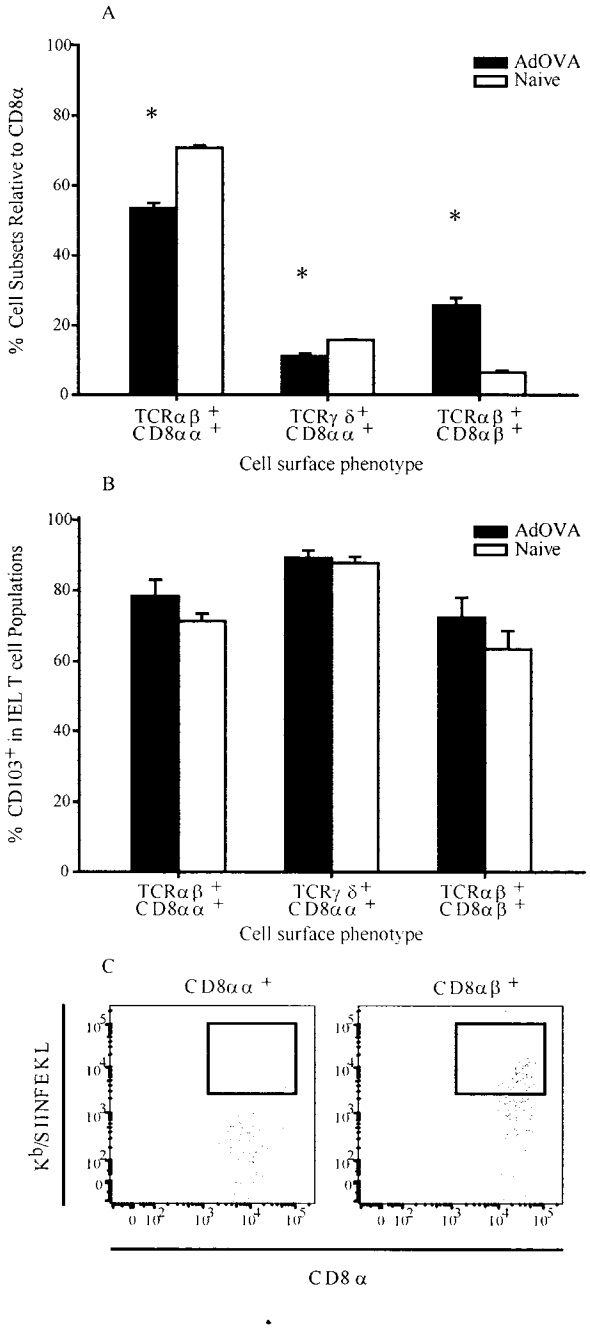
Given such phenotypic and functional disparities in the IEL populations of the large and small intestine, and the relative lack of knowledge regarding IELs in the large intestine, flow cytometric analysis was used to characterize the phenotype of $CD8^+$ T cells within the colonic epithelium 14 days following colonic Ad delivery (Fig. 6A). Ad infection in mice was accompanied by 19.1 % increase in relative frequency of $TCR\alpha\beta^+$ $CD8\alpha\beta^+$ T cells and a concomitant decline of 17.3 % and 4.6% in T cells expressing $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ and $TCR\gamma\delta^+$ $CD8\alpha\alpha^+$, respectively, when compared to non-immunized mice. Overall, the low percentage of TCRγδ-bearing $CD8\alpha\alpha^+$ T cells was characteristic of colonic IEL. Therefore, changes in the overall composition of the $CD8^+$ IEL T cell compartment were consistent with increased antigen exposure following acute viral infection.

The mucosal lymphocyte integrin $CD103(\alpha_E)\beta_7$ is important for IEL localization and/or function in both the small and large intestines. CD103 deficiency results in decreased numbers of small intestinal $TCR\alpha\beta^+$ $CD8^+$ T cells in both IEL and LP populations (Schon 1999). CD103 has been shown to be involved in the protective immunity to parasitic infection with *Trichuris trichiura* (Schon, Arya et al. 1999; Betts, Brenchley et al. 2003). Also, the entry of spleen-resident $CD8^+$ T lymphocytes into the small intestine resulted in the up-regulation of CD103. Therefore, the level of expression of CD103 was examined on the cells of three major $CD8^+$ lymphocyte subsets. The levels of expression of the epithelia-associated lymphocyte integrin $CD103(\alpha_E)\beta_7$ was not significantly different ($P > 0.05$) between Ad-inoculated and naïve mice (Fig. 6B). This was consistent with the observation that spleen- and gut-derived T cells migrating into the

intestinal epithelium increase the display of CD103 sometime after entering the intestine (Ericsson, Svensson et al. 2004; Masopust, Vezys et al. 2006). Also, all antigen-specific T cells belonged to TCR$\alpha\beta^{+}$CD8$\alpha\beta^{+}$ subset of large intestinal T cells (Fig. 6C). Therefore, the greater proportion of the TCR$\alpha\beta^{+}$CD8$\alpha\beta^{+}$ T cell subset associated with the large intestinal epithelium was most likely the consequence of expansion of and/or recruitment of antigen-specific T cells to the IEL compartment following exposure to the antigen in the course of *i.r.* AdOVA priming; this notion is consistent with the result of T cell transgenic engraftment experiments (*Section 3.1*).

Figure 6. The composition of intraepithelial CD8$^+$ T cell subsets in the mouse large intestine. Fourteen days following intrarectal (*i.r.*) AdOVA immunization, IEL from the colons of mice were isolated and reacted with anti-CD8α, anti-CD8β, anti-TCRβ, anti-TCRγδ and anti-CD103 fluorochrome-conjugated antibodies, and analyzed by flow cytometry. Panel *A* shows the proportions of CD8αα$^+$ TCRαβ$^+$, CD8αα$^+$ TCRγδ$^+$ and CD8αβ$^+$ TCRαβ$^+$ within CD8$^+$ large intestinal IEL in naïve (open bars) and AdOVA (closed bars) immunized mice. $P < 0.05$ in the group immunized with AdOVA compared with naïve controls was determined by Student's t test and is denoted by an asterisk. In panel *B*, different intraepithelial (IEL) T cell subsets from the large intestine were identified as CD103$^+$ in naïve (open bars) and AdOVA (closed bars) inoculated mice. Fourteen days post-immunization, IEL isolated from AdOVA immunized mice were reacted with fluorescently-labelled anti-CD8α, anti-CD8β antibodies and tetramers. Labelled cells were detected by flow cytometry. In panel *C*, the representative flow cytometry plots of tetramer binding by CD8αα$^+$ and CD8αβ$^+$ IEL populations of immunized mice are displayed. Data represent the mean ± SEM of 4 mice *per* group and one of two independent experiments.

Figure 6


A
AdOVA
Naive
% Cell Subsets Relative to CD8α
100
80
60
40
20
0
*
*
*
TCRαβ + CD8αα +
TCRγ δ + CD8αα +
TCRαβ + CD8αβ +
Cell surface phenotype
B
AdOVA
Naive
% CD103+ in IEL T cell Populations
100
80
60
40
20
0
TCRαβ + CD8αα +
TCRγ δ + CD8αα +
TCRαβ + CD8αβ +
Cell surface phenotype
C
CD8αα +
CD8αβ +
Kb/SIINFEKL
CD8α

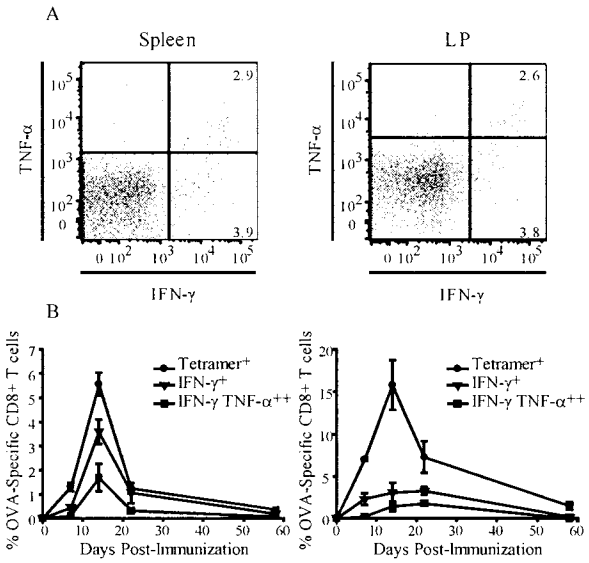
3.5 Functional activity of SIINFEKL-specific $CD8^+$ T cells

Antigen-specific $CD8^+$ T cells induced and activated during vaccination should produce cytokines. It has been reported previously that antigen-specific $CD8^+$ T cells producing a multitude of cytokines might be the hallmark of successful immunization leading to enhanced protection from pathogens (Seder, Darrah et al. 2008). Various intracellular cytokines can be detected immunochemically using staining methods that rely on the ability of fungal metabolite Brefeldin A to inhibit protein transport processes during T cell activation, which leads to the accumulation of cytokines in the Golgi complex and endoplasmic reticulum. Thus, upon SIINFEKL re-stimulation, cytokine-containing, antigen-specific T cells can be detected by fluorescence flow cytometry using appropriate fluorochrome-conjugated antibodies. In order to analyze the effector functions of the SIINFEKL-specific $CD8^+$ T cells elicited by *i.r.* vaccination, cells isolated from the spleens and LP were assessed for the presence of cytoplasmic, SIINFEKL-induced IFN-γ, TNF-α, and IL-2 on days 7 through 58 post-immunization. The percentage of cytokine-producing T cells to SIINFEKL-peptide was obtained in both AdOVA- and Addl70-inoculated mice and the background cytokine accumulation, as determined by stimulation of these cells with an irrelevant peptide, was subtracted. The final values of SIINFEKL-induced cytokine accumulation were then computed by subtracting the values of cytokine accumulation in Addl70-immunized mice from those levels obtained in AdOVA-immunized mice. $CD8^+$ effector T cells from the spleen (Fig. 7A) and LP (Fig. 7B) were diminished in their accumulation of both IFN-γ and TNF-α. As such, at any time examined post-immunization, not more than 84% of splenic and

45% of LP tetramer-binding $CD8^+$ T cells were accumulating IFN-γ. Even smaller fractions, 30% and 25% of K^b/SIINFEKL-binding $CD8^+$ T cells from the splenic and LP compartments, respectively, accumulated TNF-α. $CD8^+$ T cells induced by rectal Ad priming did not secrete IL-2 (data not shown). Therefore, colonic administration of Ad-based vectors resulted in the induction of antigen-specific $CD8^+$ T cells with a diminished ability to produce cytokines. An explanation for this finding might be provided by studies of chronic LCMV infection or infections resulting in prolonged antigen exposure, such as Ad5 or adeno-associated virus delivered intramuscularly (*i.m.*) (Fuller, Khanolkar et al. 2004; Yang, Millar et al. 2006; Lin, Hensley et al. 2007; Tatsis, Fitzgerald et al. 2007). These infections result in low levels of cytokine production by $CD8^+$ T cells in response to antigen-specific stimulation, which was attributable to $CD8^+$ T cells dysfunction due to the persistence of the antigen.

Figure 7. Cytokine production and the physical presence of epitope-specific CD8$^+$ T cells. To further define the kinetics of virus-specific CD8$^+$ T cell responses in the spleen and LP populations of mice, the percentages of SIINFEKL-specific CD8$^+$ T cells were measured by flow cytometry at the indicated times following intrarectal (*i.r.*) AdOVA immunization by fluorescent tetramer staining and the corresponding intracellular cytokine detection of IFN-γ and TNF-α by fluorescently-labelled antibodies. In panel *A*, representative plots of IFN-γ- and TNF-α-containing CD8$^+$ T cells are shown for spleen and LP populations following *in vitro* restimulation with SIINFEKL peptide 14 days after AdOVA immunization. The values in the upper right and lower right quadrants indicate the percentages of gated CD8$^+$ T cell that produced both IFN-γ and TNF-α, or only IFN-γ, respectively. In panel B, the percentage of SIINFEKL-specific IFN-γ$^+$ and IFN-γ TNF-α^{++} CD8$^+$ T cells is shown in the spleen and LP cell populations. Within the same figure, the percentage of fluorescent tetramer-binding CD8$^+$ T cells determined on the same day is shown to demonstrate the disparity between the amounts of antigen-specific C8$^+$ T cells detected by tetramer-binding and those producing IFN-γ and TNF-α. Data represent the mean ± SEM of 4-5 mice *per* group at each time post-immunization. The results are representative of two independent experiments.

Figure 7


A
Spleen
LP
TNF-α
IFN-γ
2.9
3.9
2.6
3.8
B
% OVA-Specific CD8+ T cells
Days Post-Immunization
Tetramer+
IFN-γ+
IFN-γ TNF-α++

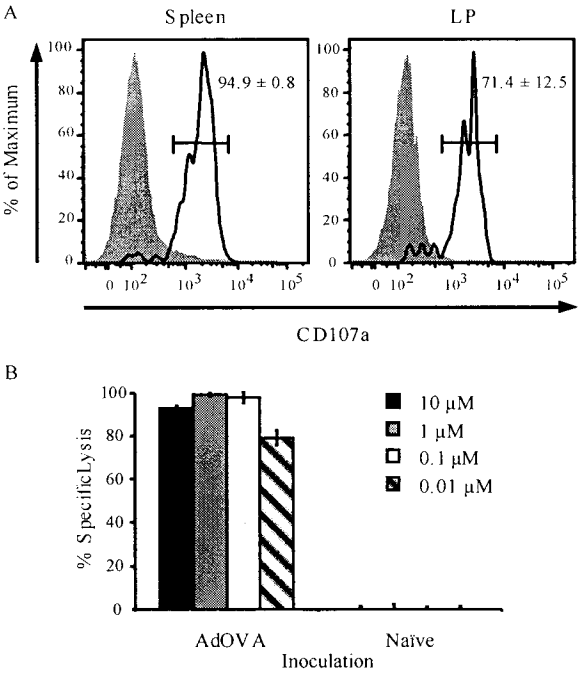
Cytotoxicity effected *via* the perforin-granzyme, known as granule-mediated cytotoxicity, and Fas-FasL pathways, known as receptor-mediated cytotoxicity, is one of the main effector functions of $CD8^+$ T cells required to control many viral infections. Intracellular cytokine staining procedures incorporating the detection of CD107a, also known as lysosomal-associated membrane protein 1 (LAMP-1), allow the indirect measurement of the granule-mediated cytotoxic potential of IFN-γ^+ $CD8^+$ T cells by detecting the ability of these cells to degranulate upon activation (Betts, Brenchley et al. 2003). This method measures the levels of exposure of CD107a, present in the membrane of cytotoxic granules, onto the cell surface as a result of degranulation, through the binding of anti-CD107a fluorochrome-conjugated antibodies during a 5-hour stimulation period with the peptide. Thus, the capability of SIINFEKL-specific $CD8^+$ T cells to degranulate upon cognate recognition of antigen can then be judged by the measurement of CD107a by flow cytometry. Approximately 95% of splenic and 71% of LP IFN-γ^+ $CD8^+$ T cells co-stained for CD107a and degranulated upon stimulation (Fig. 8A). Therefore, rectal vaccination with Ad-based vectors induced $CD8^+$ T cells that had cytolytic capability.

Also, cytotoxicity can be directly evaluated either *in vitro*, using assays, such as cytoplasmic protein-^{51}Cr-release, or *in vivo*, by detecting the killing of systemically injected, peptide-loaded target cells. Using the latter assay method, target cells were fluorescently labelled with (1,3-dichloro-9,9-dimethylacridin-2-one)-succinimidyl ester (DDAO-SE) or various concentrations of CFSE or a combination of both. This staining procedure allowed the simultaneous detection by flow cytometry of 5 different

populations of cells which were left untreated or were exposed to various concentrations of the SIINFEKL peptide. The disappearance of the fluorescent (target) cells exposed to the SIINFEKL peptide in an immunized host, compared to naïve mice, was determined 14 days after AdOVA immunization. Twelve hours following labelled cell injection, the disappearance of peptide-exposed cells was observed in the spleens of immunized animals only (Fig. 8B), and cells exposed to as little as 0.01μM of peptide were efficiently eliminated by SIINFEKL-specific $CD8^+$ T cells (Fig. 8B). Cytotoxic activity in the intestinal compartment was not assessed *in vivo* due to the limited tropism of naïve lymphocytes for the gastrointestinal mucosa (Mackay, Marston et al. 1992), while determination of the *ex vivo* cytotoxicity by cytoplasmic protein-^{51}Cr-release of LP lymphocytes was not possible due to contamination of the cell population by endogenous microbes. Collectively, these data indicated that, while colonic Ad immunization led to the induction of T_{EM} cells that were compromised in their ability to produce cytokines upon *in vitro* re-stimulation, these $CD8^+$ T cells were, nonetheless, functionally competent *in vivo*.

Figure 8. Functional characterization of cytotoxic T lymphocytes induced by colonic AdOVA delivery into mice. To further assess the effector function of antigen-specific $CD8^+$ T cells, their cytolytic activity was measured 14 days following intrarectal (*i.r.*) immunization with AdOVA. The ability of splenic and LP $CD8^+$ T cells to degranulate was monitored by the appearance of cell surface CD107a following restimulation with SIINFEKL on IFN-γ^+ $CD8^+$ (open areas) and total $CD8^+$ (shaded areas) T cells. Cytotoxic cells were enumerated by flow cytometry. In panel *A*, the percentage of degranulating IFN-γ^+ $CD8^+$ T cells is indicated on each flow cytometry plot and represents the mean ± SEM of 5 mice per group. Fourteen days following *i.r.* AdOVA immunization, direct assessment of the *in vivo* cytotoxic potential of OVA-specific $CD8^+$ T cells was conducted by monitoring the survival of differentially-labelled target cells exposed to titrated doses of SIINFEKL peptide relative to those exposed to irrelevant peptide. The different populations were combined at 3×10^6 cells each and injected *via* the tail vein and disappearance of labelled cells was analyzed 12 hours post-injection. The cytotoxicity was evaluated by FACS analysis. In panel *B*, the histogram represents the percentage specific lysis for each group of 3 mice and presented as mean ± SEM. The difference between AdOVA-immunized and naïve group of mice is significant at $P < 0.001$ by Student's t test for each dose of SIINFEKL peptide. The results are representative of three independent experiments.

Figure 8


A
Spleen
LP
% of Maximum
94.9 ± 0.8
71.4 ± 12.5
CD107a
B
% Specific Lysis
10 µM
1 µM
0.1 µM
0.01 µM
AdOVA
Naïve
Inoculation

3.6 Proliferative and protective capacity of T_{EM} $CD8^+$ T cells in intrarectally primed mice

A hallmark of a successful immunization regimen is the priming of the $CD8^+$ T cell population such that it can undergo an appreciable secondary expansion upon *in vivo* exposure to the antigen (Kaech, Wherry et al. 2002). For example, vaccinia virus challenge results in a robust expansion of antigen-specific $CD8^+$ lymphocytes in immunized mice between 3-7 days post-immunization, and clearance of the virus is mediated by antigen-specific $CD8^+$ T cells. Also, since mice can be inoculated with vaccinia virus *via* different routes, such as *i.r.*, intravaginally (*i.vag.*), intraperitoneally (*i.p.*) and intranasally (*i.n.*), vaccinia virus can be used as a potent means to secondarily deliver antigen. In order to evaluate the ability of rectal Ad immunization to induce protective mucosal immune responses, *i.r.* challenge was chosen (Belyakov, Moss et al. 1999). However, this challenge route did not result in expansion of colonic $CD8^+$ T cells at indicated time points and no virus was detected in the ovaries of naïve mice (data not shown). Thus, to evaluate the ability of SIINFEKL-specific T cells to proliferate upon secondary exposure to the antigen, vaccinia virus expressing ovalbumin (VaccOVA) was administered *i.vag.* 30 days after colonic immunization with AdOVA or Addl70 (Jiang, Patrick et al. 2005). In these experiments, the possibility of a response to Ad5 was eliminated by using Vacc vector. Five days following *i.vag.* challenge with the vaccinia virus vector, cells from the spleen, LP and vaginal mucosa were isolated, and the frequencies of antigen-specific $CD8^+$ T cells in AdOVA and Addl70 immunized mice were compared. To detect the increase in antigen-specific $CD8^+$ T cells, cells isolated

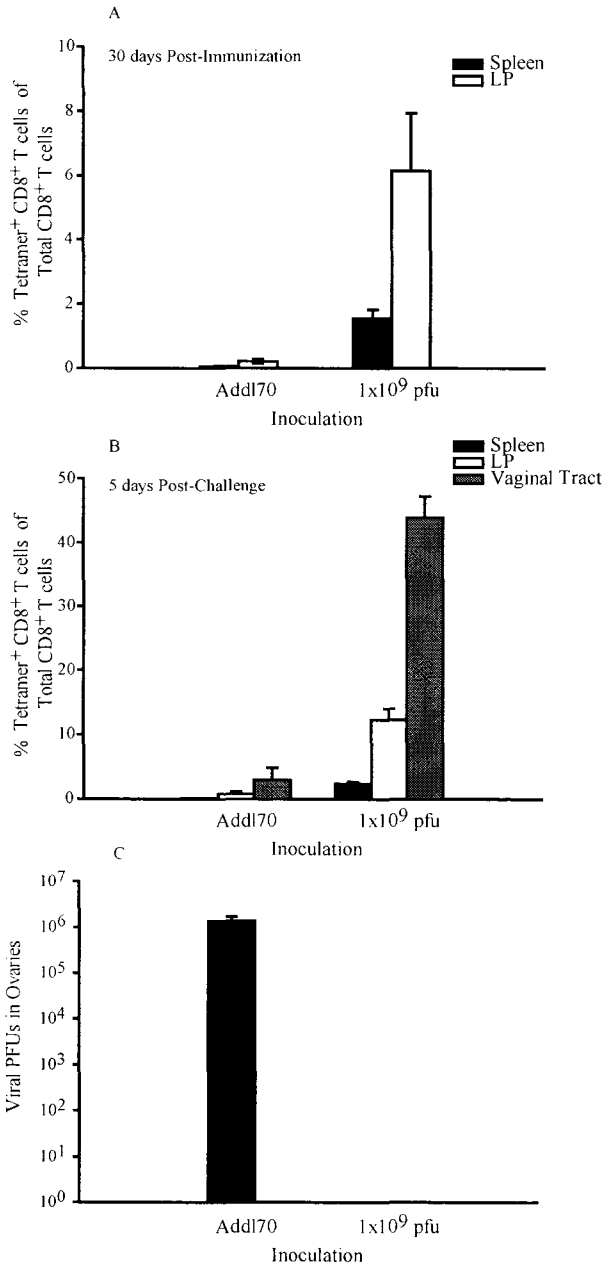
from different organs were stained with fluorescently-labeled tetramers and the fluorescence intensity was analyzed by flow cytometry. SIINFEKL-specific $CD8^+$ T cells demonstrated proliferation capacity in the vaginal and colonic mucosae (Fig. 9A). Unlike LCMV infection, *i.r.* delivery of AdOVA did not result in the recruitment to and/or expansion of $CD8^+$ T cells in the vaginal tract. The frequency of $CD8^+$ T cells in the vaginal mucosa prior to any vaginal infection was very low, and the tetramer technology used to detect antigen-specific $CD8^+$ T cells could not be used reliably to estimate the frequencies of antigen-specific T cells in the vaginal tract 30 days following AdOVA immunization; this was due to relatively high background fluorescence of the methodology. However, upon vaginal vaccinia virus vector infection, $CD8^+$ T cells in AdOVA immunized mice were dramatically expanded in and/or recruited to the vaginal tract, and the frequency of SIINFEKL-specific $CD8^+$ T cells reached greater than 40% of all vaginal $CD8^+$ T cells (Fig. 9B). The frequencies of tetramer-binding $CD8^+$ T cells in the LP doubled upon vaginal VaccOVA infection (from 6.2% to 12.3%). Therefore, rectal AdOVA immunization resulted in a robust, amnestic antigen-specific $CD8^+$ T-cell response upon secondary mucosal viral challenge. The dramatic localization of SIINFEKL-specific T cells in the vaginal tract following challenge demonstrated the ability of rectal replication-deficient Ad vector immunization to prime functional memory $CD8^+$ T cell responses that can disseminate to distant mucosal sites.

The clearance of vaccinia virus infection is mediated by $CD8^+$ T cells. Therefore, to determine the level of protective efficacy of the mucosal $CD8^+$ T cell response following rectal Ad immunization, mice that had been primed intrarectally with AdOVA were

challenged *i.vag.* with VaccOVA. In addition, unlike *i.p.* vaccinia challenge, vaginal delivery of vaccinia virus has been identified as a means of measuring the degree of mucosal $CD8^+$ T cell responses. Six days after VaccOVA inoculation, mice were sacrificed, their ovaries were removed, homogenized and analyzed for the presence of vaccinia virus using CV-1 cell monolayers. Following mucosal AdOVA immunization, a 6-log reduction in virus titer occurred, when compared to mice immunized with control Add170 vector (Fig. 9C). Therefore, Ad-induced effector-memory $CD8^+$ T cells were functional upon live virus challenge at the vaginal mucosa.

Figure 9. Recall proliferation and induction of protective immunity in the intravaginal recombinant vaccinia challenge model following rectal immunization. To determine the protective efficacy of the mucosal immune response following intrarectal (*i.r.*) AdOVA immunization, primed mice were challenged intravaginally (*i.vag.*) with recombinant vaccinia virus carrying the gene encoding OVA (VaccOVA) 30 days following priming. To determine the magnitude of the $CD8^+$ T cell response before and after challenge, cells isolated from the indicated tissues were detected by fluorescent tetramer binding and analyzed by flow cytometry. In panel A, the histogram represents the percentage (mean ± SEM) of tetramer-binding $CD8^+$ T cells in the spleen (closed bars) and LP (open bars) 30 days after immunization. In panel *B*, the histogram shows the percentage of antigen-specific $CD8^+$ T cells isolated from spleen (closed bars), LP (open bars) and vaginal tract (VG, shaded bars) 5 days after *i.vag.* vaccinia challenge. Data represent the mean ± SEM of 4 mice *per* group and time point. Vaccinia virus titers in mouse ovaries were determined on CV-1 monolayers 5 days post-challenge in Addl70- and AdOVA-immunized mice. In panel *C*, the histogram shows the ovarian vaccinia virus titer (mean ± SEM) from Addl70-immunized (closed bars) and AdOVA-immunized (open bars) mice. The difference between AdOVA-immunized (n = 11) and Addl70-immunized (n = 7) mice is significant ($P < 0.001$) by log-transformed Student's t test. The results are representative of three independent experiments.

Figure 9


A
30 days Post-Immunization
Spleen
LP
% Tetramer+ CD8+ T cells of Total CD8+ T cells
10
8
6
4
2
0
Addl70
1x10^9 pfu
Inoculation
B
5 days Post-Challenge
Spleen
LP
Vaginal Tract
% Tetramer+ CD8+ T cells of Total CD8+ T cells
50
40
30
20
10
0
Addl70
1x10^9 pfu
Inoculation
C
Viral PFUs in Ovaries
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Addl70
1x10^9 pfu
Inoculation

3.7 Titration of doses of systemically administered AdOVA

Previous studies suggested that mucosal immunization elicited protective immunity by providing protection from mucosal challenge that is superior to that induced by systemic immunization. In such studies, the presence of antibodies in vaginal secretions following mucosal immunization correlated with the selective localization of mainly IgA-producing plasma cells in mucosal sites (McDermott 1990). In contrast, systemic immunization resulted in the selective localization of the IgG-secreting plasma cells in the systemic compartment. Similarly, in murine *Mycobacterium tuberculosis* (*M. tuberculosis*) and herpes simplex virus type 2 (HSV-2) mucosal challenge models, mucosal immunization was associated with increased presence of antigen-specific T cells at the mucosal sites following mucosal, but not systemic immunization (Gallichan and Rosenthal 1996; Santosuosso, McCormick et al. 2005; Santosuosso, McCormick et al. 2007). Although initially identified as a mucosal challenge designed to evaluate mucosal protection, live vaccinia virus administered via the vaginal tract is not localized to the vaginal tract and disseminates to systemic sites. Therefore, to evaluate the importance of the route of the immunization for the establishment of protection from vaginal vaccinia challenge, immunization studies with Ad-based vectors delivered systemically (subcutaneously (*s.c.*) or intramuscularly (*i.m.*)) were conducted. To establish the viral doses that induced systemic and mucosal immune responses comparable to those elicited by *i.r.* inoculation, doses of AdOVA (1×10^5 pfu, 1×10^6 pfu, 1×10^7 pfu) were tested. Thirty days after immunization, the mice were sacrificed and the frequencies of antigen-specific $CD8^+$ T cells in their splenic and LP cell populations were examined by fluorescent tetramer

binding. Mice vaccinated *i.m.* with 1×10^6 pfu of AdOVA displayed similar frequencies of SIINFEKL-specific T cells in their spleens (3%) and LP cells (6%) (Fig. 10A) as compared to those present in *i.r.* inoculated mice 30 days after immunization. Similar studies assessing tetramer-binding were undertaken following administration of different doses of AdOVA delivered *s.c.* Although mice vaccinated with 1×10^7 pfu of AdOVA *s.c.* displayed frequencies of antigen-specific T cell in their spleens that were comparable to those observed following either *i.r.* or *i.m.* immunization, the frequencies SIINFEKL-specific T cells observed in the LP following *s.c.* delivery were less than those observed using other immunization routes, reaching 2.3% at day 30 (Fig. 10B). The ability to accurately access the frequencies of antigen-specific cells in the vaginal tract was limited by very low total numbers of $CD8^+$ T cells present before the challenge. Therefore, *i.m.* delivery of 1×10^6 pfu of AdOVA resulted in mucosal and systemic levels of SIINFEKL-specific T cells equivalent to those achieved by rectal AdOVA immunization 30 days post-immunization, while *s.c.* delivery of 1×10^7 pfu of AdOVA led to the induction of a antigen-specific $CD8^+$ T cell response of similar magnitude to *i.r.* delivered AdOVA in the spleen but not LP. Overall, the *i.m.* route of systemic AdOVA delivery was superior to *s.c.* at inducing the $CD8^+$ T cell response in large intestinal mucosa; however, both systemic deliveries, albeit at different viral dosages, could effectively prime $CD8^+$ T cells in systemic compartments.

3.8 Proliferative and protective capacity of CD8$^+$ T cells induced by i.m. *and* s.c. *immunization*

Many previous studies, especially those that utilized non-replicating vaccines, demonstrated the compartmentalization of the immune response based on the route of immunization and the failure of systemic immunizations to provide protection at mucosal sites (Kuznetsov, Stepanov et al. 2004; Santosuosso, McCormick et al. 2007). Therefore, the proliferation potential of systemically induced CD8$^+$ T cells following mucosal viral challenge was further evaluated. Mice given different doses of AdOVA either *i.m.* or *s.c.* were challenged vaginally with VaccOVA 30 days after immunization. Analogous to *i.r.* Ad-based immunization, *i.m.* AdOVA delivery resulted in a robust, amnestic antigen-specific CD8$^+$ T-cell response six days after the challenge, as judged by large increase in frequencies of tetramer-binding CD8$^+$ T cells, which was especially apparent in the vaginal mucosa (Fig. 10C). The frequencies of K^b/SIINFEKL-positive T cells in the vaginal tract were comparable, irrespective of the dose used for primary inoculation. While *s.c.* immunization resulted in rapid, amnestic CD8$^+$ T cell response upon vaginal challenge with VaccOVA, the magnitude of the SIINFEKL-specific immune response detected in the vaginal mucosa was less than that observed in the *i.m.* or *i.r.* immunized groups (Fig. 10D). In addition, similar expansion frequencies of antigen-specific CD8$^+$ T cells were detected in the vaginal tracts of mice immunized with higher doses of AdOVA *s.c.* (1×10^6 pfu and 1×10^7 pfu). Therefore, at 30 days, systemic AdOVA immunization that achieved detectable CD8$^+$ T cell priming had led to the expansion of antigen-specific

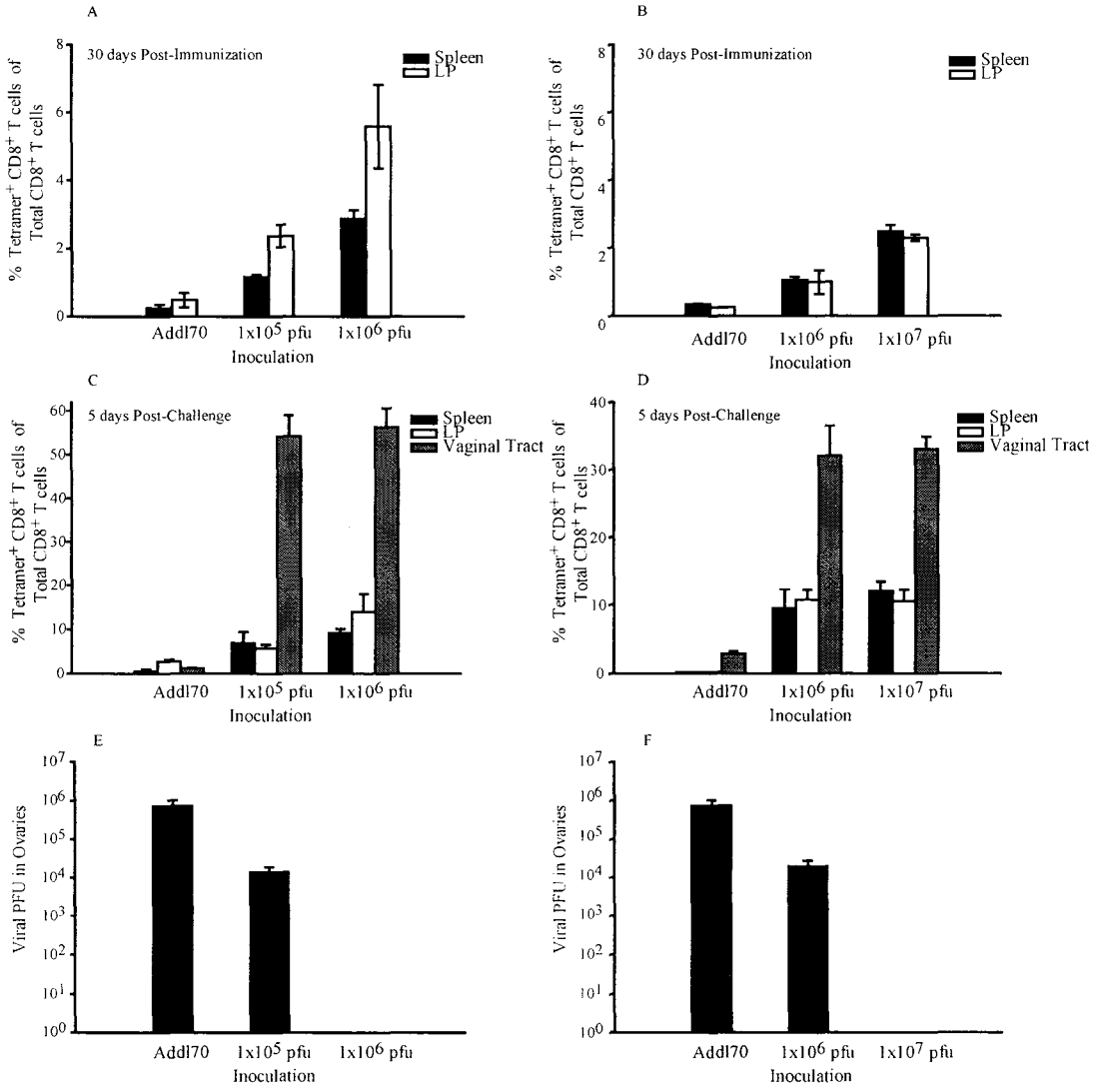
$CD8^+$ T cells in mucosal and systemic sites following vaginal challenge with VaccOVA, a finding similar to that observed following rectal AdOVA immunization.

Lastly, the protective efficacy of the systemic and mucosal immune responses elicited by different doses used for parenteral AdOVA priming was evaluated by *i.vag.* challenge with VaccOVA 30 days after immunization. Six days after VaccOVA inoculation, mice were sacrificed, their ovaries and spleens were removed, homogenized and analyzed for the presence of vaccinia virus using CV-1 cell monolayers. Following *i.m.* AdOVA immunization, a 2-log reduction in the virus titers compared to mice immunized with control Ad vector occurred in mice that received 1×10^5 of AdOVA, while no virus was detected in ovaries of mice immunized with higher doses of AdOVA (Fig. 10E). In contrast, *s.c.* AdOVA delivery resulted in a 2-log reduction in the virus titers when the dose of inoculation reached 1×10^6 pfu, and no virus was detected in those inoculated with 1×10^7 pfu (Fig. 10F). Therefore, at 30 days post-immunization, memory $CD8^+$ T cells induced by parenteral immunization were able to achieve the protective efficacy similar to that induced by mucosal immunization upon live virus challenge at the vaginal mucosa. Also, all doses of systemically-administered Ad that led to measurable priming resulted in robust, amnestic $CD8^+$ T cell responses, and progressive increase in protection could be seen with increasing dose of immunizing virus.

Figure 10. Studies of dose-response, proliferative and protective capacity of $CD8^+$ T cell elicited by systemically administered AdOVA. To establish the dose of systemically delivered AdOVA that leads to comparable level of systemic and mucosal $CD8^+$ T cell responses, 1×10^5, 1×10^6, and 1×10^7 pfu of AdOVA were delivered either intramuscularly (*i.m.*) or subcutaneously (*s.c.*). The percentage of tetramer-binding T cells in the spleen, LP and vaginal tract was evaluated using flow cytometry on days 30 post-immunization and on day 5 post-challenge. In panel *A*, the histogram represents the percentage (mean ± SEM) of SIINFEKL-specific $CD8^+$ T cells in the spleen and LP as enumerated by tetramer-binding 30 days following *i.m.* delivery of 1×10^5 and 1×10^6 pfu of AdOVA. In panel *B*, histogram depicts the percentage (mean ± SEM) of antigen-specific $CD8^+$ T-cell response in the spleen and LP as determined by tetramer-binding studies 30 days following *s.c.* delivery of 1×10^6 and 1×10^7 pfu of AdOVA. In panel *C* the histogram shows the percentage of tetramer-binding lymphocytes isolated from spleen, LP and vaginal tract (VG) of mice immunized *i.m.* with AdOVA and analyzed by tetramer staining for the presence of antigen-specific $CD8^+$ T cells 5 days after intravaginal (*i.vag.*) challenge with VaccOVA. The histogram in panel D denotes recall frequencies of antigen-specific $CD8^+$ T cells isolated from indicated tissues of *s.c.* immunized mice as determined by surface staining with tetramers 5 days after *i.vag.* challenge with recombinant vaccinia virus carrying the gene encoding OVA (VaccOVA). The bars represent the mean (± SEM) of 4-6 mice per group. To determine the protective efficacy of *i.r.* AdOVA immunization, vaccinia virus titers in mouse ovaries were determined on CV-1 cell monolayers 5 days post-challenge. In panel *E*, the histograms

display the titers (mean ± SEM) of vaccinia virus from homogenates of ovaries isolated from mice immunized *i.m.* with Adl70 and AdOVA 5 days after VaccOVA challenge are shown. In panel *F*, the histogram shows the viral titers in the ovaries of *s.c.* immunized mice on day 5 after *i.vag.* vaccinia challenge in Adl70-immunized and AdOVA-immunized mice as determined by titration on CV-1 cell monolayers. The bars represent the mean (± SEM) of 4-6 mice per group. The difference among AdOVA immunized mice and between AdOVA-immunized and Addl70-immunized mice is significant at $P < 0.001$ by log-transformed Student's t test. The results are representative of three independent experiments.

Figure 10


A
30 days Post-Immunization
Spleen
LP
% Tetramer+ CD8+ T cells of Total CD8+ T cells
Addl70
1x10^5 pfu
1x10^6 pfu
Inoculation
B
30 days Post-Immunization
Spleen
LP
% Tetramer+ CD8+ T cells of Total CD8+ T cells
Addl70
1x10^6 pfu
1x10^7 pfu
Inoculation
C
5 days Post-Challenge
Spleen
LP
Vaginal Tract
% Tetramer+ CD8+ T cells of Total CD8+ T cells
Addl70
1x10^5 pfu
1x10^6 pfu
Inoculation
D
5 days Post-Challenge
Spleen
LP
Vaginal Tract
% Tetramer+ CD8+ T cells of Total CD8+ T cells
Addl70
1x10^6 pfu
1x10^7 pfu
Inoculation
E
Viral PFU in Ovaries
Addl70
1x10^5 pfu
1x10^6 pfu
Inoculation
F
Viral PFU in Ovaries
Addl70
1x10^6 pfu
1x10^7 pfu
Inoculation

3.9 Gene transfer into the large intestine using lentivirus vectors

Previously, intratracheal *(i.t.)* delivery of lentivirus vectors pseudotyped with Ebola Zaire glycoprotein (EboZ) resulted in efficient gene transfer into the lung/tracheal epithelium as compared to commonly used vesicular stomatitis virus glycoprotein (VSVG) envelope that led to very limited gene transfer to lung epithelium (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003). Therefore, *i.r.* administration of EboZ- and VSVG-based lentiviral vectors expressing *E.coli* β-galactosidase (β-gal) was carried out to evaluate the utility of these constructs for gene transfer into the murine colorectal mucosa. Since EboZ, the mucin-rich region deletion-mutant of EboZ (EboZNTDL6), and VSVG envelope proteins confer specific tropism to the vectors, titers established by limiting dilution using appropriate target cell lines *in vitro* were different and, thus, were not used for normalizing the number of viral particles of vectors. Thus, the same volume (100 μl) of concentrated virus produced from the same number of cells transfected under the similar conditions was used to inoculate each animal. To test the ability of pseudotyped lentivirus vectors to infect the colonic epithelium, C57BL/6 mice were inoculated *i.r.* following 45% vol/vol ethanol enema with EboZβ-gal (5×10^7 TU/ml) (Fig. 1A) or EboZNTDL6β-gal (1×10^8 TU/ml) (Fig. 11B) vectors encoding β-gal. Mice were euthanized at various times (days 5, 10, 15 and 20). Colonic cryosections were assessed for β-gal activity by incubation with X-Gal developing solution in order to evaluate the kinetics of transgene expression. At all times, PBS-treated control animals demonstrated low levels of β-gal expression due to the presence of endogenous β-gal in the intestine (Fig. 11A). While minimal β-gal expression was seen on days 5 and 10 post-

inoculation with the EboZ-pseudotyped vector, cell-associated β-gal expression clusters were observed in the colorectal epithelium on day 15 post-inoculation. The gene expression subsided and was no longer detectable by day 20. Administration of EboZNTDL6 β-gal expressing vector following ethanol pretreatment did not result in measurable infection of the colonic epithelium at all time points examined. The background levels of β-gal staining were observed at all times when mice were given the EboZ vector without the ethanol pretreatment (Fig. 12). Unlike Ad delivery, which resulted in very extensive X-Gal staining of colonic epithelium on day 2 post-inoculation (Zhu, Thomson et al. 2008), inoculation with EboZ-pseudotyped lentivirus vectors resulted in 3-6 loci of cell-associated β-gal expression. In addition, C57BL/6 mice were inoculated *i.r.* with VSVGβ-gal (8×10^8 TU/ml) vectors under identical conditions and evaluated for β-gal expression 15 days later when EboZ β-gal expression was observed. No β-gal expression was seen 15 days post-delivery (data not shown). Therefore, these data indicated that *i.r.* delivery of EboZ-, but not EboZNTDL6- or VSVG-pseudotyped lentivirus vectors, led to the colonic β-gal gene expression 15 days post-inoculation. Similar to Ad virus inoculation, 45% ethanol pre-treatment was essential for filovirus-pseudotyped lentivirus-mediated gene-transfer to the colonic epithelium. The difference in kinetics of gene expression following *i.r.* lentivirus- and Ad-based gene delivery might reflect the differential tropism of these heterologous constructs for cells present in the colonic mucosa.

Figure 11. In vivo *gene transfer to C57BL/6 mice.* Animals receiving phosphate buffered saline (PBS) or EboZ pseudotyped vectors encoding β-gal intrarectally (*i.r.*) were euthanized at the times indicated, the colons were resected, and frozen in OCT. Cryosections of the colons were prepared, fixed and assessed for β-gal activity by overnight incubation with X-Gal developing solution. In panel *A*, representative sections of the colons isolated from mice given EboZβ-gal *i.r.* on days 5-20 post-infection are shown. In panel *B*, representative sections for colons isolated from mice given EboZNTDL6β-gal *i.r.* on days 5-20 post-infection are shown. No visually discernable expression was seen in animals treated with PBS at all time points. Arrow indicates β-gal-positive cells.

Figure 11

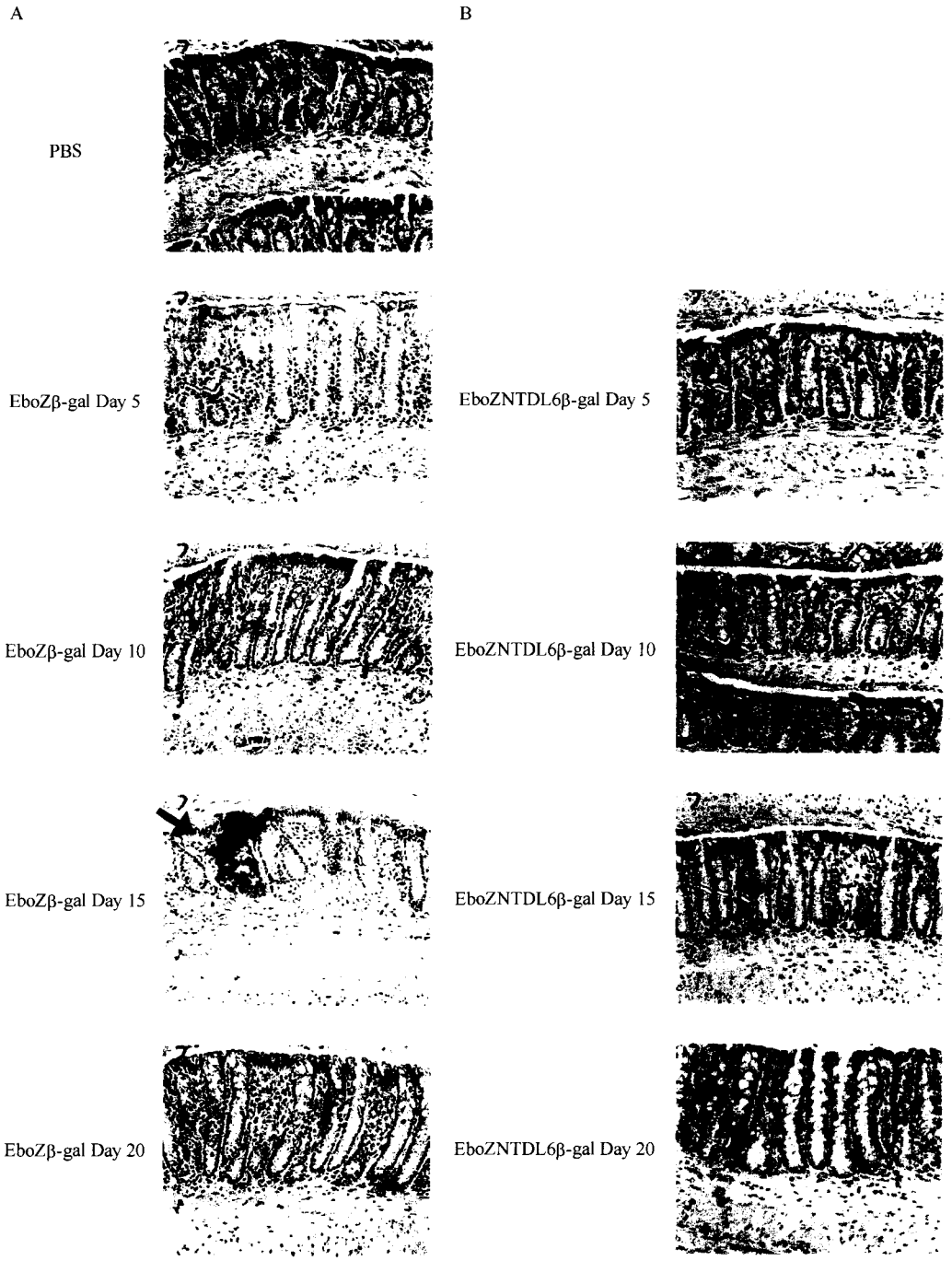
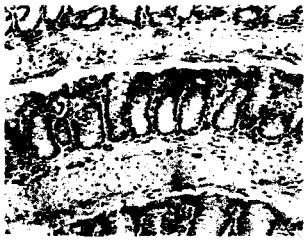

A
B
PBS
EboZβ-gal Day 5
EboZNTDL6β-gal Day 5
EboZβ-gal Day 10
EboZNTDL6β-gal Day 10
EboZβ-gal Day 15
EboZNTDL6β-gal Day 15
EboZβ-gal Day 20
EboZNTDL6β-gal Day 20

Figure 12. In vivo *gene transfer of VSVG-pseudotyped lentiviral vectors to C57BL/6 mice.* Animals receiving phosphate buffered saline (PBS) or VSVG-pseudotyped vector expressing β-gal intrarectally (*i.r.*) were euthanized at times indicated, the colons were resected, and frozen in OCT. Cryosections of colons were prepared, fixed and assessed for β-gal activity by overnight incubation with X-Gal developing solution. Representative sections for colons isolated from mice given EboZβ-gal *i.r.* on days 5-15 post-infection are shown. No visually discernable expression was seen in animals treated with PBS at all times.

Figure 12

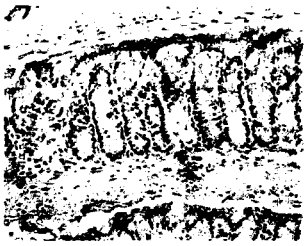
PBS

EboZβ-gal Day 5



EboZβ-gal Day 10

EboZβ-gal Day 15



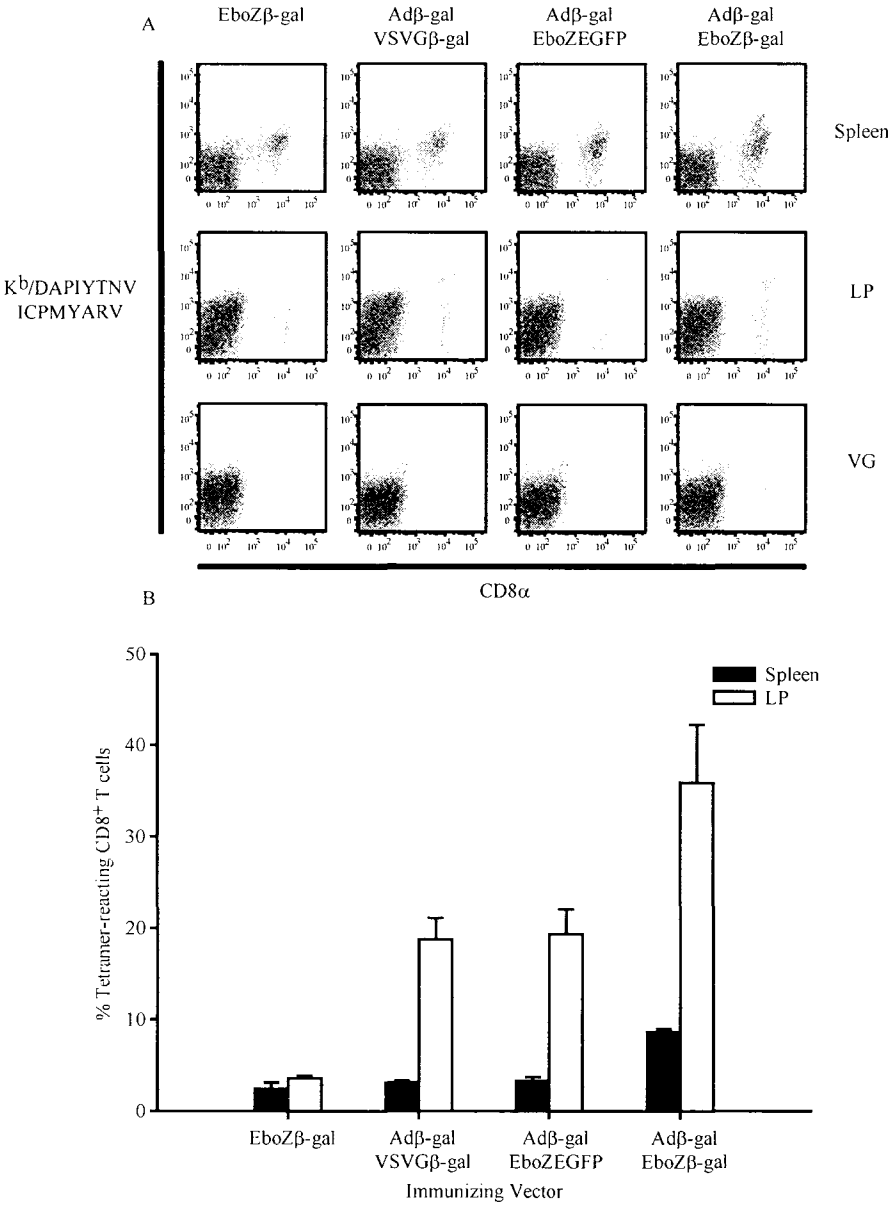
3.10 Induction of large intestinal and systemic CD8$^+$ T cell responses by intrarectal priming and boosting

Prime-boost vaccination is an important tool for increasing the immune response to immunogens (Woodland 2004). In the present study, EboZ-pseudotyped lentiviral constructs were evaluated for the purpose of enhancing CD8$^+$ T cell responses induced by *i.r.* Ad immunization. Therefore, C57BL/6 mice were left either untreated or immunized *i.r.* with 2.5×10^9 pfu of Adβ-gal and, 30 days later, either given 1×10^7 TU of EboZβ-gal (untreated group) or boosted *via* the same route with 1×10^7 TU of EboZEGFP, VSVGβ-gal or EboZβ-gal. EboZEGFP and VSVGβ-gal were used as control vectors to ensure that delivery of either EboZ-pseudotyped lentivirus vector encoding different transgene or VSVG-pseudotyped vector, infection with which did not result in visually discernable expression, did not boost β-gal-specific responses non-specifically. For the purposes of quantification of mucosal and systemic immune responses in mice, K^b/DAPIYTNV and K^b/ICPMYARV fluorescently-labelled tetramers were utilized to detect β-gal-specific CD8$^+$ T cell in the splenic and colonic LP populations by flow cytometric analyses (Fig. 13). In all treated groups, the highest percentage of DAPIYTNV- and ICPMYARV-binding CD8$^+$ T cells was observed in the LP compartment, a finding that was consistent with the antigen-driven migration of activated effector T cells to the colonic mucosa. Single *i.r.* EboZβ-gal immunization resulted in 3.6% of β-gal-specific CD8$^+$ T cells in the LP. Mice immunized *i.r.* with Adβ-gal and then inoculated with either VSVGβ-gal or EboZEGFP, displayed similar frequencies of tetramer-reactive CD8$^+$ T cells in the LP, 18.7% and 19.4%, respectively. Mice primed

i.r. with Adβ-gal and then boosted with EboZβ-gal had 35.9% β-gal-specific $CD8^+$ T cells in the LP. While the frequencies of tetramer-reactive $CD8^+$ T cells in the spleens of mice given EboZβ-gal, or a combination of Adβ-gal and either VSVGβ-gal or EboZEGFP did not exceed 3.3%, these increased to 8.6% in the mice primed with Adβ-gal and boosted with EboZβ-gal. Therefore, filovirus-pseudotyped lentivirus delivery enhanced the numbers of β-gal-specific $CD8^+$ T cells induced by *i.r.* Ad immunization. These results suggested that VSVG-pseudotyped lentiviral constructs did not boost colonic immune responses induced by Ad immunization and were consistent with the data obtained from *Section 3.9* indicating that VSVGβ-gal delivery did not result in β-gal expression in the colonic mucosa. Similar to studies reported in *Section 3.6*, *i.r.* Adβ-gal prime and EboZβ-gal boost did not result in the recruitment to and/or expansion of $CD8^+$ T cells in the vaginal tract. Therefore, these experiments demonstrated that EboZ inoculation at the colonic mucosa results in efficient boosting of mucosal immune responses elicited by *i.r.* administration of Ad-based vectors.

Figure 13. Characterization of β-galactosidase-specific CD8$^+$ T cell response in mice given Ad or EboZ-β-gal, Adβ-gal and EboZEGFP, Adβ-gal and VSVGβ-gal, or Adβ-gal and EboZβ-gal by the intrarectal route. Mice were first immunized with Ad virus encoding β-gal or left untreated. Thirty days later, untreated mice were inoculated intrarectally (*i.r.*) with EboZβ-gal, while Adβ-gal inoculated mice were given EboZEGFP, VSVGβ-gal or EboZβ-gal. Sixty days after *i.r.* Adβ-gal immunization, splenic and LP T cells were isolated, reacted with fluorescent K^b/DAPIYTNV and K^b/ICPMYARV tetramers and co-reacted with fluorescently-labelled antibodies specific for CD8α. The fluorescent cells were analyzed by flow cytometry. In panel *A*, representative flow cytometry plots of tetramer binding by CD8α$^+$ splenic, LP and VG populations of immunized mice are displayed. In panel *B*, the histogram represents the percentage (mean ± SEM) of tetramer-binding CD8$^+$ T cells of total CD8$^+$ T cells from 3-5 animals per group. The results are representative of two independent experiments.

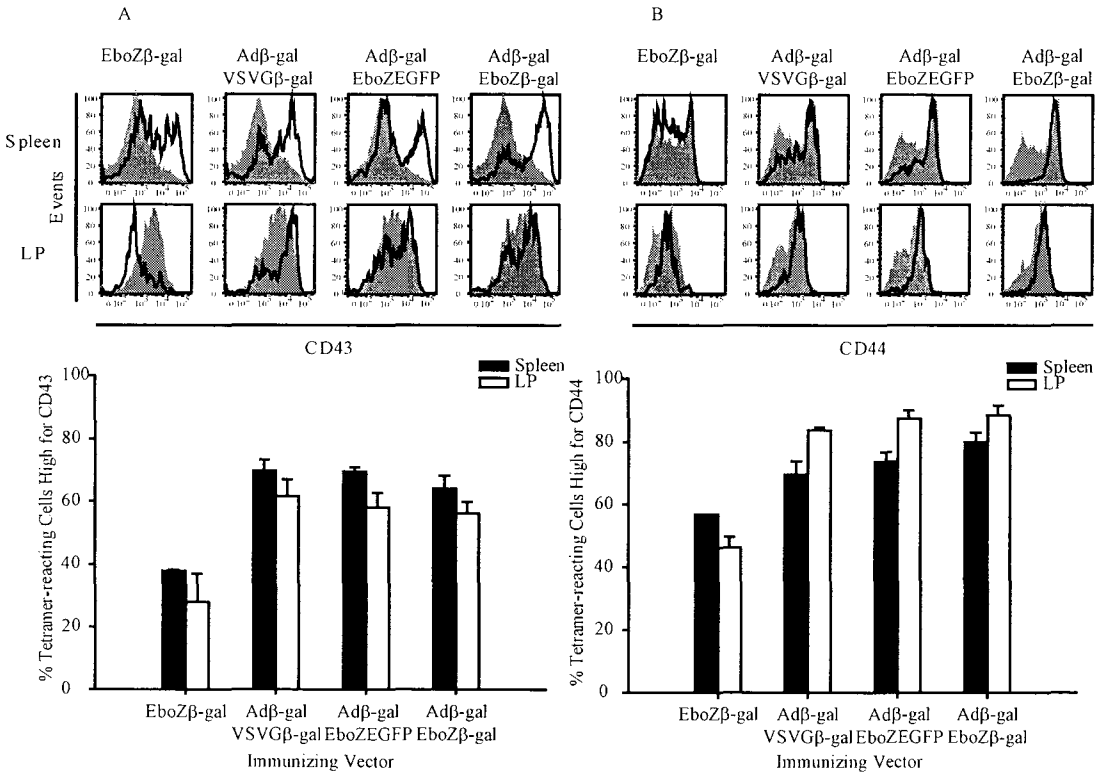
Figure 13


A
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
Spleen
LP
VG
Kb/DAPIYTNV ICPMYARV
CD8α
B
Spleen
LP
% Tetramer-reacting CD8+ T cells
50
40
30
20
10
0
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
Immunizing Vector

Phenotyping of antigen-specific $CD8^+$ T cells by detection of different surface activation and memory markers is an important tool for qualitative assessment of antigen-specific $CD8^+$ T cells (Seder, Darrah et al. 2008). Therefore, to further analyze the $CD8^+$ T cell populations induced by *i.r.* administration of Ad- and lentivirus-based vectors, the expression of CD43 (Fig. 14A) and CD44 (Fig. 14B) on K^b/DAPIYTNV- and ICPMYARV-tetramer-reactive $CD8^+$ T cells was evaluated by flow cytometric analyses at day 30 after the boost (60 days after primary immunization) in all groups of mice. The expression of CD43 isoform 1B11, which is expressed on $CD8^+$ T cells following antigen activation, was up-regulated on splenic and colonic LP β-gal-specific $CD8^+$ T cells after *i.r.* Ad inoculation, and its high levels were maintained irrespective of whether animals were boosted or not. In addition, the tetramer-positive $CD8^+$ T cells isolated from the spleen and colonic LP expressed high levels of the memory T cell marker, CD44 in primed and primed-boosted groups of animals. The expression of CD43 and CD44 was lower on splenic and LP $CD8^+$ T cells isolated 30 days after *i.r.* inoculation with EboZβ-gal, a finding that was consistent with the low levels of priming in this group. Based on the markers examined, these results demonstrated that EboZβ-gal boosting did not alter memory phenotype and activation status of the tetramer-reactive $CD8^+$ T cell population elicited in the course of *i.r.* priming with Adβ-gal.

Figure 14. Phenotypic characterization of β-galactosidase-specific CD8$^+$ T cells elicited after intrarectal inoculation with either EboZβ-gal, Adβ-gal and EboZEGFP, Adβ-gal and VSVGβ-gal, or Adβ-gal and EboZβ-gal. Sixty days following intrarectal (*i.r.*) inoculation with Ad-based vectors, β-gal-specific splenic and LP CD8α T cells were detected using fluorescent tetramers and co-reacted with fluorescently-labelled antibodies specific for CD43 and CD44. Cells were analyzed by flow cytometry. Representative data from one mouse for each tissue and different virus inoculation are shown for CD43 (panel *A*) and CD44 (panel *B*). The solid histograms correspond to total CD8$^+$ T cells and the open histograms correspond to tetramer-reactive T cells. Tetramer-binding populations were classified as expressing high and low fluorescence with respect to the aforementioned cell surface phenotypes. The percentages of CD8$^+$ T cells with a high level of expression of CD43 (panel *A*) and CD44 (panel *B*) are shown as mean ± SEM of 3-5 mice *per* group and time point. The results are representative of two independent experiments.

Figure 14


A
B
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
Spleen
LP
Events
CD43
CD44
Spleen
LP
% Tetramer-reacting Cells High for CD43
% Tetramer-reacting Cells High for CD44
0
20
40
60
80
100
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
Immunizing Vector

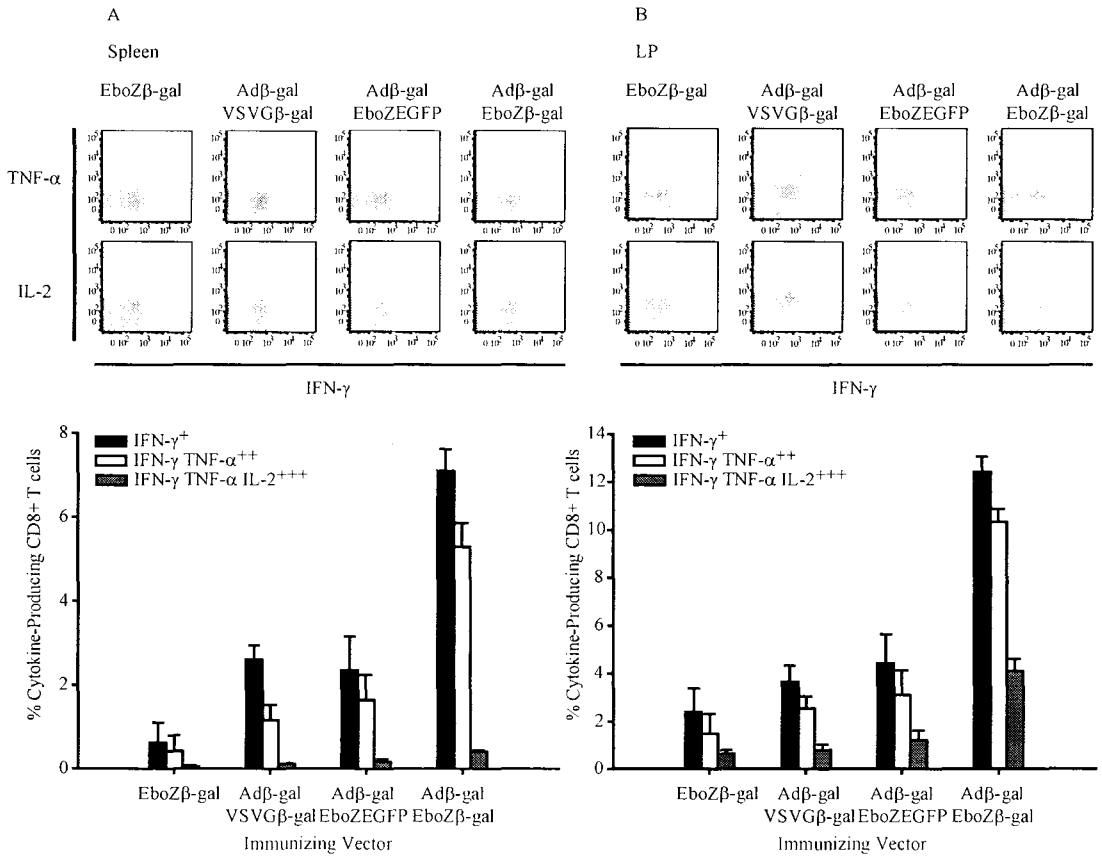
3.11 Functional activity of β-gal-specific CD8$^+$ T cells

Ability to produce cytokines is one of the most important functions of effector-memory CD8$^+$ T cells (Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004). In order to analyze the effector functions of the β-gal-specific CD8$^+$ T cells elicited by *i.r.* vaccination, cells isolated from the spleens (Fig. 15A) and the colonic LP (Fig. 15B) 60 days post-primary Ad inoculation were re-stimulated *in vitro* for 5-hours in the presence of DAPIYTNV and ICPMYARV peptides and assessed by flow cytometry for their ability to secrete IFN-γ, TNF-α, and IL-2 by flow cytometry. EboZβ-gal immunization resulted in low levels of IFN-γ-producing CD8$^+$ T cells in the spleen and LP thirty days post-immunization. At 60 days post-primary inoculation with Adβ-gal, mice that had been inoculated also with VSVGβ-gal or EboZEGFP had similar levels of IFN-γ - producing CD8$^+$ T cells, being 2.6% and 2.3%, in spleen and 3.7% and 4.4% in the LP, respectively. Lastly, animals primed with Adβ-gal and boosted with EboZβ-gal had the most IFN-γ-producing CD8$^+$ T cells in the spleen and LP populations, 7.1% and 12.4%, respectively, a finding consistent with the ability of EboZ to boost Ad-induced responses. Consistent with the data obtained with other antigens delivered *via i.r.* Ad-based immunization, even smaller fractions of β-gal-specific CD8$^+$ T cells produced TNF-α and IL-2 with all of the different virus deliveries examined. Similar to parenteral and *i.r.* Ad immunization models, antigen-specific CD8$^+$ effector T cells from the spleen and LP were diminished in their capacity to produce cytokines when the frequencies of cytokine-producing cells were compared to the frequencies of tetramer-reactive populations with difference being greatest in the LP. Overall, these results indicated that *i.r.* EboZ boosted

the cytokine-producing $CD8^+$ T cells induced by *i.r.* Ad priming and that these $CD8^+$ T cells appeared to be partially functionally impaired in terms of their cytokine production following restimulation.

Figure 15. Cytokine production of epitope-specific CD8$^+$ T cells. To further define virus-specific CD8$^+$ T cell responses in the splenic and LP populations of mice, the percentages of β-gal-specific CD8$^+$ T cells were measured by flow cytometry at 60 days following intrarectal (*i.r.*) Ad-based immunization by intracellular cytokine detection of IFN-γ, TNF-α and IL-2 by fluorescently-labelled antibodies. In panels *A* and *B*, representative plots of IFN-γ-, TNF-α-, and IL-2-containing CD8$^+$ T cells are shown for spleen (panel *A*) and LP (panel *B*) populations following *in vitro* restimulation with DAPIYTNV and ICPMYARV peptides 60 days after Adβ-gal immunization. In panels *A* and *B*, the percentage (mean ± SEM) of IFN-γ-, TNF-α- and IL-2-containing CD8$^+$ T cells are shown for splenic (panel *A*) and LP (panel *B*) populations following *in vitro* restimulation with DAPIYTNV and ICPMYARV peptides 60 days after initial Ad immunization. Data represent 3-4 animals per group. The results are representative of two independent experiments.

Figure 15

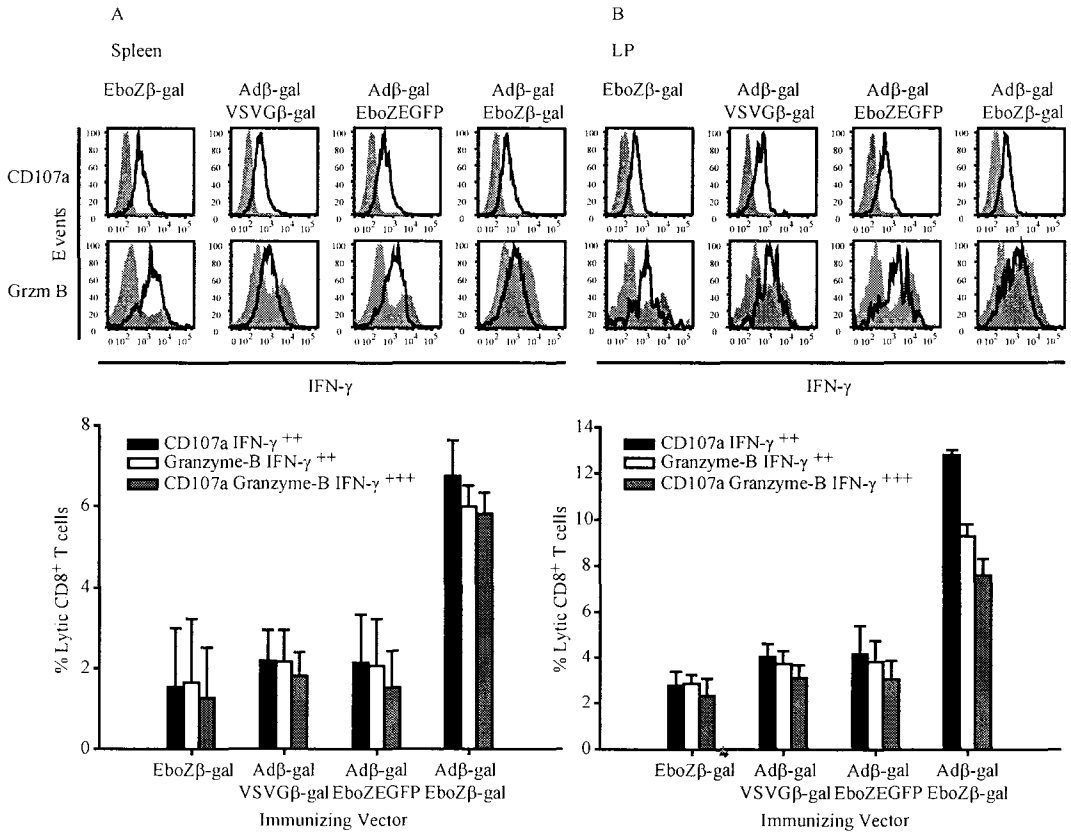

A
Spleen
B
LP
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
TNF-α
IL-2
IFN-γ
IFN-γ+
IFN-γ TNF-α++
IFN-γ TNF-α IL-2+++
% Cytokine-Producing CD8+ T cells
Immunizing Vector

Cytolytic potential is a hallmark of the antigen-specific effector-memory $CD8^+$ T cells induced during the course of different viral infections (Seder, Darrah et al. 2008). The granule-mediated cytotoxic potential of IFN-γ^+ $CD8^+$ T cells can be measured by detecting the ability of these cells to degranulate upon activation using the method incorporating fluorescently-labelled anti-CD107a antibodies during the 5 h stimulation with DAPIYTNV and ICPMYARV peptides in the presence of Monensin, which is followed intracellular cytokine staining. Therefore, 60 days post-primary Ad delivery, the splenic (Fig. 16A) and LP (Fig. 16B) cell populations isolated from mice inoculated with EboZβ-gal, Adβ-gal and either VSVGβ-gal, EboZEGFP, or EboZβ-gal were tested for the ability to degranulate upon the stimulation with DAPIYTNV and ICPMYARV and examined for the presence of CD107a using flow cytometric analyses. Most IFN-γ-producing $CD8^+$ T cells, irrespective of the prime-boost regimen, degranulated upon peptide stimulation. Also, the functionally lytic $CD8^+$ T cells induced by different viral infections have been shown to contain granzyme-B when examined directly *ex vivo*. In addition, *de novo* synthesis of granzyme B by effector cells is not required for perforin/granzyme-B mediated cytotoxicity (Slifka, Rodriguez et al. 1999; Tatsis, Fitzgerald et al. 2007). Therefore, antigen-specific IFN-γ-producing $CD8^+$ T cells from splenic and LP populations were examined for presence of granzyme-B by flow cytometric analyses 60 days post-primary inocultion. The majority of antigen-specific, IFN-γ-producing $CD8^+$ T cells isolated from spleen (Fig. 16A) and LP (Fig. 16B) of mice inoculated with EboZβ-gal, Adβ-gal and either VSVGβ-gal, EboZEGFP, or EboZβ-gal contained granzyme-B. Indeed, most granzyme-B containing $CD8^+$ T cells in splenic and

LP populations had contained CD107a. Therefore, majority of IFN-γ-producing $CD8^+$ T cells in the spleen and LP showed lytic potential by containing granzyme-B and degranulating upon the exposure to the antigen.

Figure 16. Functional characterization of cytotoxic T lymphocytes induced by colonic Ad and EboZ-pseudotyped lentivirus gene delivery into mice. To further assess the effector function of antigen-specific $CD8^+$ T cells, their cytolytic activity was measured 60 days following intrarectal (*i.r.*) immunization with Adβ-gal. The ability of splenic and LP $CD8^+$ T cells to degranulate was monitored by the appearance of cell surface CD107a detected by flow cytometry following re-stimulation with DAPIYTNV and ICPMYARV peptides of IFN-γ^+ $CD8^+$ (open areas) and total $CD8^+$ (shaded areas) T cells. In addition, IFN-γ^+ $CD8^+$ (open areas) and total $CD8^+$ (shaded areas) T cells were examined also for the presence of granzyme B (GrzmB). Cytotoxic cells were analyzed by fuorescence flow cytometry. In panels *A* and *B*, representative plots of degranulating and granzyme B-containing IFN-γ^+ $CD8^+$ T cells are shown for splenic (panel *A*) and LP (panel *B*) populations following *in vitro* re-stimulation with DAPIYTNV and ICPMYARV peptides 60 days after Adβ-gal immunization. In panels *A* and *B*, the histograms represent the percentages $CD107a^+$ and granzyme B^+ IFN-γ^+ $CD8^+$ T cells from 3-4 mice and presented as mean ± SEM. The results are representative of two independent experiments.

Figure 16


A
Spleen
B
LP
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
CD107a
Grzm B
Events
IFN-γ
CD107a IFN-γ ++
Granzyme-B IFN-γ ++
CD107a Granzyme-B IFN-γ +++
% Lytic CD8+ T cells
Immunizing Vector

3.12 Proliferative and protective capacity of T_{EM} $CD8^+$ T cells in intrarectally primed and boosted mice

Expansion of T cells upon repeated *in vivo* exposure to the antigen is a hallmark of successful prime-boost strategy (Kaech, Wherry et al. 2002). For example, intravaginal vaccinia virus challenge resulted in a robust expansion of antigen-specific $CD8^+$ lymphocytes in immunized mice between 3-7 days post-immunization, and clearance of the virus was mediated by antigen-specific $CD8^+$ T cells. β-galactosidase-specific $CD8^+$ T cells induced during the course of inoculation with a single or different combinations of Ad and EboZ- and VSVG-pseudotyped lentiviruses were evaluated for their ability to proliferate upon challenge with vaccinia virus expressing β-gal (Vaccβ-gal) administered *i.vag.* 60 days after primary *i.r* immunization with Ad β-gal. Six days following *i.vag.* challenge with the vaccinia virus vector, cells from the spleen, LP and vaginal mucosa were isolated, and the frequencies of antigen-specific $CD8^+$ T cells in different mice were compared by staining with fluorescently-labeled K^b/DAPIYTNV and K^b/ICPMYARV tetramers. The fluorescent cell populations were subjected to flow cytometric analyses. β-galactosidase-specific $CD8^+$ T cells demonstrated proliferation capacity, as judged by enumeration of tetramer-reacting $CD8^+$ T cells, in the spleen, LP and vaginal tract (Fig. 17A), which was especially prominent in mice immunized with Adβ-gal and further inoculated with either VSVGβ-gal, or EboZEGFP, or EboZβ-gal. While the frequency of $CD8^+$ T cells in the vaginal mucosa prior to any *i.vag.* infection was very low [the tetramer technology used to detect antigen-specific $CD8^+$ T cells could not be used reliably to estimate the frequencies of antigen-specific T cells in the vaginal tract 60 days

following primary Ad inoculation], $CD8^+$ T cells in Adβ-gal-immunized mice were dramatically expanded in and/or recruited to the vaginal tract. The frequency of β-gal-specific $CD8^+$ T cells reached 32.5% and 35.4% in VSVGβ-gal and EboZEGFP groups, respectively. However, the frequency of β-gal-specific $CD8^+$ T cells exceeded 65% of all vaginal $CD8^+$ T cells in the group that was primed with Adβ-gal and boosted with EboZβ-gal. In this group, the frequencies of tetramer-binding $CD8^+$ T cells in the spleen and LP has increased upon *i.vag.* Vaccβ-gal infection from day 60 to day 6 post-challenge (from 8.6% to 12.0% and from 36% to 40%, respectively). Therefore, *i.r.* Adβ-gal prime and EboZβ-gal boost resulted in a robust expansion of antigen-specific $CD8^+$ T-cell response upon mucosal Vaccβ-gal challenge. The results of these experiments also indicated that the $CD8^+$ T cells induced by *i.r.* Ad-immunization could be expanded as many as 60 days after immunization.

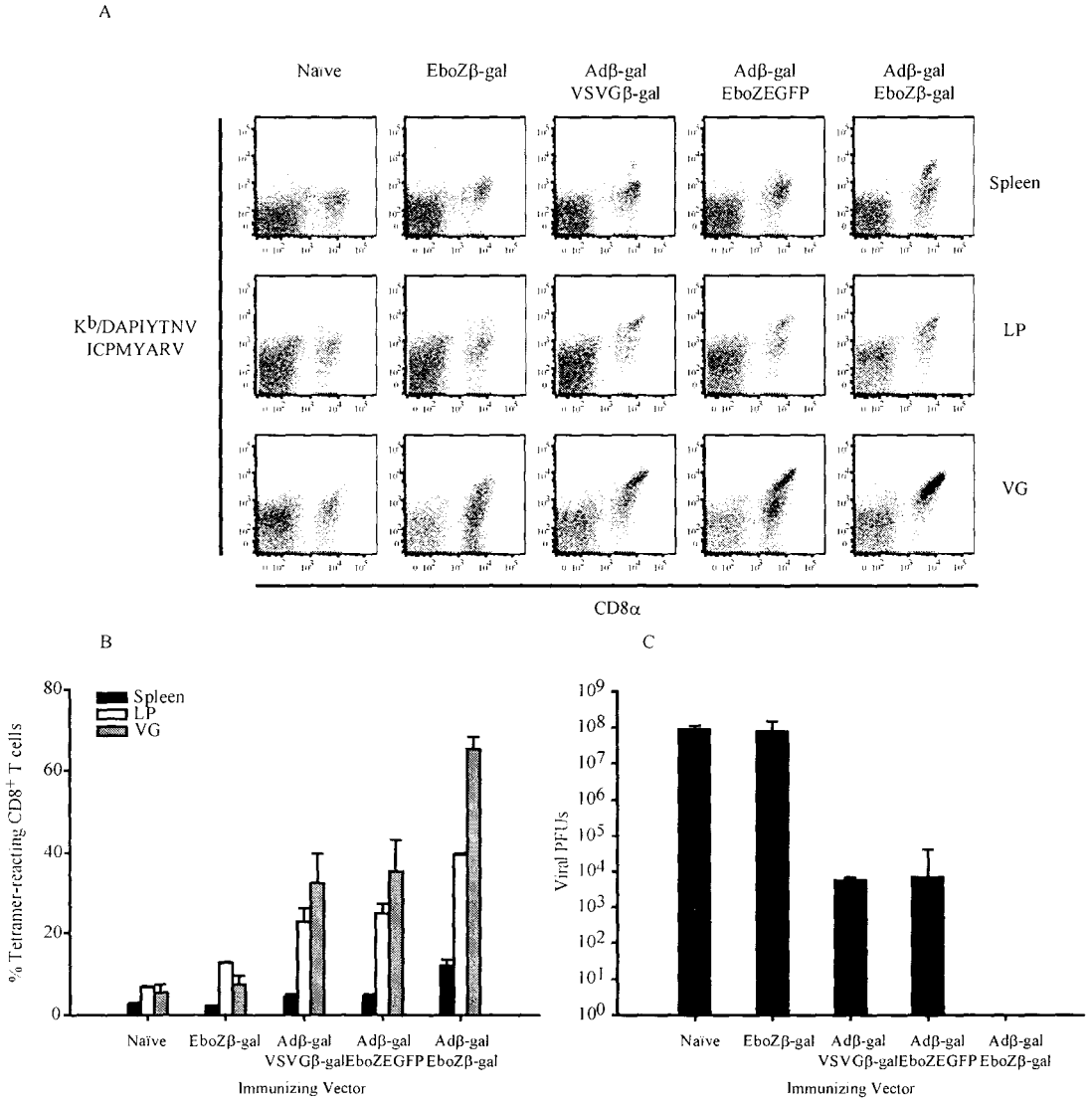
The ultimate goal of the prime-boost regimen was to induce antigen-specific $CD8^+$ T cells that can provide immunity from live viral challenge (Woodland 2004). To confirm the functionality of the $CD8^+$ T cell response induced by *i.r.* Ad priming and filovirus-pseudotyped lentivirus boosting, mice that had been inoculated *i.r.* with EboZβ-gal, Adβ-gal and either VSVGβ-gal, EboZEGFP or EboZβ-gal were challenged *i.vag.* with 2×10^7 pfu of Vaccβ-gal. Six days after Vaccβ-gal inoculation, the mice were sacrificed, their ovaries were removed, homogenized and analyzed for the presence of vaccinia virus using CV-1 cell monolayers. Following mucosal Adβ-gal prime and EboZβ-gal boost, a 9-log reduction in virus titers as compared to naïve mice occurred, while a 4-log reduction occurred in Adβ-gal mice also given either VSVGβ-gal or EboZEGFP (Fig.

17B). Therefore, Ad-based priming and filovirus-pseudotyped lentivirus boosting protected the mice from Vaccβ-gal challenge that was only partially cleared by a single *i.r.* Adβ-gal immunization.

Figure 17. Recall proliferation and induction of protective immunity in the intravaginal recombinant vaccinia challenge model following rectal treatment with either EboZβ-gal, Adβ-gal and EboZEGFP, Adβ-gal and VSVGβ-gal, or Adβ-gal and EboZβ-gal. To determine the protective efficacy of the mucosal immune response following intrarectal (*i.r.*) Adβ-gal and EboZ-pseudotyped lentivirus administration, primed and boosted mice were challenged intravaginally (*i.vag.*) with recombinant vaccinia virus carrying the gene encoding β-gal 60 days following priming. To determine the magnitude of the $CD8^+$ T cell response after challenge, cells isolated from the indicated tissues were reacted with fluorescent tetramers and analyzed by flow cytometry. In panel *A*, representative flow cytometry plots of tetramer binding by $CD8\alpha^+$ splenic, LP and vaginal tract (VG) populations of immunized mice are displayed. In panel *B*, the histograms represent the percentages (mean ± SEM) of tetramer-binding $CD8^+$ T cells in the spleen (closed bars), LP (open bars) and VG (shaded bars) 6 days after *i.vag.* vaccinia challenge. Data represent the mean ± SEM of 3-4 mice *per* group and time point. Vaccinia virus titers in mouse ovaries were determined, using CV-1 cell monolayers, 6 days post-challenge in mice previously given either EboZβ-gal, Adβ-gal and EboZEGFP, Adβ-gal and VSVGβ-gal or Adβ-gal and EboZβ-gal. In panel *C*, the histogram graph shows the ovarian vaccinia virus titer (mean ± SEM) in mice previously administered either EboZβ-gal, Adβ-gal and EboZEGFP, Adβ-gal and VSVGβ-gal or Adβ-gal and EboZβ-gal. The difference between naïve and the groups of mice treated with either Adβ-gal and EboZEGFP, Adβ-gal and VSVGβ-gal or Adβ-gal and EboZβ-gal was significant at $P < 0.001$ by log-transformed Student's t test. The difference between EboZβ-gal and the

groups of mice treated with either Adβ-gal and EboZEGFP, Adβ-gal and VSVGβ-gal, or Adβ-gal and EboZβ-gal was significant at $P < 0.001$ by log-transformed Student's t test. The difference between the groups of mice treated Adβ-gal and EboZβ-gal and the groups of mice treated with either Adβ-gal and EboZEGFP or Adβ-gal and VSVGβ-gal was significant at $P < 0.001$ by log-transformed Student's t test. The results are representative of two independent experiments.

Figure 17


A
Naïve
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
Spleen
LP
VG
Kb/DAPIYTNV
ICPMYARV
CD8α
B
Spleen
LP
VG
% Tetramer-reacting CD8+ T cells
Naïve
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
Immunizing Vector
C
Viral PFUs
Naïve
EboZβ-gal
Adβ-gal VSVGβ-gal
Adβ-gal EboZEGFP
Adβ-gal EboZβ-gal
Immunizing Vector

3.13 Characterization of AdgB-induced immune response

Based on the studies using OVA as a model antigen (*Section 3.2*), it was determined that $CD8^+$ T cell response to the antigen reached a maximum approximately 14 days following *i.r.* immunization. To evaluate the immune response to viral antigen as opposed to a model antigen, mice were inoculated *i.r.* with 2.5×10^9 pfu of Ad virus carrying the glycoprotein B (gB) gene (AdgB) of herpes simplex virus type 1 (HSV-1), the expression of which was driven by the simian virus 40 (SV40) promoter. [In the AdOVA virus used in *Sections 1-8*, OVA expression was controlled by the murine cytomegalovirus (mCMV) promoter; therefore, the frequencies of antigen-specific T cells induced following administration of these viruses might be different, although dose response studies to investigate this were not performed.] It was previously determined that gB-specific $CD8^+$ T cell response during HSV-1 infection in C57BL/6 mice was focused almost entirely at the immunodominant $gB_{498\text{-}505}$ peptide SSIEFARL (Wallace, Keating et al. 1999). Therefore, to analyze the antigen-specific $CD8^+$ T cell response to glycoprotein B, fluorochrome-labelled tetramers incorporating $gB_{498\text{-}505}$ peptide SSIEFARL were used to detect splenic, blood and LP $CD8^+$ T cell populations on day 14 post-immunization. Tetramer-binding cells were detected by flow cytometric analyses. Intrarectal AdgB immunization led to the induction of gB-specific $CD8^+$ T cells in all compartments examined with a maximal frequency (19.8 %) of antigen-specific T cells observed in the colonic LP, an intermediate frequency (14.3 %) observed in blood and the lowest frequency (6.1 %) observed in the spleen (Fig. 18A). The highest percentage of tetramer-binding T cells observed in the colonic LP was consistent with antigen-driven

migration of lymphocytes to the site of Ad infection, a finding similar to that obtained following AdOVA immunization. Additionally, the high frequencies of antigen-specific T cells detected in circulation 14 days post-immunization might have reflected the migration of $CD8^+$ T cells to different effector sites in the body. Such migration of effector T cells to many lymphoid and extra-lymphoid sites occurred quickly following number of different acute infections, such as following influenza virus infection (Lawrence and Braciale 2004). These results were consistent also with the results from *Section 3.1* and other published experiments, wherein systemically-engrafted T cells traveled to many different sites following mucosal and systemic viral infections (Masopust, Vezys et al. 2001; Yang, Millar et al. 2006). Therefore, *i.r.* AdgB immunization had resulted in the induction of gB-specific $CD8^+$ T cells in the mucosal and systemic sites with most antigen-specific T cells found in the LP.

The surface phenotype characterization initially helped to define different memory T lymphocyte populations induced by immunization and remains important tool for qualitative assessment of $CD8^+$ T cells (Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004). Therefore, 14 days post-immunization with AdgB, K^b/SSIEFARL tetramer-binding $CD8^+$ T cells from the spleen, the blood and the LP were examined for surface expression of CD44 and CD127 by flow cytometric analyses. Tetramer-binding $CD8^+$ T cell populations in all sites examined demonstrated the effector memory phenotype with high levels of expression of CD44 and low levels of expression of CD127 (Fig. 18B). These results were consistent with the phenotype of antigen-specific T cells observed following AdOVA priming (*Section 3.3*). Therefore, *i.r.* immunization with AdgB led to

the induction of systemic and mucosal gB-specific $CD8^+$ T cells with an effector-memory phenotype.

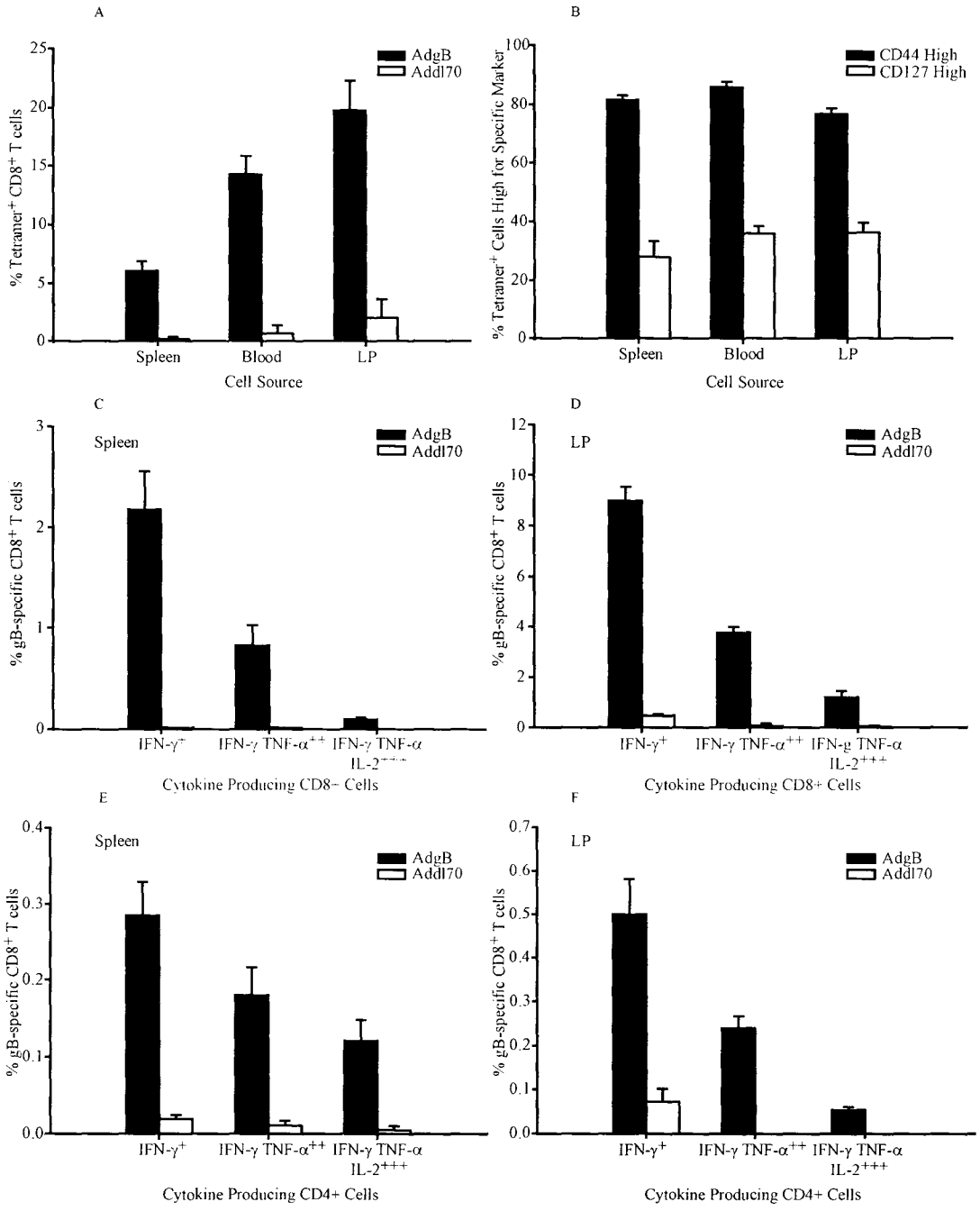
Immunization regimens that induced higher frequencies of antigen-specific $CD8^+$ T cells producing a variety of cytokines are associated with better protection from *Leishmenia major* in mice (Seder, Darrah et al. 2008). For example, in humans, the higher frequencies of $CD8^+$ T cells that produce multiple cytokines had been observed in HIV non-progressors compared to progressors (Betts, Brenchley et al. 2003). Therefore, to analyze the potential functionality of $CD8^+$ T cells induced by *i.r.* priming, as judged by cytokine expression, the frequency of gB-specific T cells in the spleen and the LP was characterized utilizing intracellular cytokine staining (ICS) following SSIEFARL peptide stimulation 14 days after AdgB immunization. Approximately 3-fold more cytokine producing cells were found in the mucosal compartment of LP (Fig. 18D) compared to the spleen (Fig. 18C) with 9% of IFN-γ secreting $CD8^+$ T cells in the LP. This finding was consistent with exposure to the antigen at this mucosal site and with the results from studies using the OVA antigen model (*Section 3.5*). Additionally, while up to 9% of the LP cells produced IFN-γ, smaller fractions of these cells produced TNF-α or IL-2. This observation had been linked to functional exhaustion of $CD8^+$ T cells observed in systemic models of Ad-based priming (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006) and was consistent with data obtained following *i.r.* AdOVA immunization (*Section 3.5*).

Although Ad-based immunization was reported previously to be more effective at inducing antigen-specific $CD8^+$ T cells than $CD4^+$ T cells, such $CD4^+$ T cells could still

be detected at different times after Ad immunization (Yang, Millar et al. 2007). Since the immunodominant MHC class II gB epitope not been elucidated, $CD4^+$ T cells were enumerated by flow cytometry using intracellular cytokine staining (ICS) procedure following a 12-hour stimulation with whole HSV-2 gB protein 14 days post-immunization. Since the overall amino acid homology between gB from HSV-1 and HSV-2 is 85%, gB antigen from HSV-1, administered using different immunization platforms, can be used to effectively immunize against HSV-2. Antigen-specific $CD4^+$ T cells were detected in splenic (Fig. 18E) and LP (Fig. 18F) populations with greater frequencies of IFN-γ secreting $CD4^+$ T cells (0.5%) found in the LP. Small but detectable frequencies of TNF-α- and IL-2-producing $CD4^+$ T cells were found also in both the systemic and mucosal compartments. Since the conditions for stimulation of $CD4^+$ T cells using intact protein might not have been optimal and/or the concentration of processed MHC class II immunodominant gB peptide(s) might have been low, the frequency of gB-specific $CD4^+$ T cells detected might have underestimated the magnitude of $CD4^+$ T cell response following Ad immunization. Additionally, the diminished ability of Ad-induced $CD4^+$ T cells to produce cytokines and the hierarchy of cytokine-producing T cell populations was similar to that observed in gB- and OVA-specific $CD8^+$ T cells. Therefore, *i.r.* AdgB immunization induced antigen-specific $CD4^+$ cells with the mucosal LP response being more prevalent due to the antigen exposure at that site.

Figure 18. Characterization of herpes simplex virus gB-specific CD4$^+$ and CD8$^+$ T cell response in mice immunized intrarectally with AdgB. At 14 days after intrarectal (*i.r.*) AdgB immunization, splenic, blood and LP T cells were isolated, reacted with fluorescent H2-K^b gB$_{498\text{-}505}$ tetramers and co-reacted with fluorescently-labelled antibodies specific for CD44 and CD127. Cells were enumerated by flow cytometry. Tetramer-binding populations were classified as high and low fluorescence with respect to the above cell surface phenotype. Panel *A* represents the percentage (mean ± SEM) of tetramer-binding CD8$^+$ T cells of total CD8$^+$ T cells from 3-5 animals per group. In panel *B*, the percentages of CD8$^+$ T cells with a high level of expression of CD44 and CD127 are shown as mean ± SEM of 3-5 mice *per* group and time point. To further define virus-specific CD8$^+$ T cell responses in the spleen and LP populations of mice, the percentages of gB-specific CD4$^+$ and CD8$^+$ T cells were measured by flow cytometry at 14 days following *i.r.* AdgB immunization by intracellular cytokine detection of IFN-γ, TNF-α and IL-2 using fluorescently-labelled antibodies. In panels *C* and *D*, the percentages (mean ± SEM) of IFN-γ-, TNF-α- and IL-2-containing CD8$^+$ T cells are shown for splenic (panel *C*) and LP (panel *D*) populations following *in vitro* restimulation with herpes simplex virus type 1 (HSV-1) SSIEFARL peptide 14 days after AdgB immunization. These data represent 4 animals per group. In panels *E* and *F*, the percentages (mean ± SEM) of IFN-γ-, TNF-α- and IL-2-containing CD4$^+$ T cells are shown for splenic (panel *E*) and LP (panel *F*) populations following *in vitro* restimulation with HSV-2 gB-protein 14 days after AdgB immunization. Data represents 4 animals per group. The results are representative of two independent experiments.

Figure 18


A
% Tetramer+ CD8+ T cells
AdgB
Addl70
Spleen
Blood
LP
Cell Source
B
% Tetramer+ Cells High for Specific Marker
CD44 High
CD127 High
Spleen
Blood
LP
Cell Source
C
Spleen
% gB-specific CD8+ T cells
AdgB
Addl70
IFN-γ+
IFN-γ TNF-α++ IFN-γ TNF-α IL-2+++
Cytokine Producing CD8- Cells
D
LP
% gB-specific CD8+ T cells
AdgB
Addl70
IFN-γ+
IFN-γ TNF-α++ IFN-g TNF-α IL-2+++
Cytokine Producing CD8+ Cells
E
Spleen
% gB-specific CD8+ T cells
AdgB
Addl70
IFN-γ+
IFN-γ TNF-α++ IFN-γ TNF-α IL-2+++
Cytokine Producing CD4+ Cells
F
LP
% gB-specific CD8+ T cells
AdgB
Addl70
IFN-γ+
IFN-γ TNF-α++ IFN-γ TNF-α IL-2+++
Cytokine Producing CD4+ Cells

3.14 Protective efficacy of i.r. AdgB immunization in the context of mucosal HSV-2 challenge

In order to evaluate the protective efficacy of mucosal, gB-specific responses, *i.r.* and *i.vag.* murine HSV-2 infection models were chosen. It was shown previously that *i.r.* AdgB immunization was 100% effective in protecting the mice from lethal *i.r.* (2×10^6 pfu) and *i.vag.* (2×10^5 pfu) HSV-2 challenge two to three weeks after immunization (Zhu, Thomson et al. 2008). These HSV-2 challenge times were chosen arbitrarily without the prior knowledge of the magnitude and kinetics of T cell response induced by *i.r.* Ad immunization. Since mucosal and systemic rectal Ad-induced $CD8^+$ T cell responses were maximal at day 14 post-immunization, the protection from lesser but still lethal rectal (1×10^5 pfu) or vaginal (1×10^4 pfu) HSV-2 inoculations was examined 30 days after AdgB immunization, a time when the primary effector T cell-response subsided (Fig. 19B). Intrarectal AdgB immunization led to 80% or 89% of mice being protected following HSV-2 vaginal or colonic challenge, respectively, as compared to only 0-19% in the control Addl70-immunized group. While a low level of protection was observed in Addl70-immunized group, these levels were above those observed in naïve mice. This might be due to the stimulation of the innate components of the immune system by *i.r.* Ad-delivery method. In addition, while *i.r.* Addl70-immunized mice, on average, showed advanced pathology, reaching a score of 5 in mice challenged *i.r.* with HSV-2 and score of 4.8 in mice challenged *i.vag.* with HSV-2, it remained low in mice immunized *i.r.* with AdgB, reaching the score of 1 in mice challenged *i.r.* with HSV-2 and score of 0.9 in mice challenged *i.vag.* with HSV-2 (Fig. 19C). Therefore, *i.r.* AdgB immunization led to

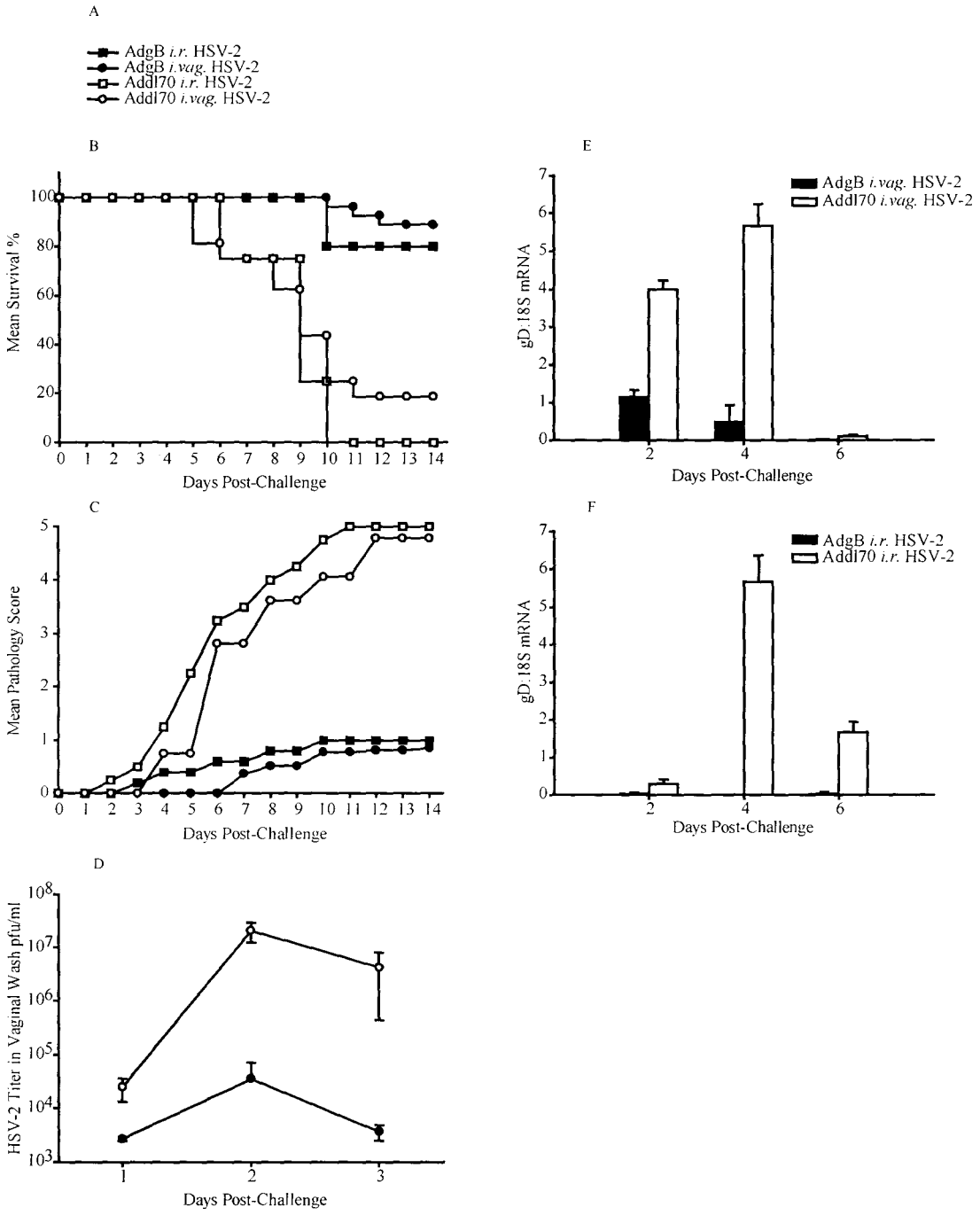
the induction of an immune response that protected the majority of mice from both vaginal and colonic HSV-2 challenge.

While most AdgB-immunized mice survived lethal *i.r.* or *i.vag.* HSV-2 challenges, the measure of the viral titers also provided information about the productivity of the HSV-2 infection and the efficacy of AdgB immunization. Viral titers in the vaginal washes on days 1-3 following *i.vag.* HSV-2 challenge were determined using Vero cell monolayers (Fig. 19D). Additionally, the levels of HSV-2 glycoprotein D (gD) RNA transcript from vaginal (Fig. 19E) or intestinal tissues (Fig. 19F), as detected by RT-PCR, following *i.r.* or *i.vag.* HSV-2 challenges, respectively, were examined also on days 2-6 post-challenge. While very little virus was detected in the vaginal washes of AdgB-immunized mice following *i.vag.* HSV-2 challenge, mice immunized with Addl70 vector were unable to control the infections, which resulted in significantly higher viral titers (Fig. 19D) detected in the vaginal washes of these mice compared to AdgB immunized mice. Similarly, higher levels of the gD RNA transcript were detected in vaginal (Fig. 19E) and colonic tissues (Fig. 19F) of control Ad-immunized groups as compared to AdgB-inoculated mice. Therefore, *i.r.* AdgB-immunization resulted in efficient viral clearance at the site of infection, as detected by the low HSV-2 titers found in the vaginal washes and low levels of the gD RNA transcript detected in both vaginal and colonic tissues of the mice inoculated *i.vag.* or *i.r.* with lethal HSV-2, respectively.

Figure 19. Examination of protection from intrarectal and intravaginal herpes simplex virus type 2 infection in mice rectally immunized with AdgB. To assess whether intrarectal (*i.r.*) AdgB immunization can result in protection from mucosal HSV-2 challenge, mice immunized with 2.5×10^9 pfu of AdgB were challenged either *i.r.* or intravaginally (*i.vag.*) with HSV-2 30 days post-immunization. In panel *A*, the legend for data in panels *B-D* is shown. Closed squares represent the data for AdgB immunized mice challenged *i.r.* with HSV-2, and open squares represent the data for Addl70 immunized mice challenged *i.r.* with HSV-2. Closed circles denote AdgB immunized mice infected *i.vag.* with HSV-2, and open circles represent the data for Addl70 immunized mice infected *i.vag.* with HSV-2. In panel *B*, the mean pathology score of *i.r.* immunized mice is shown for each group of 20-25 mice. In panel *B*, the mean survival percentage of *i.r.* immunized mice from days 0 to day 14 following HSV-2 challenge is depicted for each group of 20-26 mice. In panel *C*, the mean genital pathology scores of *i.r.* immunized mice are shown for each group of 20-26 mice. In panel *D*, the viral titers (mean ± SEM) in the vaginal washes of mice on days 1-3 following *i.vag.* HSV-2 challenge. In panel *E*, the histogram represents the percentage (mean ± SEM) of gD:18S mRNA levels as determine by RT-PCR in the vaginal tissues of AdgB- (closed bars) and Addl70-immunized (open bars) mice challenged *i.vag.* with HSV-2. In panel *F*, the gD:18S mRNA levels (mean ± SEM) as determine by RT-PCR in the rectal tissues of AdgB- (closed bars) and Addl70-immunized (open bars) mice challenged *i.r.* with HSV-2. Data represent the mean ± SEM of 3-4 mice *per* group and time point. The difference in gD:18S mRNA levels between AdgB-immunized and Addl70-immunized groups of

mice was significant at $P < 0.05$ by Student's t test. The results are representative of two independent experiments.

Figure 19


A
AdgB i.r. HSV-2
AdgB i.vag. HSV-2
Addl70 i.r. HSV-2
Addl70 i.vag. HSV-2
B
Mean Survival %
Days Post-Challenge
C
Mean Pathology Score
Days Post-Challenge
D
HSV-2 Titer in Vaginal Wash pfu/ml
Days Post-Challenge
E
AdgB i.vag. HSV-2
Addl70 i.vag. HSV-2
gD.18S mRNA
Days Post-Challenge
F
AdgB i.r. HSV-2
Addl70 i.r. HSV-2
gD.18S mRNA
Days Post-Challenge

3.15 Role of T lymphocytes in mediating protection from HSV-2 challenge

Different components of adaptive immune system play important roles in the clearance of many mucosal and systemic viral infections. Specifically, studies examining the role of HSV-2-specific antibodies in protecting mice from HSV-2 challenge concluded that while the absence of most antibodies resulted in the delay of viral clearance, antibody-deficient mice survived the *i.vag.* HSV-2 challenge (Tatsis, Fitzgerald et al. 2007; Tengvall, O'Hagan et al. 2008). Additionally, $CD3^+$ T cells were shown to be vital for protection of mice from *i.vag.* HSV-2 challenge as their depletion completely abrogated the protection in immunized mice (Milligan, Dudley-McClain et al. 2004). While the role of different cytokines in protection from HSV-2 challenge has not been extensively studied, IFN-γ had been shown to be vital for protection of mice from lethal *i.vag.* HSV-2 challenge, while the absence of TNF-α did not affect the survival of immunized mice (Milligan, Dudley-McClain et al. 2004). To examine the role of $CD4^+$ and $CD8^+$ T cells in conferring mice with protective immunity to mucosal HSV-2 infection, the levels of vaginal and rectal protection in CD4 and CD8 gene-deleted mice, operationally-termed "knockout" animals, were examined 30 days following *i.r.* AdgB immunization. The protective efficacy of AdgB immunization was evaluated by survival studies and the detection of HSV-2 in vaginal secretions or gD RNA transcripts in the large intestines by RT-PCR on days 2-6 following mucosal HSV-2 delivery. These and others studies demonstrated that the protection was abrogated completely in CD4 knockout (and in MHC class II knockout animals; data not shown), but not in vaginal (Fig. 20B) and rectal acute lethality models in CD8 knockout animals (Fig. 20C). Advanced pathology scores

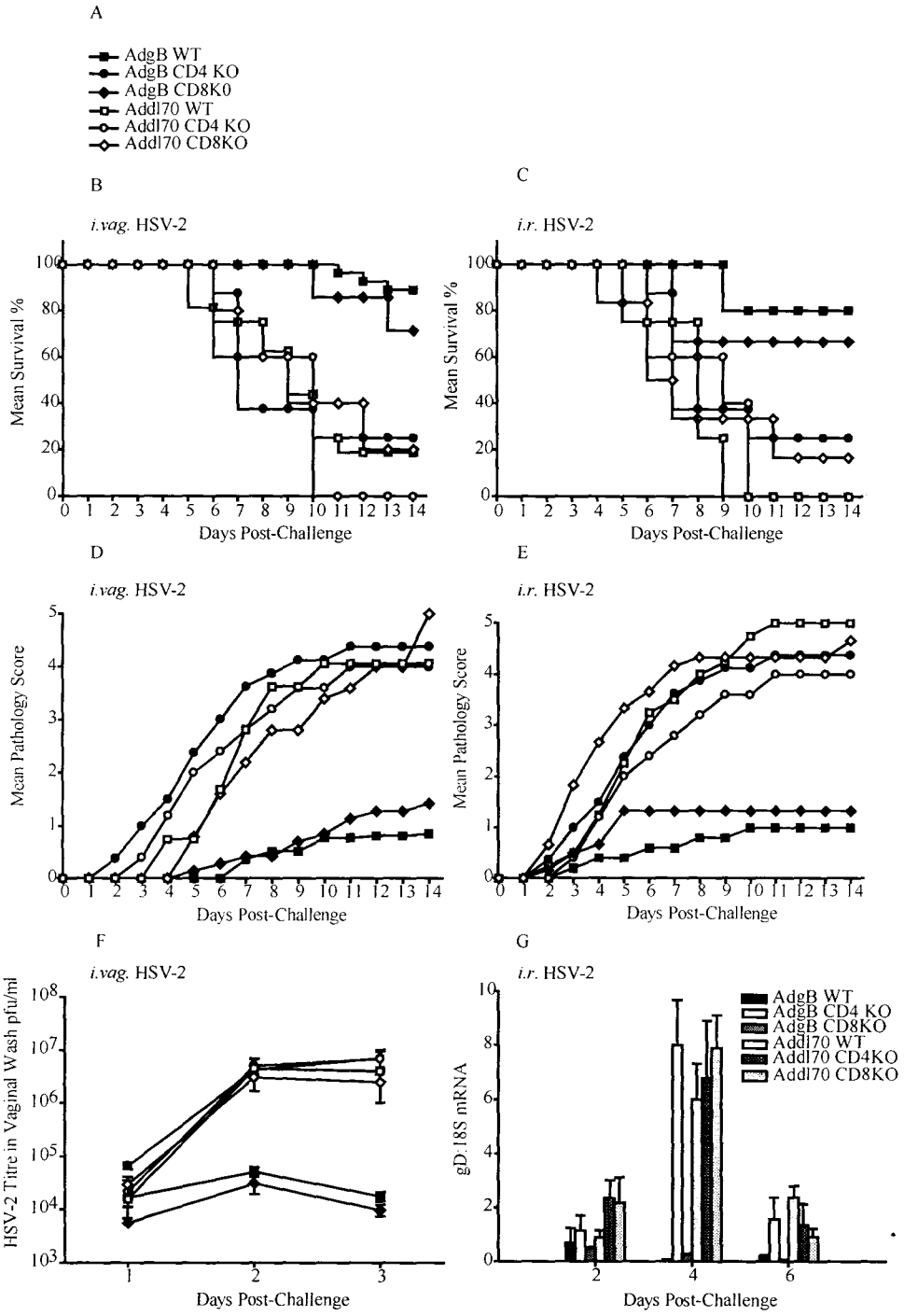
in AdgB-immunized CD4 knockout animals reflected the high mortality of these mice following either *i.vag.* (Fig. 20D) or *i.r.* (Fig. 20E) HSV-2 challenge. Similarly, AdgB-immunized CD4 knockout animals had high viral titers detected in vaginal washes on days 1-3 post-challenge (Fig. 20F) and high levels of RNA gD transcript (Fig. 20G) in colonic tissues, compared to CD4 wild type AdgB-immunized animals. While it is most likely that lack of $CD4^+$ T cells resulted in this abrogation of protection in AdgB-immunized CD4 knockout animals, it was not possible to rule out the possibility that the expression of $CD4^+$ on cells other than T cells might have resulted in abrogation of protective effect of AdgB immunization. Additionally, the thymic selection of T cells in CD8 knockout mice resulted in the selection of $CD8^+$ T cells that qualitatively resemble the $CD4^+$ T cells, thus suggesting that there might be a functional compensation in CD8 knockout mice (Liu, Halbert et al. 2004). However, based on the result of the present experiments, these cells were unable to compensate for the lack of $CD4^+$ cells. This complication might have been circumvented by the use β2-microglobulin knockout mice, in which $CD8^+$ T cells cannot develop due β2-microglobulin requirement for MHC class I expression. However, these animals were not available at the time of the study. The MHC class II knockout mice used in these experiments did not display the mucosal symptoms of HSV-2 infection and succumbed to HSV-2-induced paralysis. However, MHC class II knockout mice were generated with a 129Svj background and were crossed to C57BL/6 background less than 12 breedings at the time of the experiments. Therefore, the results were more difficult to interpret as it was not possible to attribute the lack of protection in these animals to deficient MHC class II presentation. Taken together, the

results indicate that $CD4^+$ cells were vital for the protection of AdgB immunized mice from lethal *i.r.* and *i.vag.* HSV-2 challenges.

Figure 20. Protection from intrarectal and intravaginal HSV-2 challenge in CD4 and CD8 gene-deleted mice rectally immunized with AdgB. CD4 and CD8 gene deleted (knockout, KO), as well as wild-type (WT) C57BL/6 mice, were immunized with 2.5×10^9 pfu of AdgB and challenged either intrarectally (*i.r.*) or intravaginally (*i.vag.*) with 2×10^5 pfu or 1×10^4 pfu, respectively, of HSV-2 on day 30 post-immunization. In panel *A*, the legend for data in panels *B-F* is shown. Closed squares represent the data for AdgB-immunized mice, and open squares represent the data for Addl70-immunized mice. Closed circles represent the data for CD4 knockout, AdgB immunized mice, and open circles represent the data for CD4 knockout, Addl70-immunized mice. Closed diamonds denote the data for CD8 knockout, AdgB-immunized mice, and open diamonds represent the data for CD8 knockout, Addl70-immunized mice. In panel *B*, the mean survival percentages of AdgB-immunized, wild-type, CD4 and CD8 knockout mice, from days 0 to day 14 following *i.vag.* HSV-2 challenge, are depicted for each group of 6-8 mice. In panel *C*, the mean survival percentages of AdgB-immunized, wild-type, CD4 and CD8 knockout mice, on days 0 to 14 following *i.r.* HSV-2 challenge, are shown for each group of 6-8 mice. In panel *D* and *E*, the mean pathology scores of *i.r.* immunized mice challenged *i.vag.* or *i.r.* with HSV-2, respectively, are shown for each group of 6-8 mice. In panel *F*, viral titers (mean ± SEM) in the vaginal washings from mice on days 1-3 following *i.vag.* HSV-2 challenge are shown. In panel *G*, the histogram represents the percentage (mean ± SEM) of gD:18S mRNA levels as determine by RT-PCR in the

vaginal tissues of different AdgB- and Addl70-immunized mice challenged *i.r.* with HSV-2. Data represent the mean ± SEM of 3-4 mice *per* group and time point. The difference in gD:18S mRNA levels between AdgB-immunized and Addl70-immunized groups of wild-type and CD8 knockout mice was significant at $P < 0.05$ by Student's t test. The results are representative of two or three independent experiments.

Figure 20


A
AdgB WT
AdgB CD4 KO
AdgB CD8K0
Addl70 WT
Addl70 CD4 KO
Addl70 CD8KO
B
i.vag. HSV-2
Mean Survival %
Days Post-Challenge
C
i.r. HSV-2
Mean Survival %
Days Post-Challenge
D
i.vag. HSV-2
Mean Pathology Score
Days Post-Challenge
E
i.r. HSV-2
Mean Pathology Score
Days Post-Challenge
F
i.vag. HSV-2
HSV-2 Titre in Vaginal Wash pfu/ml
Days Post-Challenge
G
i.r. HSV-2
gD:18S mRNA
AdgB WT
AdgB CD4 KO
AdgB CD8KO
Addl70 WT
Addl70 CD4KO
Addl70 CD8KO
Days Post-Challenge

In addition to the complications associated with functional compensation, AdgB immunization studies conducted in knockout animals did not allow elucidation of the role of different cell subsets at the time of priming and challenge. Therefore, to more thoroughly establish the role of T-cell-mediated mechanisms in the mucosal HSV-2 protection, passive transfer of rat monoclonal antibody was used to deplete $CD4^+$ and $CD8^+$ subsets at the time of priming and challenge. $CD4^+$ and $CD8^+$ cell depletion before and after immunization was carried out by *i.p.* injection anti-CD4 and anti-CD8 antibodies on days -3, -1, and +1, while the depletion at challenge involved the administration of the same antibodies over a more extended period (on days -3, -1, +1, +3, +5, +7). As a control group, irrelevant rat IgG was administered *i.p.*. The effectiveness of $CD4^+$ and $CD8^+$ cell depletion at the time of immunization and challenge was evaluated by flow cytometric analyses of cells present in the splenic, LP and vaginal sites. The depletion regimens chosen resulted in complete absence of $CD4^+$ and $CD8^+$ cells 2 days after AdgB immunization (Fig. 21A) and after *i.vag.* (Fig. 21B) or *i.r.* (Fig. 21C) HSV-2 challenge, as detected by flow cytometry. The depletion of $CD4^+$ cells at the time of immunization led to increased mortality of AdgB-immunized animals in *i.vag.* and *i.r.* HSV-2 challenge models, thus indicating that priming of $CD4^+$ T cells was essential in establishing the immunity to the virus (Fig. 22B and 22C). Additionally, extensive depletion (day -3, -1, +1, +3, +5, +7) of $CD4^+$ T cells at the time of challenge also resulted in the abrogation of protection in both models (Fig. 22B and 22C). This loss in protection was reflected also in greater levels of pathology in both vaginal (Fig. 22D) and rectal (Fig. 22E) sites. Additionally, the viral titers in the vaginal washes (Fig. 22F)

and the levels gD RNA transcripts in the colonic tissues (Fig. 22G) of $CD4^+$ T cell depleted mice were increased compared to non-depleted AdgB-immunized mice. The less aggressive depletion regimen used on days -3, -1, +1 (i.e., before and after the time of challenge) resulted in the eventual recruitment of $CD4^+$ cells to the mucosal sites and some degree of protection in AdgB-immunized mice was observed (data not shown). In contrast, the depletion of $CD8^+$ subsets at either immunization or challenge did not affect the protective outcome of the *i.r.* Ad-immunization (Fig. 23B and 23C) and, consequently, did not impact upon the pathology scores (Fig. 23D and 23E), vaginal HSV-2 titers (Fig. 23F) and gD RNA transcript levels (Fig. 23G). Therefore, the depletion of $CD4^+$ cells, but not $CD8^+$ T cells, either at the time of immunization or at the time of challenge, resulted in the complete absence of protection in mice immunized *i.r.* with AdgB and challenged *i.r.* or *i.vag.* with HSV-2. These data indicted also that the recruitment to and/or expansion of $CD4^+$ cells in the vaginal tract might not be vital for the mediating HSV-2 protection at early times after challenge.

Figure 21. Efficiency of $CD4^+$ and $CD8^+$ cell depletion before and after intrarectal AdgB immunization and intrarectal and intravaginal HSV-2 challenge. Animals immunized intrarectally (*i.r.*) with 2.5×10^9 pfu of AdgB, as well as those *i.r.* immunized and challenged intravaginally (*i.vag.*) or *i.r.* with 2×10^5 pfu or 1×10^4 pfu, respectively, of HSV-2 on day 30 post-immunization, were depleted of $CD4^+$ or $CD8^+$ cells either before and after immunization (on days -3, -1, +1) or challenge (on days -3, -1, +1) by passive transfer of rat anti-mouse CD4- or CD8 antibodies or irrelevant (control) rat IgG. At 2 days after *i.r.* AdgB immunization or *i.vag.* or *i.r.* HSV-2 challenge, splenic, LP and vaginal tract cell populations were isolated and reacted with fluorescently-labelled antibodies specific for CD3, CD4 and CD8. Fluorescent cells were enumerated by flow cytometry. In panel *A*, the representative plots of $CD4^+$ and $CD8^+$ cells, gated on $CD3^+$ cells, are shown for splenic and LP cell populations isolated 2 days following *i.r.* AdgB immunization. In panel *B*, the representative plots of $CD4^+$ and $CD8^+$ cells, gated on $CD3^+$ cells, are shown for splenic, LP and vaginal tract cell populations isolated 2 days following *i.vag.* HSV-2 challenge in AdgB-immunized mice. In panel *C*, the representative plots of $CD4^+$ and $CD8^+$ cells, gated on $CD3^+$ cells, are shown for splenic and LP cell populations isolated 2 days following *i.r.* HSV-2 challenge in AdgB-immunized mice.

Figure 21

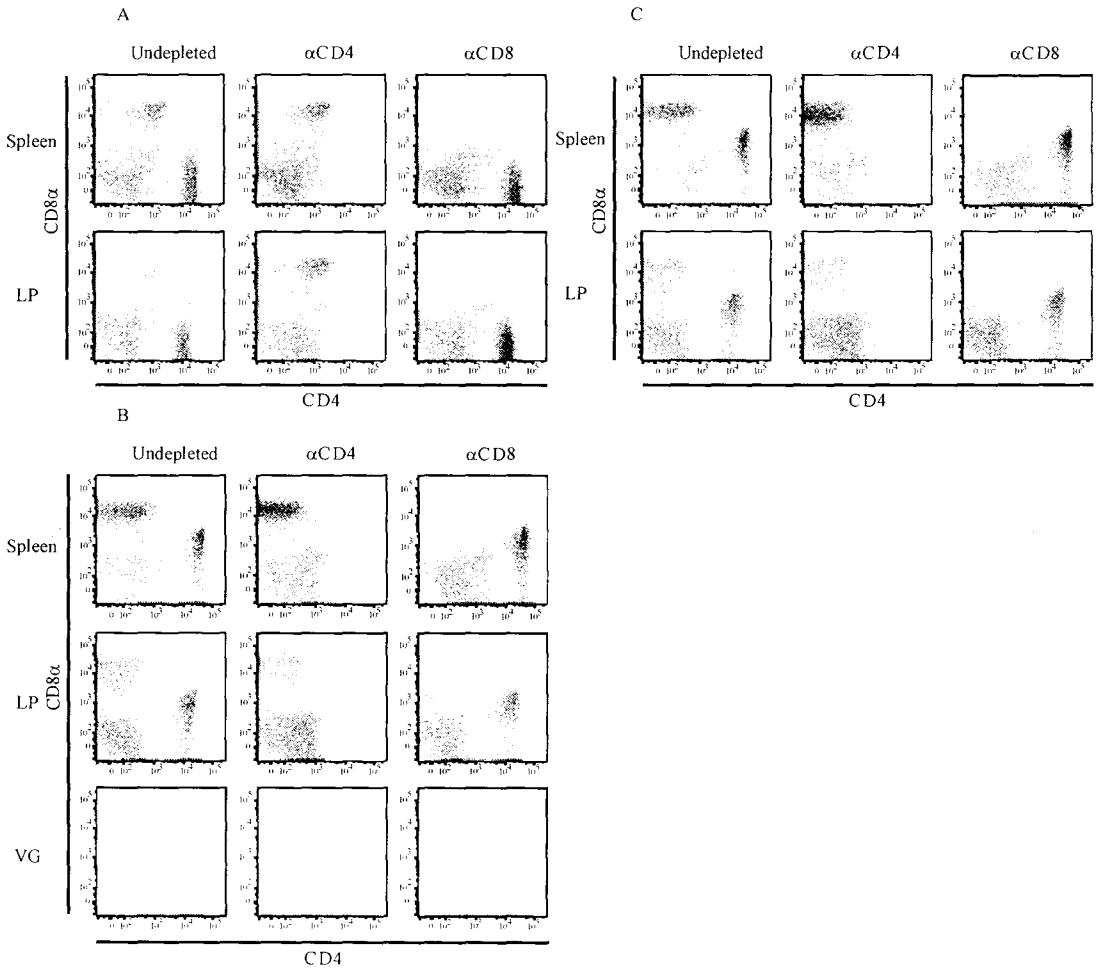

A
Undepleted
αCD4
αCD8
Spleen
LP
CD8α
CD4
B
Undepleted
αCD4
αCD8
Spleen
LP
VG
CD8α
CD4
C
Undepleted
αCD4
αCD8
Spleen
LP
CD8α
CD4

Figure 22. HSV-2 protection studies in animals depleted of $CD4^+$ cells at the time of immunization or at the time of challenge. Animals immunized intrarectally (*i.r.*) with 2.5 $\times 10^9$ pfu of AdgB and challenged *i.r.* or intravaginally (*i.vag.*) with 2×10^5 pfu or 1×10^4 pfu, respectively, of HSV-2 on day 30 post-immunization were depleted of $CD4^+$ cells either before and after the immunization (on days -3, -1, +1) or the challenge (on days -3, -1, +1, +3, +5, +7) by passive transfer of rat anti-mouse CD4 antibodies or irrelevant (control) rat IgG. In panel *A*, the legend for data in panels *B-F* is shown. Closed squares represent the data for rat IgG-treated AdgB-immunized mice, and open squares represent the data for rat IgG-treated Addl70-immunized mice. Closed circles represent the data for AdgB immunized mice depleted of $CD4^+$ cells at the time of immunization, and open circles represent the data for Addl70 immunized mice depleted of $CD4^+$ cells the time of immunization. Closed diamonds denote the data for AdgB-immunized mice depleted of $CD4^+$ cells before and after the challenge, and open diamonds represent the data Addl70-immunized mice depleted of $CD4^+$ cells before and after the challenge. In panels *B* and *C*, the mean survival percentages of CD4-depleted and control antibody-treated AdgB-immunized mice, from days 0 to day 14 following *i.vag.* or *i.r.* HSV-2 challenge, respectively, are depicted for each group of 6-8 mice. In panels *D* and *E*, the mean pathology score of *i.r.* immunized mice challenged *i.vag.* or *i.r.* with HSV-2, respectively, is shown for each group of CD4-depleted and control IgG-treated groups of 6-8 mice. In panel *F*, viral titers (mean ± SEM) in the vaginal washes of CD4-depleted and control IgG-treated mice on days 1-3 following *i.vag.* HSV-2 challenge are shown. In panel *G*, the histogram represents the percentages (mean ± SEM) of gD:18S mRNA levels as

determined by RT-PCR in the vaginal tissues from AdgB- and Addl70-immunized mice subjected to different depletion regimens and challenged *i.r.* with HSV-2. Data represent the mean ± SEM of 3-4 mice *per* group and time point. The difference in gD:18S mRNA levels between AdgB-immunized and Addl70-immunized groups of IgG-treated mice was significant at $P < 0.05$ by Student's t test. The results are representative of three independent experiments.

Figure 22

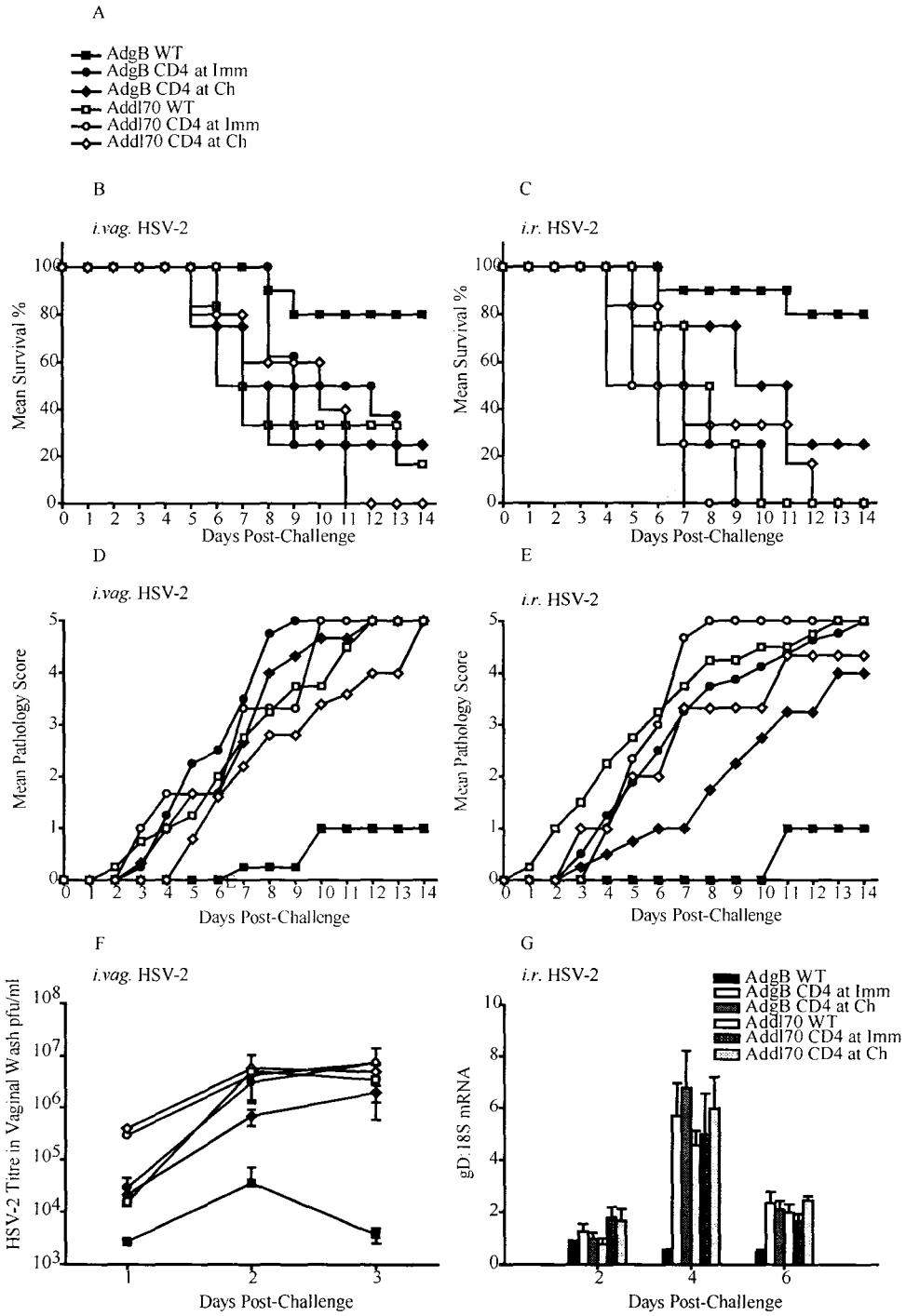
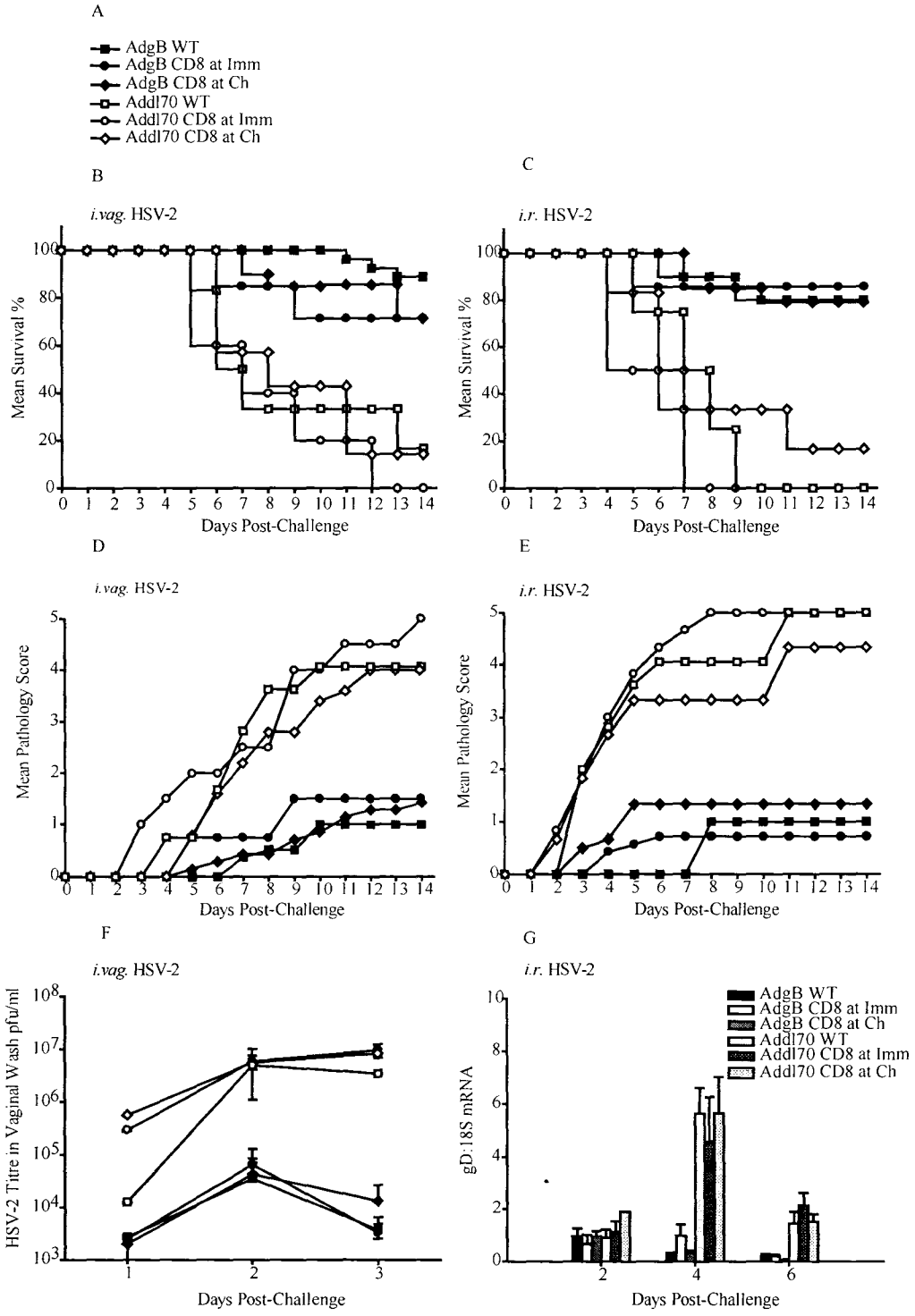

A
AdgB WT
AdgB CD4 at Imm
AdgB CD4 at Ch
Addl70 WT
Addl70 CD4 at Imm
Addl70 CD4 at Ch
B
i.vag. HSV-2
Mean Survival %
Days Post-Challenge
C
i.r. HSV-2
Mean Survival %
Days Post-Challenge
D
i.vag. HSV-2
Mean Pathology Score
Days Post-Challenge
E
i.r. HSV-2
Mean Pathology Score
Days Post-Challenge
F
i.vag. HSV-2
HSV-2 Titre in Vaginal Wash pfu/ml
Days Post-Challenge
G
i.r. HSV-2
gD:18S mRNA
Days Post-Challenge

Figure 23. HSV-2 protection studies in animals depleted of $CD8^+$ cells at the time of immunization or at the time of challenge. Animals immunized intrarectally (*i.r.*) 2.5×10^9 pfu of AdgB and challenged *i.r.* or intravaginally (*i.vag.*) with 2×10^5 pfu or 1×10^4 pfu, respectively, of HSV-2 on day 30 post-immunization were depleted of $CD8^+$ cells either before and after the time of immunization (on days -3, -1, +1) or the time of challenge (on days -3, -1, +1, +3, +5, +7) by passive transfer of rat anti-mouse CD8 antibodies or irrelevant (control) rat IgG. In panel *A*, the legend for data in panels *B-F* is shown. Closed squares represent the data for rat IgG-treated AdgB-immunized mice, and open squares represent the data for rat IgG-treated Addl70-immunized mice. Closed circles represent the data for AdgB immunized mice depleted of $CD8^+$ cells at the time of immunization, and open circles represent the data for Addl70 immunized mice depleted of $CD8^+$ cells the time of immunization. Closed diamonds denote the data for AdgB-immunized mice depleted of $CD8^+$ cells before and after the challenge, and open diamonds represent the data Addl70-immunized mice depleted of $CD8^+$ cells before and after the challenge. In panels *B* and *C*, the mean survival percentages of CD8-depleted and control antibody-treated AdgB-immunized mice, from days 0 to day 14 following *i.vag.* or *i.r.* HSV-2 challenge, respectively, are depicted for each group of 6-8 mice. In panels *D* and *E*, the mean pathology score of *i.r.* immunized mice challenged *i.vag.* or *i.r.* with HSV-2, respectively, is shown for each group of CD8-depleted and control IgG-treated groups of 6-8 mice. In panel *F*, viral titers (mean ± SEM) in the vaginal washes of CD8-depleted and control IgG-treated mice on days 1-3 following *i.vag.* HSV-2 challenge are shown. In panel *G*, the histogram represents the percentage (mean ± SEM)

of gD:18S mRNA levels as determined by RT-PCR in the vaginal tissues of AdgB- and Addl70-immunized mice subjected to different depletion regimens and challenged *i.r.* with HSV-2. Data represent the mean ± SEM of 3-4 mice *per* group and time point. The difference in gD:18S mRNA levels between AdgB-immunized and Addl70-immunized groups of mice was significant at $P < 0.05$ by Student's t test. The results are representative of three independent experiments.

Figure 23

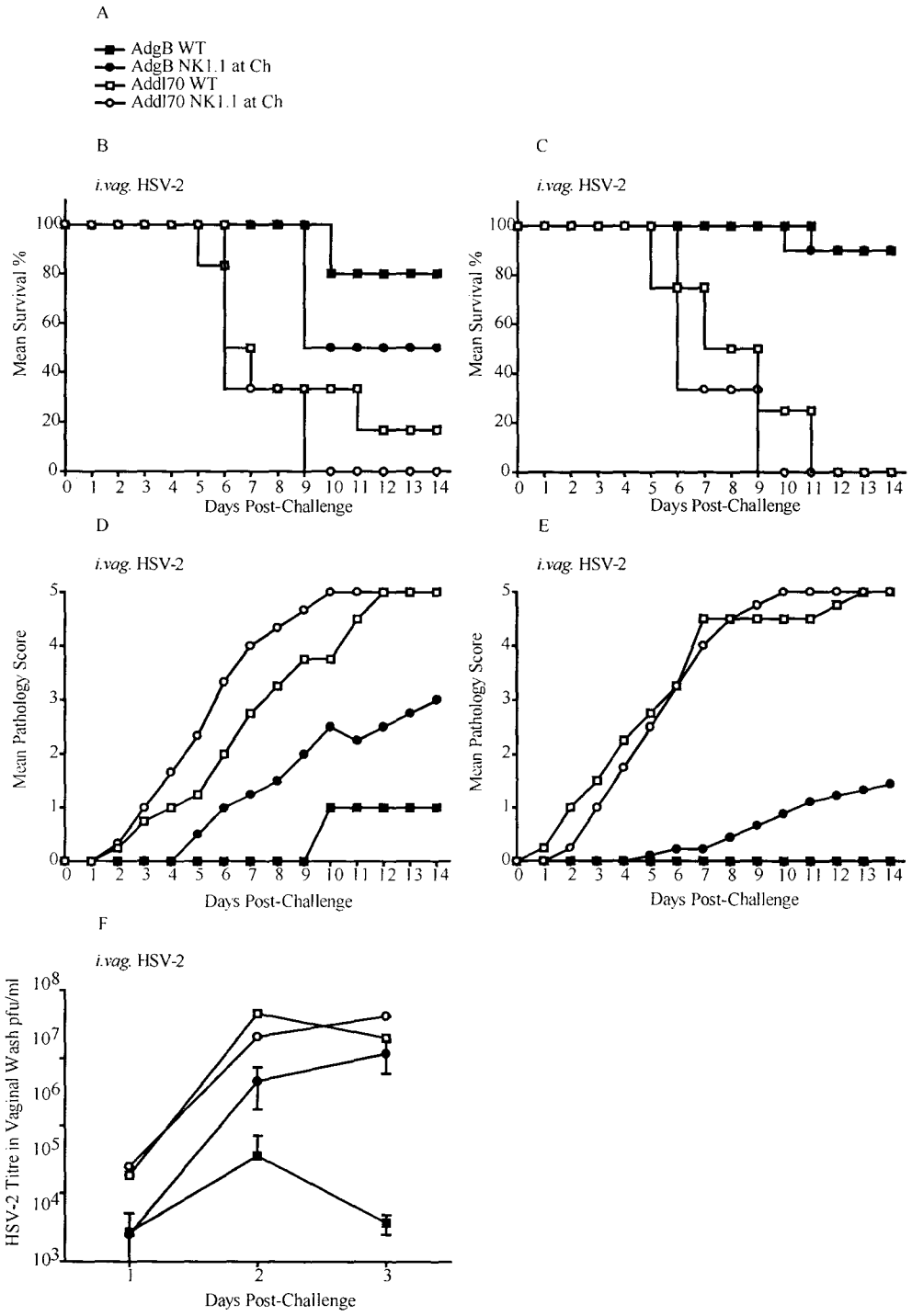

A
AdgB WT
AdgB CD8 at Imm
AdgB CD8 at Ch
Addl70 WT
Addl70 CD8 at Imm
Addl70 CD8 at Ch
B
i.vag. HSV-2
Mean Survival %
Days Post-Challenge
C
i.r. HSV-2
Mean Survival %
Days Post-Challenge
D
i.vag. HSV-2
Mean Pathology Score
Days Post-Challenge
E
i.r. HSV-2
Mean Pathology Score
Days Post-Challenge
F
i.vag. HSV-2
HSV-2 Titre in Vaginal Wash pfu/ml
Days Post-Challenge
G
i.r. HSV-2
gD:18S mRNA
AdgB WT
AdgB CD8 at Imm
AdgB CD8 at Ch
Addl70 WT
Addl70 CD8 at Imm
Addl70 CD8 at Ch
Days Post-Challenge

3.16 Role of NK cells in mediating mucosal protection from HSV-2 challenge

Some studies indicate that NK cells can provide protection from lethal vaginal HSV-2 challenge in CCR5 and IL-15 knockout mice (Kaushic, Ashkar et al. 2003; Thapa, Kuziel et al. 2007). However, the role of NK cells in fully competent C57BL/6 mice was never examined. Therefore, NK.1.1 cell depletions by *i.p.* injection of monoclonal anti-NK1.1 before and after the challenge (day -3, -1, +1, +3, +5, +7) were carried out for both rectal and vaginal HSV-2 lethality models. Control AdgB-immunized animals were administered irrelevant mouse IgG2a *i.p.* The administration of NK1.1 antibodies decreased the effectiveness of *i.r.* Ad immunization from over 90% to approximately 50% in the vaginal HSV-2 challenge model (Fig. 24B). However, NK1.1 depletion in the rectal model did not alter the protective capacity of Ad-based priming (Fig. 24C). The diminished protection observed in NK1.1-depleted, *i.vag.* challenged mice was reflected also as increased pathology scores (Fig. 24D) and vaginal HSV-2 viral titers (Fig. 24F) compared to intact animals. The pathology scores remained low in NK1.1-depleted, *i.r.* challenged mice (Figure 24E). This difference in protection might arise from site-specific difference in the function of NK cells at different mucosal surfaces. However, this difference also might be the consequence of the modulation of the innate mucosal environment by ethanol pretreatment, which masked the role of innate cells such as NK cells, in the protection of mice from rectal HSV-2 infection.

Figure 24. HSV-2 protection studies in animals depleted of NK1.1$^+$ cells at the time of immunization or at the time of challenge. Animals immunized intrarectally (*i.r.*) 2.5×10^9 pfu of AdgB and challenged *i.r.* or intravaginally (*i.vag.*) with 2×10^5 pfu or 1×10^4 pfu, respectively, of HSV-2 on day 30 post-immunization, were depleted of NK1.1 cells before and after the time of challenge (on days -3, -1, +1, +3, +5, +7) by passive transfer of mouse anti-NK1.1 antibodies or treated with irrelevant (control) mouse IgG2a. In panel *A*, the legend for data in panels *B-F* is shown. Closed squares represent the data for mouse IgG2a-treated AdgB-immunized mice, and open squares represent the data for mouse IgG2a-treated Addl70-immunized mice. Closed circles represent the data for AdgB-immunized mice depleted of NK1.1$^+$ cells at the time of challenge, and open squares represent the data for Addl70-immunized mice depleted of NK1.1$^+$ cells the time of challenge. In panels *B* and *C*, the mean survival percentages of NK1.1-depleted and control antibody-treated AdgB-immunized mice, from days 0 to day 14 following *i.vag.* or *i.r.* HSV-2 challenge, respectively, are depicted for each group of 6-8 mice. In panels *D* and *E*, the mean pathology score of *i.r.* immunized mice challenged *i.vag.* or *i.r.* with HSV-2, respectively, is shown for each group of NK1.1-depleted and control IgG-treated groups of 6-8 mice. In panel *F*, viral titers (mean ± SEM) in the vaginal washes of NK1.1-depleted and control IgG-treated mice on days 1-3 following *i.vag.* HSV-2 challenge. The results are representative of three independent experiments.

Figure 24


A
AdgB WT
AdgB NK1.1 at Ch
Addl70 WT
Addl70 NK1.1 at Ch
B
i.vag. HSV-2
Mean Survival %
Days Post-Challenge
C
i.vag. HSV-2
Mean Survival %
Days Post-Challenge
D
i.vag. HSV-2
Mean Pathology Score
Days Post-Challenge
E
i.vag. HSV-2
Mean Pathology Score
Days Post-Challenge
F
i.vag. HSV-2
HSV-2 Titre in Vaginal Wash pfu/ml
Days Post-Challenge

CHAPTER 4 DISCUSSION

4.1 Summary of the study

It has long been established that specific immunologic T cell responses in mucosal tissues are critical components of protection against numerous pathogens and other potential intruders that initiate infection at mucosal surfaces. It has proven difficult to design appropriate vaccines that target the induction of specific mucosal protection. Adenovirus serotype 5 (Ad5) vectors, that are replication deficient and encode various transgenes, have proven to be highly immunogenic, inducing protective, transgene-specific, humoral and $CD8^+$ T cell responses in rodents and non-human primates, and in preclinical studies (reviewed in (Tatsis and Ertl 2004)). Thus, to further explore the utility of Ad vectors specifically for the purposes of mucosal vaccine design, the quantitative and qualitative induction of antigen-specific mucosal and systemic $CD8^+$ T-cell responses following intrarectal (*i.r.*) administration of Ad-based vectors expressing different model and viral antigens was analyzed herein. These studies provided a number of important insights. First, antigen-specific $CD8^+$ T cells induced by mucosal vaccination with adenovirus vectors displayed an effector phenotype characterized by the increased expression of CD43 and low levels of CD127 expression. Although $CD8^+$ T cells elicited in the course of colonic priming were impeded in their ability to secrete cytokines, these T cells displayed an array of *in vivo* functions that are typical of memory T cells with efficient recall and protective capacities. When compared with other systemic routes of Ad-based immunization, *i.r.* immunization primed similar quantities of colonic LP $CD8^+$ T cells comparable to the intramuscular (*i.m.*) route of vector

administration. Subcutaneous (s.*c.*) delivery of Ad-based vectors was the least effective route of priming mucosal $CD8^+$ T cell responses. Additionally, regardless of the route, Ad-based vector immunization led to protection against intravaginal (*i.vag.*) vaccinia challenge, when high enough levels of antigen-specific $CD8^+$ T cells in systemic and mucosal compartments were achieved. Importantly, colonic $CD8^+$ T cell responses induced by *i.r.* Ad primary immunization could be further enhanced by *i.r.* delivery of EboZ-pseudotyped lentiviral vectors, which on their own did not effectively prime antigen-specific $CD8^+$ T cell responses in the colonic lamina propria (LP). This heterologous vector *i.r.* prime and boost regimen led to a dramatic expansion of antigen-specific $CD8^+$ T cells in the vaginal tract and protection from an *i.vag.* vaccinia challenge. Further, *i.r.* administration of Ad vector encoding herpes simplex virus HSV-1 glycoprotein B led to protection from either intravaginal or intrarectal challenge with HSV-2. This protection appeared to be mediated by $CD4^+$ T cells in both HSV-2 challenge models and by $NK1.1^+$ cells in the *i.vag.* HSV-2 challenge model. Therefore, these studies demonstrated that *i.r.* Ad-based vaccine delivery is an effective means of priming colonic $CD4^+$ and $CD8^+$ T cell responses that can protect mice from different viral infections. In addition, these responses can be further amplified by *i.r.* delivery of heterologous EboZ-pseudotyped lentiviral vectors expressing the antigens. In conclusion, the Ad- and lentivirus-based vaccines evaluated during the course of the present studies proved to be effective mucosal vaccines that can elicit potent cellular immune responses to the delivered antigen and can protect the mice from different experimental viral infections. Although these studies are not directly applicable to humans, when used with

appropriate delivery vehicle, these vectors could one day be evaluated as mucosal vaccines in humans. Additionally, correlates of protection in murine HSV-2 infection model may not be identical to those in humans, especially since the murine model does not fully recapitulate the range of symptoms associated with HSV-2 infection in humans. The role of NK and $CD4^+$ cells in protection from HSV-2 infection in humans merits further evaluation.

4.2 Intrarectal delivery of viruses

Adenoviral-based gene transfer *via* the intestinal lumen is limited severely by numerous intestinal defense mechanisms including tight junctions, numerous antimicrobial peptides and immunoglobulins of the brush border glycocalyx and mucus (Nagler-Anderson 2001; Wirtz and Neurath 2003). However, luminal gene delivery to the mouse colon, using adenovirus vectors, has been carried out previously and led to the expression of the transgene by colonic epithelial cells (Wirtz, Galle et al. 1999; Wirtz and Neurath 2003; Vallance, Gunawan et al. 2005). Some transgene might be expressed also in the LP of the colon. In addition, low levels of gene expression were observed in the liver and spleen following *i.r.* administration of the Ad-based vectors (Wirtz, Galle et al. 1999). The highest levels of transgene-expression were observed within the first 48-72 hours after Ad immunization. However, low but detectable levels of gene expression could still be observed up to 8 days after inoculation (Wirtz, Galle et al. 1999; Vallance, Gunawan et al. 2005). Intrarectal Ad delivery method was further optimized by Zhu *et al* (Zhu, Thomson et al. 2008). This method involved pre-treatment of murine colonic

mucosa with absolute ethanol diluted in water (45% (v/v) ethanol solution). This pre-treatment has a mucolytic effect and leads to increased Ad-based gene transfer to the colonic epithelia. It has been reported previously that the *i.r.* delivery of 100 µl of aqueous 50% vol/vol ethanol solution alone did not induce detectable inflammation in different strains of mice (Fiorucci, Antonelli et al. 2002). However, *in vitro* experiments demonstrated that a 5 minute incubation with as little as 2.5% ethanol in media results in reduced viability of epithelial colorectal adenocarcinoma Caco-2 cells (Banan, Smith et al. 1998). Therefore, although 100 µl enema of aqueous 45% v/v ethanol solution delivered prior to the delivery of Ad-based vectors did not result in histopathological changes in the colon, this pre-treatment might have some immunomodulatory effect.

Many studies have demonstrated that acute and chronic alcohol consumption results in changes in inflammatory and immune responses, such as the alterations in the numbers and functionality of the lymphocytes, monocytes and neutrophils (Cook 1998). In particular, maturation, endocytic and phagocytic activity, migration and cytokine secretion patterns of these cell subsets are affected (Cook, Keiner et al. 1990; Zuiable, Wiener et al. 1992; Silvain, Patry et al. 1995). For example, chronic alcohol ingestion leads to increased leukocyte maturation, cytokine and chemokine secretion due to increases in nuclear factor κB activation (Bautista 2002). Also, a single intragastric delivery of 0.25-0.6 ml of 32% vol/vol ethanol solution decreased basal and polyinosinic-polycytidylic acid upregulated splenic natural killer (NK) cell lytic activity in mice 18 hours following ethanol administration (Collier and Pruett 2000; Du, Zhao et al. 2008). These findings suggest that *i.r.* ethanol pretreatment might have an influence on the

intensity of the immune T cell response observed following colonic vector-based priming; however, with the limitations of the antigen delivery system we employed, these effects on T cells cannot be examined separately from those induced by the delivery of vector alone.

The same methodology was then used to deliver the HSV-2 to the murine colon, which resulted in a productive infection characterized by symptoms similar to those seen following *i.vag.* HSV-2 infection, including redness and swelling and the appearance of genital ulcerations with severe redness and hair loss of genital and surrounding tissue. Previously, Phillips *et al.* observed that BALB/c mice become susceptible to rectal HSV-2 infection following topical application of nonoxynol-9, which caused epithelium destruction (Phillips and Zacharopoulos 1998). Similarly, abrasion using aluminum oxide-coated cylinder can also lead to establishment of HSV-2 infection in the rectal mucosa (Zeitlin, Hoen et al. 2001). Therefore, it appears that either insult or removal of the mucus to expose the underlying epithelium is required in order to achieve productive HSV-2 infection in the colon.

In addition, the methodology of ethanol pre-treatment was used to *i.r.* deliver EboZ-pseudotyped lentiviral vectors. Gene transfer to the colonic mucosa using EboZ-pseudotyped lentiviral vectors was only achieved following the pre-treatment with 45% v/v aqueous ethanol solution, with no detectable transgene expression to the colonic epithelium seen without this pretreatment. When VSVG-pseudotyped first-generation lentiviral vectors were delivered *i.r.* following 45% v/v aqueous ethanol enema, no transgene β-galactosidase activity was observed. However, it has been reported that

VSVG-pseudotyped third generation, self-inactivating lentiviral vectors can be used to infect colonic epithelia and LP when used at doses of virus of 100-1000 ng of p24 (corresponding to 10^7-10^8 TU of virus) following the treatment with polybrene, a substance that is known to increase the efficiency of retroviral infection (Matsumoto, Kimura et al. XXXX). While the *in vivo i.r.* delivery of VSVG-pseudotyped lentivirus vectors had not been carried out previously, the delivery of VSVG-pseudotyped vectors to the lung *via i.t.* instillation did not result in detectable gene expression (Kobinger, Weiner et al. 2001). However, it has been shown to lead to protection from mucosal challenge with *M. tuberculosis* when vectors expressing mycobacterial antigens were administered (Hashimoto, Nagata et al. 2008). VSVG-pseudotyped lentiviruses have also been delivered intratracheally following pre-treatment with lysophosphatidylcholine, a mild detergent (Kremer, Dunning et al. 2007). When used, it has been shown to lead to VSVG-mediated gene transfer to the lung and the transgene was detected for a longer time compared to the EboZ-lentivirus mediated gene transfer with the same pre-treatment. Although VSVG-pseudotyped lentivirus vectors when delivered alone did not efficiently infect mucosal epithelia, these can infect mucosal epithelia *in vitro* and *in vivo* when delivered in conjunction with detergents or other pre-treatments.

4.3 Kinetics of gene expression of antigen following vector-based gene delivery to the mucosa

The kinetics and extent of gene expression following administration of the same vector at different mucosal surfaces appears to be determined, at least in part, by the turnover

rate of the epithelial cells. For example, *i.t.* Ad-based gene delivery resulted in β-gal expression detectable by microscopic examination for up to 10-12 days later, while the rapid turn-over of epithelial cells in the gut effectively limits gene expression to 4 days if evaluated by microscopic examination and for up to 8 days, when β-gal expression was evaluated by quantitative methodology in tissue homogenates (Wirtz, Galle et al. 1999; Wirtz and Neurath 2003; Zhu, Thomson et al. 2008). Similarly, while *i.t.* EboZ-pseudotyped lentivirus gene delivery resulted in β-gal expression that was detectable between 28 and 63 days following administration, *i.r.* delivery of EboZ-pseudotyped lentivirus resulted in transgene expression detectable only at 2 weeks after priming (Kobinger, Weiner et al. 2001; Medina, Kobinger et al. 2003). The difference in the kinetics of gene expression following the mucosal Ad- and lentivirus-based gene delivery might be due to the difference in tropism of these vectors for the different epithelial cell subsets. Therefore, the kinetics and the longevity of the gene expression in the mucosal sites appeared to be determined by the rate of the epithelial turnover and the tropism of the virus for different populations of cells in the mucosal epithelium.

4.4 Difficulties associated with obtaining single cell suspensions from mucosal tissues

The deficiencies associated with the experimental procedures used in these studies to obtain single cell suspensions from mucosal tissue must be considered when evaluating the data presented in this thesis. In order to create single cell suspensions from systemic lymphoid organs or fluids, such as spleen, or lymph nodes, simple compressive rupture of the organ was sufficient to release lymphocytes in quantities for all further studies.

However, in order to obtain the single cell suspensions from the mucosal organs, extensive washing, digestion and enrichment procedures were needed. While all of these were essential to obtain the cells, these practices might have impacted the functionality or phenotype of the cells, and, thus, the outcome of the specific experiments might not be attributed solely to effects of immunization. In some cases, the purity of mucosal cell suspensions was lower as judged by flow cytometric analysis, thus, resulting in difficulties associated with the interpretation of the flow cytometric assays. This deficiency was further complicated by the use of reagents which are normally optimized for the assessment of cells isolated from discrete lymphoid organs. While the histological or immunofluorescence methods might have been appropriate to circumvent some of these problems, these methods did not allow visualizing more than 3 thus fluorescently defined phenotypes simultaneously. In conclusion, the isolation procedure of mucosal cell suspension not only might have impacted the phenotype and/or functionality of the mucosal T cell subsets analyzed, but also the actual flow cytometric staining and analysis of these cells. With these limitations in mind, flow cytometric analysis of cell suspension from lymphoid and peripheral sites was carried out.

4.5 Antigen maintenance and anti-viral $CD8^+$ T cell response

Although antigen persistence and its influence on the ensuing contraction and functionality of $CD8^+$ T cells has been extensively addressed in exploring acute and chronic LCMV infections, it is only recently that this topic has been addressed with respect to Ad delivery models. Despite different routes of Ad administration, the

common feature of these models was generation of a T-effector memory (T_{EM}) population that persists well into the memory stage of the immune response and was diminished in its capacity to produce effector cytokines (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006). These features made this population analogous to the T cell phenotype associated with chronic LCMV infection (Fuller, Khanolkar et al. 2004). Therefore, both the effector phenotype and delayed contraction of $CD8^+$ T cell response observed following *i.m.* Ad priming, were attributed to prolonged (up to 20 days) transgene expression (Yang, Millar et al. 2006). Whilst reports suggest that the length of the antigen presence influences the contraction phase, which is the case in some *L. monocytogenes* models (Porter and Harty 2006), this is not a universally described phenomenon, as the delayed onset of contraction is not observed during chronic LCMV infections (Badovinac, Porter et al. 2002; Wherry, Blattman et al. 2003; Fuller, Khanolkar et al. 2004; Wherry and Ahmed 2004). Upon colonic Ad vector delivery, the dynamic nature of the epithelial cell turn-over effectively limits the antigen exposure time to 7-8 days, a time similar to that observed in respect pathogen persistence during acute LCMV, *L. monocytogenes* and pulmonary influenza virus infections (Badovinac, Porter et al. 2002; Lawrence and Braciale 2004; Wherry and Ahmed 2004). As predicted, after colonic Ad priming, $CD8^+$ T cell contraction occurred, with ~60% of antigen-specific T cells eliminated within the first 10 days following the peak of the immune response. Since it is possible that antigen persisted in the gastric mucosa or other tissues, it remains to be determined whether the partially exhausted phenotype of the $CD8^+$ T cells following *i.r.* Ad administration is dependent upon prolonged antigen exposure, high amounts of

peripherally expressed antigen and/or their tissue environment during adenovirus vector infection.

The long-term antigen persistence in models of chronic viral infections have been associated with a prolonged presence of activated effector T cells, characterized by high levels of expression of CD43 and low levels of expression of CD62L and CD127 (Krebs, Scandella et al. 2005; Yang, Millar et al. 2006). Additionally, these effector T cells have been partially compromised in their ability to produce cytokines, such as IFN-γ, TNF-α and IL-2. While this partially exhausted phenotype was first reported for chronic LCMV infection, similar findings have then been reported for *i.v.* and *i.m.*-delivered Ad vectors (Fuller, Khanolkar et al. 2004; Krebs, Scandella et al. 2005; Yang, Millar et al. 2006; Tatsis, Fitzgerald et al. 2007). Analogous findings have been described also for $CD8^+$ T cells induced by the *i.m.* immunization with recombinant adeno-associated virus vectors, although no disparity between the magnitude of the T cell response, as measured by tetramer and intracellular cytokine staining for IFN-γ and TNF-α was observed, as was the case of *i.m.* Ad infection (Lin, Hensley et al. 2007).

Following *i.m.* delivery of Ad and adeno-associated virus vectors, antigen is present locally for at least 32 and 47 days, respectively (Yang, Millar et al. 2006). Similar, partially exhausted phenotypes have been observed in these studies following *i.r.* delivery of Ad-based vectors. While the antigen has been previously shown to be expressed for 7-8 days following the *i.r.* gene delivery, it was not possible to exclude antigen persistence at the colonic or other sites, which might lead to the observed phenotype of antigen-specific $CD8^+$ T cells induced by *i.r.* Ad immunization (Wirtz, Galle et al. 1999).

4.6 Systemic vs. peripheral antigen-specific CD8$^+$ T cells

When mucosa-associated CD8$^+$ T cells were characterized in the acute model of LCMV inoculation, CD8$^+$ T cells confined to the intestinal epithelium expressed levels of surface markers characteristic of effector cells such as low levels of CD127 well into the memory stage of the immune response and showed a diminished ability to produce effector cytokines compared to the splenic T cells (Masopust, Vezys et al. 2006). In addition, when *i.p.* and *i.n.* routes of LCMV inoculation were compared, the disparity between the IFN-γ-producing and IFN-γ-, TNF-α-co-producing antigen-specific T cells was greater in the iliac lymph nodes than in the spleen, irrespective of the route of LCMV administration (Suvas, Dech et al. 2007). Also, when lung and splenic mononuclear cells were evaluated by intracellular cytokine staining for IFN-γ-producing and IFN-γ-, TNF-α-co-producing *M. tuberculosis* epitope-specific T cells, a similar greater disparity between these populations was observed in the lungs compared to the spleen (Kohlmeier, Miller et al. 2007). In addition, the studies utilizing the adoptive transfer of Sendai virus-specific splenic CD8$^+$ T lymphocytes *i.t.* noted the increased activation of these lymphocytes in the lung environment compared to the same cells transferred *i.p.*, although the functionality of these cells was not examined (Kamath, Woodworth et al. 2006). Similar observations have been made in the present studies as antigen-specific T cell populations in both the colonic LP and IEL compartments in *i.r.* Ad-based model of infection had greater disparity, as compared to the splenic cell population, between the antigen-specific tetramer-reactive and cytokine-producing CD8$^+$ T cells. A disparity in the ability to produce effector cytokines in systemic *vs.* non-lymphoid tissues was also

apparent in peripheral organs (liver and lungs) following *i.v.* Ad administration (Krebs, Scandella et al. 2005). Therefore, T cells entering many peripheral organs are reprogrammed, perhaps to limit the immunopathological effects of activation through down-modulation of inflammatory cytokine production. In fact, reports indicate that the environment within non-lymphoid organs can influence $CD8^+$ T cell differentiation, especially those displaying the memory phenotype, and future experiments must identify those factors responsible for the phenotype modulation of tissue-resident memory T cells (Springer 1994; Belyakov, Earl et al. 2003; Kassiotis and Stockinger 2004; Masopust, Vezys et al. 2006). However, most procedures for preparation of single cell suspensions from the peripheral tissues often require enzymatic digestion and enrichment. These treatments might affect the surface phenotype and/or functional characteristics of antigen-specific $CD8^+$ T cells. Therefore, these considerations have to be taken into account when interpreting the data on the functionality of lymphocytes isolated from lymphoid vs. peripheral organs.

4.7 Studies involving adoptive transfer of transgenic $CD8^+$ T cells

To study the lineage relationship among memory T cell subsets, adoptive transfer of small numbers of transgenic T cell pools, the size of which closely resembles endogenous precursor frequencies of epitope-specific cells, have been shown to result in a population that is composed predominantly of T_{EM} after viral antigen priming (Masopust, Vezys et al. 2001). Notably, in the context of *in vivo* viral infectious challenge, the generated T_{EM} cells do not revert to a T central memory (T_{CM}) phenotype and possess a limited

proliferative capacity (Masopust, Vezys et al. 2001; Tatsis, Fitzgerald et al. 2007). Furthermore, artificially increasing the number of dendritic cells resulted in more complete conversion to the T_{EM} phenotype (Masopust, Vezys et al. 2001). Together with other studies, these results indicated that production of T_{EM} cells required stronger signaling than the production of T_{CM} cells in both the *in vivo* and *in vitro* settings (Sallusto, Lenig et al. 1999; Masopust, Vezys et al. 2001; Sallusto, Geginat et al. 2004). Therefore, mucosal Ad-based immunization created a high antigen: T cell ratio that led to the formation of a predominantly T_{EM} population (Masopust, Vezys et al. 2001).

Indeed, the present studies of adoptively transferred T cells demonstrated that only the most proliferated cells were detected in the LP and IEL compartments, thus, signifying the predominant migration of those T cells that, over the course of immunization, acquired the effector phenotype. Similarly, naïve donor T cells underwent complete proliferation upon migration into the lung tissue following influenza virus infection (Lawrence and Braciale 2004); however, proliferation is not a prerequisite for migration into the mucosa. For example, partial proliferation of gut-associated donor T cells was observed at an early stage after transfer of OT-I cells into transgenic mice expressing OVA-antigen in the terminal ileal and large intestinal epithelia (Liu and Lefrancois 2004). Furthermore, there is evidence to indicate that following an undefined period of systemic proliferation, epitope-specific T cells continue to proliferate upon arrival in non-lymphoid organs (Lawrence, Ream et al. 2005). Reports utilizing similar methodologies to the studies reported in this thesis indicated that a high precursor frequency of antigen-specific T cells resulted in a significant underestimation of the proliferative potential of

monoclonal lymphocytes due to the increased competition for access to antigen displayed on antigen-presenting cells (Foulds and Shen 2006). Despite the relatively high number of donor T cells used in the current studies, due to concerns of low yield of colonic T cell subsets and the localized nature of Ad infection, the OT-I cells underwent a considerable number of cell divisions, even at early times after immunization. Therefore, Ad vectors are capable of causing strong stimulation of $CD8^+$ T cells, and facilitate maximal differentiation of the effector T cells with possible T cell exhaustion.

When T cell receptor transgenic studies were carried in the studies reported herein, the ability to transfer a large number of transgenic $CD8^+$ T cells was considered the strength of this approach due to the ability to detect the transferred $CD8^+$ T cells both before and after the infection by flow cytometry (Harty and Badovinac 2008). However, later studies that examined the titrating numbers of adoptively transferred T cells have demonstrated that transfer of antigen-specific T cells at 1,000 – 100,000-fold higher than the frequency of endogenous naïve precursor T cells prevented the endogenous T cell response to the same epitope (Masopust, Vezys et al. 2001; Probst, Dumrese et al. 2002; Kemp, Powell et al. 2004; Harty and Badovinac 2008). In addition, these T cell transgenic $CD8^+$ T cells proliferated less, reached maximal numbers earlier and showed a decreased efficiency of the generation of memory cells compared to the endogenous population. Lastly, transgenic T cell receptor cells transferred in frequencies similar to endogenous numbers resulted in the slower upregulation of CD62L and CD127 and the slower acquisition of effector function compared to transgenic T cells transferred at abnormally high frequencies. In light of this knowledge, the kinetics of proliferation and

mucosal recruitment of OT-I transgenic T cells in these studies might not entirely reflect the behavior of endogenous naïve precursor T cells.

4.8 Size vs. *proliferative capacity of antigen-specific CD8$^+$ T cell populations*

Recent studies suggest that the size of the antigen-specific population at the time of challenge, rather than its proliferative potential, correlated with protection against vaccinia virus infection (Bachmann, Wolint et al. 2005). The results of the studies presented in this thesis support these findings, although there were limitations in the execution of this experiment associated with the viral challenge model. The local nature of *i.r.* Ad immunization and the *i.vag.* vaccinia virus challenge model used in these studies relied on the ability of the effector CD8$^+$ T cells to recruit and/or expand in the vaginal tract. Since the frequencies of proliferated CD8$^+$ T cells observed in the vaginal tract were similar between the groups that were protected and those that were not, the data suggested that the overall frequencies of antigen-specific CD8$^+$ T cell present at the time of challenge might determine the protective outcome. In general, for more compartmentalized sites, it appeared to be more advantageous to rely on resident T_{EM} cells for protection, especially since the memory T cells are limited in their ability to traffic through these sites once the primary response subsided (Poussier, Edouard et al. 1992; Klonowski, Williams et al. 2004), and few if any T_{CM} cells were found in the intestine. Indeed, the preferential accumulation of effector T cells in non-lymphoid sites was observed in both mice and humans (Masopust, Vezys et al. 2001; Reinhardt, Khoruts et al. 2001; de Bree, van Leeuwen et al. 2005; Galkina, Thatte et al. 2005), thus indicating

the potential importance of T_{EM} for protection in peripheral tissues. Alternatively, the T_{CM} population was also capable of swift re-population of numerous non-lymphoid sites upon expansion, though the rate at which this process was accomplished most likely determined the protective outcome of immunization regimens in the face of mucosal infections such as human immunodeficiency virus (HIV) (Haase 2005). In fact the lag time in the $CD8^+$ T cell response in the female reproductive tract of rhesus macaques following *i.vag.* simian immunodeficiency virus (SIV) inoculation was believed to contribute to the spreading of infection and/or $CD4^+$ T cell loss (Reynolds, Rakasz et al. 2005). The results of the present experiments suggested that although *i.r.* Ad-based immunization mainly induces $CD8^+$ T cells that display the characteristics of the effector cells, these can proliferate and disseminate to distal mucosal sites. However, the protection seen in this model was a consequence of the size of the memory pool present before the infection rather that contributed by cells present in the vaginal mucosa 5-6 days after the challenge.

4.9 Homing of $CD8^+$ T lymphocytes to the vaginal mucosa during viral infections

Lack of homing of lymphocytes to the vaginal mucosa following *i.r.* Ad-based virus inoculation has been consistently observed during the studies presented in this thesis, and homing of $CD8^+$ T cells was only caused by *i.vag.* vaccinia vector infection in this site. However, this was the consequence of the relatively local nature of the replication-deficient Ad infection, since data from a number of studies showed that infection with viruses or attenuated bacteria, which still disseminate to other sites, resulted in population

of vaginal mucosa with antigen-specific $CD8^+$ T lymphocytes irrespective of whether these agents were administered either mucosally or systemically (Suvas, Dech et al. 2007; Li, Zhang et al. 2008). Also, the quantities of antigen-specific lymphocytes appeared to be greater when the infectious agent was administered *i.vag.* For example, *i.vag.* priming with attenuated strain of *L. monocytogenes* followed by *i.vag.* boosting with Ad-based vector expressing HIV-gag resulted in the greatest frequencies of gag-specific $CD8^+$ T cells in the vaginal tract (Li, Zhang et al. 2008). The vaginal tract was also effectively seeded with antigen-specific $CD8^+$ T cells, when either one of these agents was administered vaginally. Therefore, it appears that vaginal tract can be populated by $CD8^+$ T cells by non-localized dissiminating infections or *i.vag.* delivery of more localized infectious agents.

4.10 The parenteral vs. *mucosal route of immunization in the protection against mucosal infections*

The experimental models utilizing mucosal Ad delivery were reported to be far superior to systemic Ad immunization in controlling viral and bacterial mucosal infections, such as HSV-2, vaccinia virus or *M. tuberculosis* (Gallichan and Rosenthal 1996; Belyakov, Moss et al. 1999; Bogers, Bergmeier et al. 2004; Santosuosso, McCormick et al. 2005). The results of the studies reported herein contradicted these earlier findings and suggested that both mucosal and systemic Ad-based primary immunization protected mice from mucosal vaccinia vector challenge. Two explanations can be proposed to account for this finding. When evaluating the importance of mucosal

Ad-based immunization using truly localized infections, such as HSV-2 or *M. tuberculosis*, it appeared that robust local immunity induced by Ad-based immunization was important for mediating protection. However, when evaluating the protection using *i.vag.* delivery of vaccinia virus, that has tropism for systemic sites, it is likely that this infection could also be controlled by $CD8^+$ T cells present in systemic compartments, if these are present at sufficient quantities, as this infectious challenge is not restricted to the mucosal tissue. Also, the route of the systemic Ad immunization chosen is important, as *s.c.* delivery of Ad vectors generated weaker $CD8^+$ T cell responses than the delivery of the same virus *via* the *i.m.* route. Although certain viruses and bacteria that cause disseminating infections, administered *via* parenteral routes, generate a sufficiently large T_{EM} population, it remains to be examined if this population is protective at mucosal sites (Masopust, Jiang et al. 2001; Masopust, Vezys et al. 2001; Yang, Millar et al. 2006; Hapfelmeier, Muller et al. 2008) following localized mucosal challenge. Many of these viral and bacterial immunization platforms protect the mice from *i.vag.* inoculation with vaccinia virus, but, as noted above, the protection from this challenge might also be mediated by systemic $CD8^+$ T cells (Suvas, Dech et al. 2007; Li, Zhang et al. 2008). While the results of the present investigation were aimed at examining the generation of mucosal immune responses elicited by mucosal and parenteral Ad immunization, and elucidating the ability of systemic and mucosal T cells to protect the organisms from mucosal infections, the limitations associated with the design of the experiments and with the challenge models did not allow adequate assessment of this important question. However, the results of the experiments presented here demonstrated the ability of *i.r.*

delivered Ad vectors to generate tissue-resident T_{EM}. These cells might be of considerable importance to vaccine design rationale due to the potency with which these cells are able to defend mucosal surfaces, the commonest routes of entry for many pathogens.

4.11 Characterization of VSVG-pseudotyped lentivirus-induced CD8$^+$ T cell response

While immune responses to antigens encoded in EboZ-pseudotyped lentivirus vectors have not been previously examined, the immune response to antigens expressed from VSVG-pseudotyped lentivirus vector administered systemically has been examined previously (Esslinger, Chapatte et al. 2003; Garcia Casado, Janda et al. 2008). VSVG-pseudotyped lentiviral delivery results in effective systemic priming when delivered either *s.c.*, *i.p.* or intravenously *i.v.*, with *s.c.* route inoculation being more common. The *s.c.* delivered priming seems to be dependent upon the ability of VSVG-pseudotyped lentivirus vectors to transduce skin CD8$^-$DEC205$^+$ DCs that are then responsible for eliciting T cell immunity. Intravenous delivery of VSVG-pseudotyped vectors does not appear to prime antigen-specific CD8$^+$ T cells as efficiently as *s.c.* delivery and the doses of lentivirus needed to be increased 10-fold to achieve similar levels of priming (Garcia Casado, Janda et al. 2008; Lopes, Dewannieux et al. 2008). When immune responses induced by VSVG-pseudotyped lentivirus gene delivery were compared to those induced by other virus vectors, such as vaccinia virus and Ad vectors, the *s.c.* route was most commonly compared (Zuiable, Wiener et al. 1992). As such, *s.c.* lentiviral based immunization appears to be superior to *s.c.* Ad immunization. However, based on the

work done previously and presented in *Section 3.7*, it was concluded that the levels of priming achieved by *s.c.* Ad immunization were much lower than those achieved by *i.m.* immunization. Following *s.c.* VSVG-pseudotyped lentivirus delivery, the antigen was maintained in the draining lymph nodes and spleen for as long as 24-30 days as examined by CFSE proliferation or less when examined by transcript RNA PCR or transgene fluorescence (Zuiable, Wiener et al. 1992). Immune responses in the systemic compartment reached a maximum between 8-14 days post immunization, in normal and T cell transgenic mice, although some reports indicated that immune responses in some mouse strains with limited T cell repertoires are maximal at 25-30 days post-immunization (Esslinger, Chapatte et al. 2003; Kimura, Koya et al. 2007; Garcia Casado, Janda et al. 2008). The kinetics in the peripheral compartments following lentiviral based immunization remains largely undefined. Similarly, conflicting data exists in regard to the phenotype of T cells induced by *s.c.* VSVG-pseudotyped lentivirus immunization in the systemic compartments. While some reports described the antigen-specific $CD8^+$ T cells as those that are characterized by low levels of expression of CD62L and CD127, when immune response is maximal, others described these cells expressing high levels of CD127 (Esslinger, Chapatte et al. 2003; Garcia Casado, Janda et al. 2008). If, indeed, long-term antigen maintenance during priming results in exhaustion of $CD8^+$ T cells, it is unlikely that, at least at the priming stage, these cells display high levels of CD127. While cytotoxicity of $CD8^+$ T cells have been evaluated, the cytokine profile of VSVG-pseudotyped lentivirus induced $CD8^+$ T cells have not been examined. Although these cells have been reported to produce IFN-γ, the proportion of antigen-specific $CD8^+$ T

cells producing IFN-γ as well as the ability of antigen-specific $CD8^+$ T cells to produce TNF-α and IL-2 was not examined (Rowe, Lopes et al. 2009). While the utility of lentiviral immunization at inducing protection in different tumour models has been examined, the ability of VSVG-pseudotyped lentivirus-based immunizations to protect from infectious disease have not been studied extensively (Chapatte, Colombetti et al. 2006). More recently, the utility of self-inactivating vectors that do not integrate into the host genome and are maintained as episomes to prime systemic immune responses has been investigated. While the transgene expression was not as long-lasting as was the case with the integrating lentivirus vectors, upon *s.c.* delivery, these generated measurable $CD8^+$ T cell responses, albeit lower than those induced by integrating vectors that protected the mice from tumor cell (EG7.OVA) and West Nile virus challenges (Iglesias, Frenkiel et al. 2006; Negri, Michelini et al. 2007; Karwacz, Mukherjee et al. 2009). These non-integrating lentivirus vectors are very promising candidate vaccines due to the low risk of perturbation of host gene expression.

Further studies will need to be conducted to evaluate the kinetics, the cytokine profile, and phenotype of effector and memory $CD8^+$ T cells induced by immunization with VSVG-pseudotyped lentivirus constructs. Due to the yield limitations of EboZ-pseudotyped lentiviral vectors and the resulting very low magnitude of $CD8^+$ T cell response induced in the course of *i.r.* EboZ-pseudotyped lentivirus immunization, the kinetics of induction of $CD8^+$ T cells, as well as their phenotypic and functional characteristics was not determined. Therefore, the route-specific kinetics of humoral and cellular immune responses to antigens encoded by lentivirus vectors pseudotyped with

EboZ or envelopes other than VSVG needs to be determined if these vectors are ever to be used in the clinical setting for the purposes of immunization or gene transfer.

4.12 Prime-boost regimens involving lentiviral vectors

While EboZ-pseudotyped lentivirus vectors have not been evaluated in prime-boost regimens, those pseudotyped with VSVG have been reported to be not very effective in homologous prime-boost protocols. Homologous prime-boost vaccination using VSVG-pseudotyped lentiviral vectors every 21-60 days resulted in frequencies of antigen-specific $CD8^+$ T cells similar to those detected in the absence of boosting and did not increase the therapeutic efficacy of primary immunization, although homologous prime-boost delivered 150 days apart resulted in increased specific lysis of target cells (Dullaers, Van Meirvenne et al. 2006; Iglesias, Frenkiel et al. 2006). Similarly, the sequentially-administered feline immunodeficiency virus-based lentivirus vector pseudotyped with the envelope glycoprotein from *Autographa californica* delivered *i.n.* did not effectively boost antibody responses to the transgene, although virus was administered at weekly intervals which might not be appropriate for boosting (Sinn, Arias et al. 2008). $CD8^+$ T cell responses have not been examined using this vector. Heterologous prime-boost regimens involving VSVG-pseudotyped lentivirus vectors have been carried out primarily using the lentivirus gene delivery to prime and vaccinia virus to boost $CD8^+$ T cell responses (Palmowski, Lopes et al. 2004; Lopes, Dewannieux et al. 2008). For example, *s.c.* delivery of 1×10^7 TU of VSVG-pseudotyped lentiviral vector followed by *i.v.* boost with 2×10^6 pfu of vaccinia virus expressing human melanoma antigen NY-ESO-1 or

ovalbumin (OVA), 3 weeks later, resulted in 52-58% of NY-ESO-1- or OVA-specific $CD8^+$ T cells in the blood on day 8 after the boost (Lopes, Dewannieux et al. 2008). The data from the experiments presented in this thesis demonstrated that *i.r.* delivered EboZ-pseudotyped lentiviral vectors have been used successfully to boost mucosal $CD8^+$ T cell responses induced by *i.r.* Ad administration. However, the magnitude of these responses in all compartments examined was below those observed in the systemic compartments of mice given systemic lentivirus prime and vaccinia virus boost. Nevertheless, this prime-boost regiment provided the enhanced protection from *i.vag.* vaccinia challenge and resulted in extensive proliferation of $CD8^+$ T cells.

4.13 Intravaginal HSV-2 mouse model

The intravaginal mouse model of HSV-2 infection is one of the best studied models of HSV-2 infection (Tatsis, Fitzgerald et al. 2007). It provides the means to evaluate the potential antiviral therapies or vaccine candidates against HSV-2 primary infection with both clinical and virological endpoints. However, this model only allows the examination of primary genital HSV-2 infection as virus in mice does not reactive from latency. In addition, those animals that developing hind-leg paralysis do not develop symptoms associated with mucosal HSV-2 infection.

A model of vaginal HSV-2 infection reminiscing human infection is found in guinea pigs (Milligan, Meador et al. 2005; Ishikawa, Widman et al. 2008). Unlike the mouse model, genital HSV-2 infection in guinea pigs more closely resembles the course of disease progression in humans as a self-limited, vesiculoulcerative disease which is

followed by healing and establishment of virus latency. Following the recovery from primary HSV-2 infection, guinea pigs experience spontaneous recurrent genital lesions as well as asymptomatic viral shedding. Therefore, candidate treatments or vaccines can be further evaluated in controlling recurrent disease. Although this model more truly reflects the pathogenesis of HSV-2 in humans, the tools that are available to study immune responses in guinea pigs are limited. In addition, both rodent HSV-2 models rely on pre-treatment of animals with Depo-Provera® to facilitate vaginal HSV-2 infection. This treatment resulted in 100-fold increase in HSV-2 susceptibility compared to mice in diestrus, and 10-fold increase in susceptibility compared to mice treated with progesterone (Kaushic, Ashkar et al. 2003). This pre-treatment also leads to alterations in immune responses, such as decreases in the local antibody levels in mice (Gillgrass, Ashkar et al. 2003). In the course of this thesis, the murine genital HSV-2 model was used to evaluate the protective efficacy of mucosally-delivered Ad-based vectors in inducing protective immune responses. This model was further adapted as *i.r.* HSV-2 infection model, which was also used in these studies and shares some of the aforementioned limitations with *i.vag.* HSV-2 model.

4.14 Immunizing regimens leading to protection from vaginal HSV-2 infection in mice

Over the years, a variety of mucosally and systemically delivered vaccination agents have been used to immunize the mice from HSV-2 infection delivered *i.vag.* These vaccination agents can be subdivided into three groups including inactivated or killed virus vaccines, subunit vaccines and live vaccines (Koelle and Corey 2008). While the

data on the utility of inactivated or killed virus vaccines is limited, subunit vaccines based on glycoprotein B and glycoprotein D were successfully used to immunize mice against *i.vag.* HSV-2 challenge (Corey, Langenberg et al. 1999; Fields, Knipe et al. 2007). In addition, it has been shown that adoptive transfer of monoclonal antibodies specific to these glycoproteins provides protection from wild type challenge (Zhu, Thomson et al. 2008). However, subunit vaccines based on these glycoproteins in conjunction with different adjuvants that have been tested in large-scale randomized, double-blind, placebo-controlled human trials either proved ineffective at reducing likelihood of disease or exhibited trends towards protective efficiency only in HSV seronegative females (Aurelian, Smith et al. 1991; Corey, Langenberg et al. 1999; Stanberry, Spruance et al. 2002; Jones and Cunningham 2004).

Vaccines based on live attenuated viruses also hold promise as they generate broader long-lasting immune responses to a variety of epitopes. These also have some disadvantages including possibility of recombination with circulating wild-type HSV strains and instability of genotype during production (Sedarati, Javier et al. 1988). So far, a strategy that involves inactivation of viral genes involved in pathogenesis has been most commonly used to attenuate viruses based on HSV-2. The intravaginal delivery of HSV-2 virus containing partial deletion of thymidine kinase gene has been used extensively to immunize mice and leads to protection from wild-type HSV-2 infection (Zhu, Thomson et al. 2008). However, in human trials, vaccines containing extensive deletions of the same gene were poorly immunogenic (Meignier and Roizman 1985; Stanberry, Cunningham et al. 2000). In mice, a vaccines based upon the HSV-2 virus in which the

PK domain of the ribonucleotide reductase large-subunit gene was deleted has been shown to induce protective $CD4^+$ T cell responses (Aurelian, Kokuba et al. 1999; Gyotoku, Ono et al. 2002). Lastly, vaccines based on other recombinant viruses, such as Ad or vaccinia virus, incorporating HSV glycoproteins has been used to immunize mice and guinea pigs from *i.vag.* HSV-2 challenge (Allen, Weir et al. 1990; Aurelian, Smith et al. 1991; Fleck, Podlech et al. 1994; Gallichan and Rosenthal 1996; Zhu, Thomson et al. 2008). These viruses are very immunogenic and also hold promise to be successful immunizing agents in humans, assuming that concerns associated with pre-existing immunity to vectors and modes of delivery are addressed.

4.15 Rectal immunization and protection from HSV-2 infection

In recent years, immunization through rectal mucosa has been reported to be effective for inducing protection from *i.vag.* and *i.r.* HSV-2 challenge. For example, *i.r.* immunization with HSV-2 conaining partial deletion of thymidine kinase gene led to protection from *i.vag.* challenge with wild-type virus (Tengvall, O'Hagan et al. 2008). Similarly, *i.r.* immunization with HSV glycoprotein D in the presence of a mucosal adjuvant, cholera toxin, has resulted also in protection from *i.vag.* HSV-2 challenge (Tengvall, O'Hagan et al. 2008). Lastly, rectal delivery of AdgB (HSV glycoprotein B) has been shown to lead to protection from *i.r.* and *i.vag.* HSV-2 challenge 2 weeks after immunization (Zhu, Thomson et al. 2008). The data obtained in the experiments presented here further extended these observations and showed that mice immunized *i.r.* with AdgB could be protected from *i.r.* or *i.vag.* HSV-2 challenge 30 days after

immunization, and although the efficiency of the immunization waned by 70 days, 60-70% of AdgB-immunized mice were protected from *i.r.* and *i.vag.* HSV-2 challenge (data not shown). AdgB administered *via* this route, at sufficiently high doses, has been shown previously to induce antigen-specific T cells in the iliac lymph node, a lymph node draining vaginal mucosa, which might be one of the reasons for the effectiveness of this route of immunization. Immunization regimens specifically targeting iliac lymph node have been shown previously to be effective in inducing SIV-specific systemic and local immune responses and in reducing viral load in macaques following vaginal challenge with SHIV89.6p (Klavinskis, Bergmeier et al. 1996; Lehner, Wang et al. 1996; Bogers, Bergmeier et al. 2004).

The efficiency of the immunization regimen administered *via* vaginal mucosa is influenced by sex hormones. As such, *i.vag.* delivery of the vaccine at the estrous stage did not result in protection from subsequent HSV-2 challenge (Gillgrass, Ashkar et al. 2003; Tengvall, O'Hagan et al. 2008). Intrarectally-delivered vaccines, on the other hand, do not appear to be as influenced by hormonal environment, and this type of immunization might also be used in male populations (139). While the Ad-based approach utilized in the present work might not be directly translatable to human studies, the application of the appropriate delivery vehicle, such as suppository, might facilitate the use of Ad-based vaccines for *i.r.* immunization. However, due to the recent failure of the stage 2 trial of polyvalent Ad-based HIV vaccine delivered *i.m.*, special consideration must be given to the potential risk of acquisition of different infections associated with

increased inflammatory milieu, which might be even more pronounced at the mucosal sites following mucosal delivery of Ad vectors.

4.16 Role of lymphocytes in mediating the protection from HSV-2 infection

While early *i.vag.* HSV-2 infections has been shown to be controlled by innate immune responses, adaptive responses appear to be critical for clearance of the viral infection (Kaushic, Ashkar et al. 2003). The role of $CD4^+$ and $CD8^+$ T lymphocytes in mediating protection from *i.vag.* HSV-2 infection has been extensively examined. Early studies elucidated that T cells are crucial for conferring protection against HSV-2 challenge and suggested that this HSV-2 clearance might be mediated by $CD8^+$ T cells (Milligan, Dudley-McClain et al. 2004). Also, $CD8^+$ T cells have been reported to control HSV-1 reactivation from neuronal ganglia (Knickelbein, Khanna et al. 2008). Since mouse models of HSV-2 infection do not allow examination of viral reactivation from latency, this function of $CD8^+$ T cells in mice might not be crucial for HSV-2 protection. Later studies identified $CD4^+$ T cells as the primary mediators of protection from *i.vag.* HSV-2 infection and in cutaneous zosteriform HSV-1 models (Manickan and Rouse 1995; Gill and Ashkar 2009). More importantly, the protective function of $CD4^+$ T cells has been closely linked with their ability to produce IFN-γ, as IFN-γ, but not TNF-α knockout mice, succumb to *i.vag.* HSV-2 challenge (Milligan, Dudley-McClain et al. 2004). In humans, enhanced IFN-γ production by $CD4^+$ T cells following stimulation with specific immediate early protein 4 of HSV-2 has been positively correlated with a low rate of disease recurrence (Braun, Payne et al. 2006). Most recently, increased

fractions of IFN-γ-producing $CD8^+$ T cells in IL-15 transgenic animals has prompted Gill *et al.* (Gill and Ashkar 2009) to postulate that the diminished fraction of IFN-γ-producing $CD4^+$ T cells might have resulted in decreased protection observed in these animals, despite increased fractions of NK cells. These researchers were able to show that CD8 knockout and mice antibody-depleted of $CD8^+$ cells were still protected from *i.vag.* HSV-2 challenge following immunization with HSV-2 containing a partial deletion of thymidine kinase gene, while CD4 knockout animals were not protected. More importantly, adoptive transfer of $CD4^+$ T cells into the RAG-1 knockout mice transferred the protection from *i.vag.* HSV-2 challenge. The data obtained in studies utilizing *i.vag.* HSV-2 challenges corroborate findings by Gill *et al.* (Gill and Ashkar 2009) by demonstrating that the presence of $CD4^+$ T cells at the time of challenge is essential for protecting the mice from *i.vag.* HSV-2 infection.

Conflicting data exist concerning the role of lymphocytes in protection from *i.r.* HSV-2 challenge. Studies by Zhu *et al.*(Zhu, Thomson et al. 2008) , reported previously that CD8 knockout mice are not protected from *i.r.* HSV-2 challenge. However, there are deficiencies associated with studies in these knockout animals, such as a lower tolerance of CD8 knockout mice to Ad infection and presence of MHC class I-expressing $CD4^+$ T cells in these animals (Liu, Halbert et al. 2004). The studies reported in this thesis utilizing antibody depletion regimens do not have these caveats. Also, data reported in the studies presented in this thesis displays a degree of similarity with the data obtained in the *i.vag.* HSV-2 challenge model. Therefore, it appears that the presence of antigen-

specific $CD4^+$ T cells in the vaginal and rectal mucosa is essential for mediating protection from *i.vag.* and *i.r.* HSV-2 challenge.

4.17 Role of NK cells in innate and adoptive protection from HSV-2

NK and NKT cells have been reported to be extremely important in mediating the protection from *i.vag.* HSV-2 challenge in naïve and immunized mice based on the data obtained through studies in IL-15 knockout mice (Kaushic, Ashkar et al. 2003). Naïve IL-15 knockout mice were unable to survive low grade *i.vag.* HSV-2 challenge that could be tolerated by intact animals. However, when innate protection was examined in mice inoculated *i.vag.* with HSV-2 containing a partial deletion in thymidine kinase gene, depletion of NK cells with anti-asialo GM1 antibodies did not affect the viral clearance (Milligan and Bernstein 1997). Also, studies in mice immunized with HSV-2 containing a partial deletion in thymidine kinase gene showed that immunization of IL-15 knockout animals did not result in protection from *i.vag.* HSV-2 challenge (Gill and Ashkar 2007). However, when T cells were transferred from these animals into IL-15-sufficient hosts, these T cells appeared functional and mediated protection from *i.vag.* HSV-2 challenge, thus suggesting that a functional innate system is essential for control of HSV-2 infection. The studies presented in this thesis corroborated the findings of Gill *et al.* (Gill and Ashkar 2009) and demonstrated that depletion of $NK1.1^+$ cells before and after the time of *i.vag.* HSV-2 challenge resulted in diminished levels of protection in these animals, compared to animals treated with control antibodies, perhaps due to the lack of the control of *i.vag.* HSV-2 infection early after immunization. Alternatively, the protection

in mice challenged *i.r.* with HSV-2 in AdgB-immunized mice did not depend on the presence of NK1.1 cells. This difference in the importance of NK-mediated protection between *i.vag.* and *i.r.* models of HSV-2 inoculation might arise from site-specific differences in the function of NK cell at these different mucosal surfaces. However, these differences might be the consequence of modulation of innate mucosal environments by ethanol pretreatment, masking the role of innate cells, such as NK cells, in the protection of mice from *i.r.* HSV-2 infection (Collier and Pruett 2000; Du, Zhao et al. 2008). This is likely, especially in respect of data demonstrating defects in the activation of systemic NK cells following ingestion of a 32% v/v aqueous ethanol. However, NK cells might also be important in the context of *i.r.* HSV-2 infection in normal hosts.

4.18 Closing remarks

The mucosal surfaces are constantly exposed to variety of food antigens, environmental and infectious agents. Therefore, the immune system at the mucosal and systemic sites plays vital role in the maintaining the balance between protecting the mucosal surfaces from the invasion by pathogens by engagement of innate and adoptive immunity, and not mounting inappropriate immune responses to innocuous antigens. The majority of infectious agents enter the body *via* mucosal surfaces. For some of these pathogens successful vaccination platforms exist that protect the host or reduce symptoms associated with the pathogen. While vaccination has been one of the greatest achievements in controlling infectious diseases, there are many pathogens for which no vaccines exist, such as HIV, or the current vaccine is not fully protective, as is the case with *Bacille*

Calmette-Guérin vaccine against tuberculosis. In addition, most vaccines currently in use are administered parenterally, and might not induce optimal mucosal responses. Also, many vaccines are efficient in inducing potent humoral responses, but might not be optimal inducers of cellular immunity. Lastly, correlates of protection for many pathogens are not known. Studies performed in this thesis were aimed at evaluating Ad- and EboZ-plseudotyped lentivirus-based vectors as potential mucosal vaccines. Although these vectors were first developed for gene therapy applications, prolonged gene expression, ease of genetic manipulation and increased immunogenicity made these viral vectors attractive candidates for vaccine development. In the course of the studies performed in this thesis, colonic delivery of Ad-vectors was also compared to more conventional systemic routes of Ad inoculation. Rectal delivery of Ad vectors alone or in combination with EboZ-pseudotyped lentiviral vectors induced potent protective mucosal and systemic cellular responses that protected mice from mucosal vaccinia and HSV-2 challenges. HSV-2 in humans infects genital and rectal mucosa, and currently no successful immunization platform exists for this virus. Despite the deficiencies associated with the delivery mode of viruses and HSV-2 models in mice, these studies aimed at testing a potential mucosal vaccine and establishing the correlates of protection might help to design a vaccine strategy in humans, although the immune responses critical for protection in humans might be different from those important for protection in mice. Lentivirus vectors pseudotyped with different glycoproteins might one day become attractive vaccine candidates. While progress already have been made, such as the improvements in safety associated with removal of viral genes and mutations in

sequences and enzyme responsible for genomic integration, further studies will have to be carried out to improve the methods associated with production of these vectors and exploit the characteristics of the immune responses induced by these vectors, especially pseudotyped with different glycoproteins, so that one day the use of these vectors could be translated to humans. In conclusion, with the appropriate means of delivery and improvements in yield, mucosal Ad- and EboZ-pseudotyped lentivirus-based vectors might become good mucosal vaccines.

REFERENCES

Agace, W. W. (2006). "Tissue-tropic effector T cells: generation and targeting opportunities." Nat Rev Immunol 6(9): 682-92.

Agace, W. W. (2008). "T-cell recruitment to the intestinal mucosa." Trends Immunol 29(11): 514-22.

Ahmed, R. and D. Gray (1996). "Immunological memory and protective immunity: understanding their relation." Science 272(5258): 54-60.

Allen, E. M., J. P. Weir, et al. (1990). "Role of coexpression of IL-2 and herpes simplex virus proteins in recombinant vaccinia virus vectors on levels of induced immunity." Viral Immunol 3(3): 207-15.

Arimoto, Y., H. Nagata, et al. (2002). "In vivo expression of adenovirus-mediated lacZ gene in murine nasal mucosa." Acta Otolaryngol 122(6): 627-33.

Artis, D. (2008). "Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut." Nat Rev Immunol 8(6): 411-20.

Aurelian, L., H. Kokuba, et al. (1999). "Vaccine potential of a herpes simplex virus type 2 mutant deleted in the PK domain of the large subunit of ribonucleotide reductase (ICP10)." Vaccine 17(15-16): 1951-63.

Aurelian, L., C. C. Smith, et al. (1991). "Immune responses to herpes simplex virus in guinea pigs (footpad model) and mice immunized with vaccinia virus recombinants containing herpes simplex virus glycoprotein D." Rev Infect Dis 13 Suppl 11: S924-34.

Babiuk, L. A. and S. K. Tikoo (2000). "Adenoviruses as vectors for delivering vaccines to mucosal surfaces." J Biotechnol 83(1-2): 105-13.

Bachmann, M. F., P. Wolint, et al. (2005). "Recall proliferation potential of memory CD8+ T cells and antiviral protection." J Immunol 175(7): 4677-85.

Badovinac, V. P., B. B. Porter, et al. (2002). "Programmed contraction of CD8(+) T cells after infection." Nat Immunol 3(7): 619-26.

Banan, A., G. S. Smith, et al. (1998). "Protection against ethanol injury by prostaglandin in a human intestinal cell line: role of microtubules." Am J Physiol 274(1 Pt 1): G111-21.

Barouch, D. H. and G. J. Nabel (2005). "Adenovirus vector-based vaccines for human immunodeficiency virus type 1." Hum Gene Ther 16(2): 149-56.

Bautista, A. P. (2002). "Chronic alcohol intoxication primes Kupffer cells and endothelial cells for enhanced CC-chemokine production and concomitantly suppresses phagocytosis and chemotaxis." Front Biosci 7: a117-25.

Beier, D. C., J. H. Cox, et al. (1994). "Association of human class I MHC alleles with the adenovirus E3/19K protein." J Immunol 152(8): 3862-72.

Belyakov, I. M., J. D. Ahlers, et al. (1998). "The importance of local mucosal HIV-specific CD8(+) cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12." J Clin Invest 102(12): 2072-81.

Belyakov, I. M., M. A. Derby, et al. (1998). "Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective

immunity in mice against intrarectal recombinant HIV-vaccinia challenge." Proc Natl Acad Sci U S A 95(4): 1709-14.

Belyakov, I. M., P. Earl, et al. (2003). "Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses." Proc Natl Acad Sci U S A 100(16): 9458-63.

Belyakov, I. M., S. A. Hammond, et al. (2004). "Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells." J Clin Invest 113(7): 998-1007.

Belyakov, I. M., B. Moss, et al. (1999). "Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity." Proc Natl Acad Sci U S A 96(8): 4512-7.

Berzofsky, J. A., J. D. Ahlers, et al. (2001). "Strategies for designing and optimizing new generation vaccines." Nat Rev Immunol 1(3): 209-19.

Betts, M. R., J. M. Brenchley, et al. (2003). "Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation." J Immunol Methods 281(1-2): 65-78.

Biron, C. A., K. S. Byron, et al. (1989). "Severe herpesvirus infections in an adolescent without natural killer cells." N Engl J Med 320(26): 1731-5.

Bogers, W. M., L. A. Bergmeier, et al. (2004). "A novel HIV-CCR5 receptor vaccine strategy in the control of mucosal SIV/HIV infection." Aids 18(1): 25-36.

Boucher, R. C. (1999). "Status of gene therapy for cystic fibrosis lung disease." J Clin Invest 103(4): 441-5.

Brandtzaeg, P., H. Kiyono, et al. (2008). "Terminology: nomenclature of mucosa-associated lymphoid tissue." Mucosal Immunol 1(1): 31-7.

Braun, R. P., L. G. Payne, et al. (2006). "Characterization of the IFN-gamma T-cell responses to immediate early antigens in humans with genital herpes." Virol J 3: 54.

Byers, A. M., C. C. Kemball, et al. (2003). "Cutting edge: rapid in vivo CTL activity by polyoma virus-specific effector and memory CD8+ T cells." J Immunol 171(1): 17-21.

Celis, J. E. (1998). Cell biology : a laboratory handbook. San Diego, Academic Press.

Cerutti, A. and M. Rescigno (2008). "The biology of intestinal immunoglobulin A responses." Immunity 28(6): 740-50.

Chan, S. Y., C. J. Empig, et al. (2001). "Folate receptor-alpha is a cofactor for cellular entry by Marburg and Ebola viruses." Cell 106(1): 117-26.

Chapatte, L., S. Colombetti, et al. (2006). "Efficient induction of tumor antigen-specific CD8+ memory T cells by recombinant lentivectors." Cancer Res 66(2): 1155-60.

Cho, B. K., C. Wang, et al. (1999). "Functional differences between memory and naive CD8 T cells." Proc Natl Acad Sci U S A 96(6): 2976-81.

Collier, S. D. and S. B. Pruett (2000). "Mechanisms of suppression of poly I:C-induced activation of NK cells by ethanol." Alcohol 21(1): 87-95.

Cook, R. T. (1998). "Alcohol abuse, alcoholism, and damage to the immune system--a review." Alcohol Clin Exp Res 22(9): 1927-42.

Cook, R. T., J. A. Keiner, et al. (1990). "Ethanol causes accelerated G1 arrest in differentiating HL-60 cells." Alcohol Clin Exp Res 14(5): 695-703.

Coombes, J. L. and F. Powrie (2008). "Dendritic cells in intestinal immune regulation." Nat Rev Immunol 8(6): 435-46.

Corey, L., A. G. Langenberg, et al. (1999). "Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. Chiron HSV Vaccine Study Group." Jama 282(4): 331-40.

Craig, S. W. and J. J. Cebra (1971). "Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit." J Exp Med 134(1): 188-200.

Critchfield, J. W., D. Lemongello, et al. (2007). "Multifunctional human immunodeficiency virus (HIV) gag-specific CD8+ T-cell responses in rectal mucosa and peripheral blood mononuclear cells during chronic HIV type 1 infection." J Virol 81(11): 5460-71.

Cronin, J., X. Y. Zhang, et al. (2005). "Altering the tropism of lentiviral vectors through pseudotyping." Curr Gene Ther 5(4): 387-98.

Dalloul, A., E. Oksenhendler, et al. (2004). "Severe herpes virus (HSV-2) infection in two patients with myelodysplasia and undetectable NK cells and plasmacytoid dendritic cells in the blood." J Clin Virol 30(4): 329-36.

Damjanovic, D., X. Zhang, et al. (2008). "Organ distribution of transgene expression following intranasal mucosal delivery of recombinant replication-defective adenovirus gene transfer vector." Genet Vaccines Ther 6: 5.

De Boer, R. J., V. V. Ganusov, et al. (2006). "Estimating lymphocyte division and death rates from CFSE data." Bull Math Biol 68(5): 1011-31.

de Bree, G. J., E. M. van Leeuwen, et al. (2005). "Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung." J Exp Med 202(10): 1433-42.

Delenda, C. (2004). "Lentiviral vectors: optimization of packaging, transduction and gene expression." J Gene Med 6 Suppl 1: S125-38.

Doi, K., K. Nibu, et al. (2005). "Adenovirus-mediated gene transfer in olfactory epithelium and olfactory bulb: a long-term study." Ann Otol Rhinol Laryngol 114(8): 629-33.

Du, L., G. Zhao, et al. (2008). "Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection." J Immunol 180(2): 948-56.

Dullaers, M., S. Van Meirvenne, et al. (2006). "Induction of effective therapeutic antitumor immunity by direct in vivo administration of lentiviral vectors." Gene Ther 13(7): 630-40.

Eberle, R., B. Tanamachi, et al. (1997). "Genetic and functional complementation of the HSV1 UL27 gene and gB glycoprotein by simian alpha-herpesvirus homologs." Arch Virol 142(4): 721-36.

Ericsson, A., M. Svensson, et al. (2004). "CCL25/CCR9 promotes the induction and function of CD103 on intestinal intraepithelial lymphocytes." Eur J Immunol 34(10): 2720-9.

Esslinger, C., L. Chapatte, et al. (2003). "In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses." J Clin Invest 111(11): 1673-81.

Ferrari, S., D. M. Geddes, et al. (2002). "Barriers to and new approaches for gene therapy and gene delivery in cystic fibrosis." Adv Drug Deliv Rev 54(11): 1373-93.

Feuerbach, D. and H. G. Burgert (1993). "Novel proteins associated with MHC class I antigens in cells expressing the adenovirus protein E3/19K." Embo J 12(8): 3153-61.

Fields, B. N., D. M. Knipe, et al. (2007). Fields' virology. Philadelphia, Wolters Kluwer Health/Lippincott Williams & Wilkins.

Fiorucci, S., E. Antonelli, et al. (2002). "NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice." Proc Natl Acad Sci U S A 99(24): 15770-5.

Fleck, M., J. Podlech, et al. (1994). "A vaccinia virus--herpes simplex virus (HSV) glycoprotein B1 recombinant or an HSV vaccine overcome the HSV type 2 induced humoral immunosuppression and protect against vaginal challenge in BALB/c mice." Med Microbiol Immunol 183(2): 87-94.

Foulds, K. E. and H. Shen (2006). "Clonal Competition Inhibits the Proliferation and Differentiation of Adoptively Transferred TCR Transgenic CD4 T Cells in Response to Infection." J Immunol 176(5): 3037-43.

Fuller, M. J., A. Khanolkar, et al. (2004). "Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections." J Immunol 172(7): 4204-14.

Galkina, E., J. Thatte, et al. (2005). "Preferential migration of effector CD8+ T cells into the interstitium of the normal lung." J Clin Invest 115(12): 3473-83.

Gallichan, W. S. and K. L. Rosenthal (1996). "Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization." J Exp Med 184(5): 1879-90.

Garcia Casado, J., J. Janda, et al. (2008). "Lentivector immunization induces tumor antigen-specific B and T cell responses in vivo." Eur J Immunol 38(7): 1867-76.

Gherardi, M. M. and M. Esteban (2005). "Recombinant poxviruses as mucosal vaccine vectors." J Gen Virol 86(Pt 11): 2925-36.

Gill, N. and A. A. Ashkar (2007). "Adaptive immune responses fail to provide protection against genital HSV-2 infection in the absence of IL-15." Eur J Immunol 37(9): 2529-38.

Gill, N. and A. A. Ashkar (2009). "Overexpression of interleukin-15 compromises CD4-dependent adaptive immune responses against herpes simplex virus 2." J Virol 83(2): 918-26.

Gillgrass, A. E., A. A. Ashkar, et al. (2003). "Prolonged exposure to progesterone prevents induction of protective mucosal responses following intravaginal immunization with attenuated herpes simplex virus type 2." J Virol 77(18): 9845-51.

Gogev, S., N. Vanderheijden, et al. (2002). "Induction of protective immunity to bovine herpesvirus type 1 in cattle by intranasal administration of replication-defective human adenovirus type 5 expressing glycoprotein gC or gD." Vaccine 20(9-10): 1451-65.

Gowans, J. L. and E. J. Knight (1964). "The Route of Re-Circulation of Lymphocytes in the Rat." Proc R Soc Lond B Biol Sci 159: 257-82.

Gyotoku, T., F. Ono, et al. (2002). "Development of HSV-specific CD4+ Th1 responses and CD8+ cytotoxic T lymphocytes with antiviral activity by vaccination with the HSV-2 mutant ICP10DeltaPK." Vaccine 20(21-22): 2796-807.

Haase, A. T. (2005). "Perils at mucosal front lines for HIV and SIV and their hosts." Nat Rev Immunol 5(10): 783-92.

Hapfelmeier, S., A. J. Muller, et al. (2008). "Microbe sampling by mucosal dendritic cells is a discrete, MyD88-independent step in DeltainvG S. Typhimurium colitis." J Exp Med 205(2): 437-50.

Harriman, G. R., M. Bogue, et al. (1999). "Targeted deletion of the IgA constant region in mice leads to IgA deficiency with alterations in expression of other Ig isotypes." J Immunol 162(5): 2521-9.

Harty, J. T. and V. P. Badovinac (2008). "Shaping and reshaping CD8+ T-cell memory." Nat Rev Immunol 8(2): 107-19.

Hashimoto, D., T. Nagata, et al. (2008). "Intratracheal administration of third-generation lentivirus vector encoding MPT51 from Mycobacterium tuberculosis induces specific CD8+ T-cell responses in the lung." Vaccine 26(40): 5095-100.

Havenga, M. J., A. A. Lemckert, et al. (2002). "Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease." J Virol 76(9): 4612-20.

Hermans, I. F., J. D. Silk, et al. (2004). "The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo." J Immunol Methods 285(1): 25-40.

Hogquist, K. A., S. C. Jameson, et al. (1994). "T cell receptor antagonist peptides induce positive selection." Cell 76(1): 17-27.

Holmgren, J. and C. Czerkinsky (2005). "Mucosal immunity and vaccines." Nat Med 11(4 Suppl): S45-53.

Horwitz, M. S., J. Tufariello, et al. (1995). "Model systems for studying the effects of adenovirus E3 genes on virulence in vivo." Curr Top Microbiol Immunol 199 (Pt 3): 195-211.

Husband, A. J. and J. L. Gowans (1978). "The origin and antigen-dependent distribution of IgA-containing cells in the intestine." J Exp Med 148(5): 1146-60.

Hutchinson, L., F. L. Graham, et al. (1993). "Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants." Virology 196(2): 514-31.

Iglesias, M. C., M. P. Frenkiel, et al. (2006). "A single immunization with a minute dose of a lentiviral vector-based vaccine is highly effective at eliciting protective humoral immunity against West Nile virus." J Gene Med 8(3): 265-74.

Ishikawa, T., D. G. Widman, et al. (2008). "Construction and evaluation of a chimeric pseudoinfectious virus vaccine to prevent Japanese encephalitis." Vaccine 26(22): 2772-81.

Jawahar, S., C. Moody, et al. (1996). "Natural Killer (NK) cell deficiency associated with an epitope-deficient Fc receptor type IIIA (CD16-II)." Clin Exp Immunol 103(3): 408-13.

Jiang, J. Q., A. Patrick, et al. (2005). "CD8+ T-cell-mediated cross-clade protection in the genital tract following intranasal immunization with inactivated human immunodeficiency virus antigen plus CpG oligodeoxynucleotides." J Virol 79(1): 393-400.

Jones, C. A. and A. L. Cunningham (2004). "Vaccination strategies to prevent genital herpes and neonatal herpes simplex virus (HSV) disease." Herpes 11(1): 12-7.

Jung, S., D. Unutmaz, et al. (2002). "In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens." Immunity 17(2): 211-20.

Kaech, S. M., E. J. Wherry, et al. (2002). "Effector and memory T-cell differentiation: implications for vaccine development." Nat Rev Immunol 2(4): 251-62.

Kaetzel, C. S. (2005). "The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces." Immunol Rev 206: 83-99.

Kamath, A., J. S. Woodworth, et al. (2006). "Antigen-specific CD8+ T cells and the development of central memory during Mycobacterium tuberculosis infection." J Immunol 177(9): 6361-9.

Karwacz, K., S. Mukherjee, et al. (2009). "Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy." J Virol 83(7): 3094-103.

Kassiotis, G. and B. Stockinger (2004). "Anatomical heterogeneity of memory CD4+ T cells due to reversible adaptation to the microenvironment." J Immunol 173(12): 7292-8.

Kaushic, C., A. A. Ashkar, et al. (2003). "Progesterone increases susceptibility and decreases immune responses to genital herpes infection." J Virol 77(8): 4558-65.

Kemp, R. A., T. J. Powell, et al. (2004). "Cutting edge: regulation of CD8+ T cell effector population size." J Immunol 173(5): 2923-7.

Kimura, T., R. C. Koya, et al. (2007). "Lentiviral vectors with CMV or MHCII promoters administered in vivo: immune reactivity versus persistence of expression." Mol Ther 15(7): 1390-9.

Kiyono, H. and S. Fukuyama (2004). "NALT- versus Peyer's-patch-mediated mucosal immunity." Nat Rev Immunol 4(9): 699-710.

Klavinskis, L. S., L. A. Bergmeier, et al. (1996). "Mucosal or targeted lymph node immunization of macaques with a particulate SIVp27 protein elicits virus-specific CTL in the genito-rectal mucosa and draining lymph nodes." J Immunol 157(6): 2521-7.

Klenerman, P., V. Cerundolo, et al. (2002). "Tracking T cells with tetramers: new tales from new tools." Nat Rev Immunol 2(4): 263-72.

Klonowski, K. D., K. J. Williams, et al. (2004). "Dynamics of blood-borne CD8 memory T cell migration in vivo." Immunity 20(5): 551-62.

Knickelbein, J. E., K. M. Khanna, et al. (2008). "Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency." Science 322(5899): 268-71.

Kobinger, G. P., D. J. Weiner, et al. (2001). "Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo." Nat Biotechnol 19(3): 225-30.

Koelle, D. M. and L. Corey (2008). "Herpes simplex: insights on pathogenesis and possible vaccines." Annu Rev Med 59: 381-95.

Kohlmeier, J. E., S. C. Miller, et al. (2007). "Cutting edge: Antigen is not required for the activation and maintenance of virus-specific memory CD8+ T cells in the lung airways." J Immunol 178(8): 4721-5.

Kolb, M., P. J. Margetts, et al. (2001). "Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin." Am J Respir Crit Care Med 163(3 Pt 1): 770-7.

Kolls, J. K., P. B. McCray, Jr., et al. (2008). "Cytokine-mediated regulation of antimicrobial proteins." Nat Rev Immunol 8(11): 829-35.

Krebs, P., E. Scandella, et al. (2005). "Rapid functional exhaustion and deletion of CTL following immunization with recombinant adenovirus." J Immunol 174(8): 4559-66.

Kremer, K. L., K. R. Dunning, et al. (2007). "Gene delivery to airway epithelial cells in vivo: a direct comparison of apical and basolateral transduction strategies using pseudotyped lentivirus vectors." J Gene Med 9(5): 362-8.

Kuznetsov, V. A., V. S. Stepanov, et al. (2004). "Assessment of the relative therapeutic effects of vaccines on virus load and immune responses in small groups at several time points: efficacy of mucosal and subcutaneous polypeptide vaccines in rhesus macaques exposed to SHIV." J Clin Virol 31 Suppl 1: S69-82.

Lamm, M. E. (1997). "Interaction of antigens and antibodies at mucosal surfaces." Annu Rev Microbiol 51: 311-40.

Lawrence, C. W. and T. J. Braciale (2004). "Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection." J Immunol 173(2): 1209-18.

Lawrence, C. W., R. M. Ream, et al. (2005). "Frequency, specificity, and sites of expansion of CD8+ T cells during primary pulmonary influenza virus infection." J Immunol 174(9): 5332-40.

Lee, M. G., M. A. Abina, et al. (1995). "The constitutive expression of the immunomodulatory gp19k protein in E1-, E3- adenoviral vectors strongly reduces the host cytotoxic T cell response against the vector." Gene Ther 2(4): 256-62.

Lee, S. H., P. M. Starkey, et al. (1985). "Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80." J Exp Med 161(3): 475-89.

Lehner, T., Y. Wang, et al. (1996). "Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques." Nat Med 2(7): 767-75.

Lemiale, F., W. P. Kong, et al. (2003). "Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system." J Virol 77(18): 10078-87.

Li, Z., J. Dullmann, et al. (2002). "Murine leukemia induced by retroviral gene marking." Science 296(5567): 497.

Li, Z., M. Zhang, et al. (2008). "Novel vaccination protocol with two live mucosal vectors elicits strong cell-mediated immunity in the vagina and protects against vaginal virus challenge." J Immunol 180(4): 2504-13.

Lin, S. W., S. E. Hensley, et al. (2007). "Recombinant adeno-associated virus vectors induce functionally impaired transgene product-specific CD8+ T cells in mice." J Clin Invest 117(12): 3958-70.

Little, M. C., L. V. Bell, et al. (2005). "The characterization of intraepithelial lymphocytes, lamina propria leukocytes, and isolated lymphoid follicles in the large intestine of mice infected with the intestinal nematode parasite Trichuris muris." J Immunol 175(10): 6713-22.

Liu, S. L., C. L. Halbert, et al. (2004). "Jaagsiekte sheep retrovirus envelope efficiently pseudotypes human immunodeficiency virus type 1-based lentiviral vectors." J Virol 78(5): 2642-7.

Liu, Z. and L. Lefrancois (2004). "Intestinal epithelial antigen induces mucosal CD8 T cell tolerance, activation, and inflammatory response." J Immunol 173(7): 4324-30.

Looker, K. J., G. P. Garnett, et al. (2008). "An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection." Bull World Health Organ 86(10): 805-12, A.

Lopes, L., M. Dewannieux, et al. (2008). "Immunization with a lentivector that targets tumor antigen expression to dendritic cells induces potent CD8+ and CD4+ T-cell responses." J Virol 82(1): 86-95.

Mackay, C. R., W. L. Marston, et al. (1992). "Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells." Eur J Immunol 22(4): 887-95.

Macpherson, A. J., M. B. Geuking, et al. (2005). "Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria." Immunology 115(2): 153-62.

Manickan, E. and B. T. Rouse (1995). "Roles of different T-cell subsets in control of herpes simplex virus infection determined by using T-cell-deficient mouse-models." J Virol 69(12): 8178-9.

Masopust, D., J. Jiang, et al. (2001). "Direct analysis of the dynamics of the intestinal mucosa CD8 T cell response to systemic virus infection." J Immunol 166(4): 2348-56.

Masopust, D., V. Vezys, et al. (2001). "Preferential localization of effector memory cells in nonlymphoid tissue." Science 291(5512): 2413-7.

Masopust, D., V. Vezys, et al. (2006). "Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population." J Immunol 176(4): 2079-83.

Matsumoto, H., T. Kimura, et al. (2007). "Efficient Gene Transduction of Colonic Epithelial and Lamina Propria Cells by Pseudotyped Lentivirus Vectros in vitro and ex vivo." Name of conference or title of publication.

McShane, H. and A. Hill (2005). "Prime-boost immunisation strategies for tuberculosis." Microbes Infect 7(5-6): 962-7.

Medina, M. F., G. P. Kobinger, et al. (2003). "Lentiviral vectors pseudotyped with minimal filovirus envelopes increased gene transfer in murine lung." Mol Ther 8(5): 777-89.

Meignier, B. and B. Roizman (1985). "Herpes simplex virus vaccines." Antiviral Res Suppl 1: 259-65.

Mestecky, J. (2005). Mucosal immunology. Amsterdam ; Boston, Elsevier Academic Press.

Milligan, G. N. and D. I. Bernstein (1997). "Interferon-gamma enhances resolution of herpes simplex virus type 2 infection of the murine genital tract." Virology 229(1): 259-68.

Milligan, G. N., K. L. Dudley-McClain, et al. (2004). "T-cell-mediated mechanisms involved in resolution of genital herpes simplex virus type 2 (HSV-2) infection of mice." J Reprod Immunol 61(2): 115-27.

Milligan, G. N., M. G. Meador, et al. (2005). "Long-term presence of virus-specific plasma cells in sensory ganglia and spinal cord following intravaginal inoculation of herpes simplex virus type 2." J Virol 79(17): 11537-40.

Mitragotri, S. (2005). "Immunization without needles." Nat Rev Immunol 5(12): 905-16.

Monack, D. M., A. Mueller, et al. (2004). "Persistent bacterial infections: the interface of the pathogen and the host immune system." Nat Rev Microbiol 2(9): 747-65.

Monto, A. S. and S. E. Ohmit (2009). "Seasonal influenza vaccines: evolutions and future trends." Expert Rev Vaccines 8(4): 383-9.

Moro, J. R., M. Iwata, et al. (2008). "Vitamin effects on the immune system: vitamins A and D take centre stage." Nat Rev Immunol 8(9): 685-98.

Mowat, A. M. (2003). "Anatomical basis of tolerance and immunity to intestinal antigens." Nature Reviews Immunology 3(4): 331-341.

Murali-Krishna, K., J. D. Altman, et al. (1998). "Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection." Immunity 8(2): 177-87.

Nagler-Anderson, C. (2001). "Man the barrier! Strategic defences in the intestinal mucosa." Nat Rev Immunol 1(1): 59-67.

Negri, D. R., Z. Michelini, et al. (2007). "Successful immunization with a single injection of non-integrating lentiviral vector." Mol Ther 15(9): 1716-23.

Neutra, M. R. and P. A. Kozlowski (2006). "Mucosal vaccines: the promise and the challenge." Nat Rev Immunol 6(2): 148-58.

O'Riordan, C. R., A. Lachapelle, et al. (1999). "PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo." Hum Gene Ther 10(8): 1349-58.

Ophorst, O. J., S. Kostense, et al. (2004). "An adenoviral type 5 vector carrying a type 35 fiber as a vaccine vehicle: DC targeting, cross neutralization, and immunogenicity." Vaccine 22(23-24): 3035-44.

Palmowski, M. J., L. Lopes, et al. (2004). "Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response." J Immunol 172(3): 1582-7.

Pamer, E. G. (2004). "Immune responses to Listeria monocytogenes." Nat Rev Immunol 4(10): 812-23.

Perkins, S. D., L. M. O'Brien, et al. (2006). "Boosting with an adenovirus-based vaccine improves protective efficacy against Venezuelan equine encephalitis virus following DNA vaccination." Vaccine 24(17): 3440-5.

Phalipon, A. and B. Corthesy (2003). "Novel functions of the polymeric Ig receptor: well beyond transport of immunoglobulins." Trends Immunol 24(2): 55-8.

Phillips, D. M. and V. R. Zacharopoulos (1998). "Nonoxynol-9 enhances rectal infection by herpes simplex virus in mice." Contraception 57(5): 341-8.

Pierce, N. F. and J. L. Gowans (1975). "Cellular kinetics of the intestinal immune response to cholera toxoid in rats." J Exp Med 142(6): 1550-63.

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(3): 1528-36.

Poussier, P., P. Edouard, et al. (1992). "Thymus-independent development and negative selection of T cells expressing T cell receptor alpha/beta in the intestinal epithelium: evidence for distinct circulation patterns of gut- and thymus-derived T lymphocytes." J Exp Med 176(1): 187-99.

Probst, H. C., T. Dumrese, et al. (2002). "Cutting edge: competition for APC by CTLs of different specificities is not functionally important during induction of antiviral responses." J Immunol 168(11): 5387-91.

Reinhardt, R. L., A. Khoruts, et al. (2001). "Visualizing the generation of memory CD4 T cells in the whole body." Nature 410(6824): 101-5.

Reynolds, M. R., E. Rakasz, et al. (2005). "CD8+ T-lymphocyte response to major immunodominant epitopes after vaginal exposure to simian immunodeficiency virus: too late and too little." J Virol 79(14): 9228-35.

Robert-Guroff, M. (2007). "Replicating and non-replicating viral vectors for vaccine development." Curr Opin Biotechnol 18(6): 546-56.

Rojas, R. and G. Apodaca (2002). "Immunoglobulin transport across polarized epithelial cells." Nat Rev Mol Cell Biol 3(12): 944-55.

Rouse, B. T. and M. Gierynska (2001). "Immunity to herpes simplex virus: a hypothesis." Herpes 8 Suppl 1: 2A-5A.

Rowe, H. M., L. Lopes, et al. (2009). "Expression of vFLIP in a lentiviral vaccine vector activates NF-{kappa}B, matures dendritic cells, and increases CD8+ T-cell responses." J Virol 83(4): 1555-62.

Sailaja, G., H. HogenEsch, et al. (2002). "Encapsulation of recombinant adenovirus into alginate microspheres circumvents vector-specific immune response." Gene Ther 9(24): 1722-9.

Sallusto, F., J. Geginat, et al. (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annu Rev Immunol 22: 745-63.

Sallusto, F., D. Lenig, et al. (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature 401(6754): 708-12.

Sankaran, S., M. Guadalupe, et al. (2005). "Gut mucosal T cell responses and gene expression correlate with protection against disease in long-term HIV-1-infected nonprogressors." Proc Natl Acad Sci U S A 102(28): 9860-5.

Sansonetti, P. J. (2004). "War and peace at mucosal surfaces." Nat Rev Immunol 4(12): 953-64.

Santosuosso, M., S. McCormick, et al. (2007). "Mucosal luminal manipulation of T cell geography switches on protective efficacy by otherwise ineffective parenteral genetic immunization." J Immunol 178(4): 2387-95.

Santosuosso, M., S. McCormick, et al. (2005). "Adenoviral vectors for mucosal vaccination against infectious diseases." Viral Immunol 18(2): 283-91.

Santosuosso, M., S. McCormick, et al. (2006). "Intranasal boosting with an adenovirus-vectored vaccine markedly enhances protection by parenteral Mycobacterium bovis BCG immunization against pulmonary tuberculosis." Infect Immun 74(8): 4634-43.

Santosuosso, M., X. Zhang, et al. (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(12): 7986-94.

Santra, S., M. S. Seaman, et al. (2005). "Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates." J Virol 79(10): 6516-22.

Schon, M. P., A. Arya, et al. (1999). "Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice." J Immunol 162(11): 6641-9.

Schulte, R., Y. S. Suh, et al. (2009). "Mucosal prior to systemic application of recombinant adenovirus boosting is more immunogenic than systemic application twice but confers similar protection against SIV-challenge in DNA vaccine-primed macaques." Virology 383(2): 300-9.

Seaman, M. S., S. Santra, et al. (2005). "Vaccine-elicited memory cytotoxic T lymphocytes contribute to Mamu-A*01-associated control of simian/human

immunodeficiency virus 89.6P replication in rhesus monkeys." J Virol 79(8): 4580-8.

Sedarati, F., R. T. Javier, et al. (1988). "Pathogenesis of a lethal mixed infection in mice with two nonneuroinvasive herpes simplex virus strains." J Virol 62(8): 3037-9.

Seder, R. A., P. A. Darrah, et al. (2008). "T-cell quality in memory and protection: implications for vaccine design." Nat Rev Immunol 8(4): 247-58.

Shiver, J. W., T. M. Fu, et al. (2002). "Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity." Nature 415(6869): 331-5.

Silvain, C., C. Patry, et al. (1995). "Altered expression of monocyte IgA Fc receptors is associated with defective endocytosis in patients with alcoholic cirrhosis. Potential role for IFN-gamma." J Immunol 155(3): 1606-18.

Sinn, P. L., A. C. Arias, et al. (2008). "Lentivirus vector can be readministered to nasal epithelia without blocking immune responses." J Virol 82(21): 10684-92.

Sinn, P. L., E. R. Burnight, et al. (2005). "Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer." J Virol 79(20): 12818-27.

Sinn, P. L., M. A. Hickey, et al. (2003). "Lentivirus vectors pseudotyped with filoviral envelope glycoproteins transduce airway epithelia from the apical surface independently of folate receptor alpha." J Virol 77(10): 5902-10.

Slifka, M. K., F. Rodriguez, et al. (1999). "Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells." Nature 401(6748): 76-9.

Smythies, L. E., M. Sellers, et al. (2005). "Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity." J Clin Invest 115(1): 66-75.

Springer, T. A. (1994). "Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm." Cell 76(2): 301-14.

Stanberry, L. R., A. L. Cunningham, et al. (2000). "Prospects for control of herpes simplex virus disease through immunization." Clin Infect Dis 30(3): 549-66.

Stanberry, L. R., S. L. Spruance, et al. (2002). "Glycoprotein-D-adjuvant vaccine to prevent genital herpes." N Engl J Med 347(21): 1652-61.

Subramanian, R. P. and R. J. Geraghty (2007). "Herpes simplex virus type 1 mediates fusion through a hemifusion intermediate by sequential activity of glycoproteins D, H, L, and B." Proc Natl Acad Sci U S A 104(8): 2903-8.

Suvas, P. K., H. M. Dech, et al. (2007). "Systemic and mucosal infection program protective memory CD8 T cells in the vaginal mucosa." J Immunol 179(12): 8122-7.

Tatsis, N. and H. C. Ertl (2004). "Adenoviruses as vaccine vectors." Mol Ther 10(4): 616-29.

Tatsis, N., J. C. Fitzgerald, et al. (2007). "Adenoviral vectors persist in vivo and maintain activated CD8+ T cells: implications for their use as vaccines." Blood 110(6): 1916-23.

Tengvall, S., D. O'Hagan, et al. (2008). "Rectal immunization generates protective immunity in the female genital tract against herpes simplex virus type 2 infection:

relative importance of myeloid differentiation factor 88." Antiviral Res 78(3): 202-14.

Thapa, M., W. A. Kuziel, et al. (2007). "Susceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization." J Virol 81(8): 3704-13.

Torseth, J. W. and T. C. Merigan (1986). "Significance of local gamma interferon in recurrent herpes simplex infection." J Infect Dis 153(5): 979-84.

Trono, D. (2003). "Virology. Picking the right spot." Science 300(5626): 1670-1.

Vallance, B. A., M. I. Gunawan, et al. (2005). "TGF-beta1 gene transfer to the mouse colon leads to intestinal fibrosis." Am J Physiol Gastrointest Liver Physiol 289(1): G116-28.

van Lier, R. A., I. J. ten Berge, et al. (2003). "Human CD8(+) T-cell differentiation in response to viruses." Nat Rev Immunol 3(12): 931-9.

Wallace, M. E., R. Keating, et al. (1999). "The cytotoxic T-cell response to herpes simplex virus type 1 infection of C57BL/6 mice is almost entirely directed against a single immunodominant determinant." J Virol 73(9): 7619-26.

Wherry, E. J. and R. Ahmed (2004). "Memory CD8 T-cell differentiation during viral infection." J Virol 78(11): 5535-45.

Wherry, E. J., J. N. Blattman, et al. (2003). "Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment." J Virol 77(8): 4911-27.

Wherry, E. J., V. Teichgraber, et al. (2003). "Lineage relationship and protective immunity of memory CD8 T cell subsets." Nat Immunol 4(3): 225-34.

Wille-Reece, U., B. J. Flynn, et al. (2006). "Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates." J Exp Med 203(5): 1249-58.

Wirtz, S., P. R. Galle, et al. (1999). "Efficient gene delivery to the inflamed colon by local administration of recombinant adenoviruses with normal or modified fibre structure." Gut 44(6): 800-7.

Wirtz, S. and M. F. Neurath (2003). "Gene transfer approaches for the treatment of inflammatory bowel disease." Gene Ther 10(10): 854-60.

Wold, W. S., A. E. Tollefson, et al. (1995). "E3 transcription unit of adenovirus." Curr Top Microbiol Immunol 199 (Pt 1): 237-74.

Woodland, D. L. (2004). "Jump-starting the immune system: prime-boosting comes of age." Trends Immunol 25(2): 98-104.

Wool-Lewis, R. J. and P. Bates (1998). "Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines." J Virol 72(4): 3155-60.

Xiang, Z. Q., G. P. Gao, et al. (2003). "Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier." J Virol 77(20): 10780-9.

Yang, T. C., K. Dayball, et al. (2003). "Detailed analysis of the CD8+ T-cell response following adenovirus vaccination." J Virol 77(24): 13407-11.

Yang, T. C., J. Millar, 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(1): 200-10.

Yang, T. C., J. B. Millar, et al. (2007). "T-cell immunity generated by recombinant adenovirus vaccines." Expert Rev Vaccines 6(3): 347-56.

Yoshida, M., S. M. Claypool, et al. (2004). "Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells." Immunity 20(6): 769-83.

Zeitlin, L., T. E. Hoen, et al. (2001). "Tests of Buffergel for contraception and prevention of sexually transmitted diseases in animal models." Sex Transm Dis 28(7): 417-23.

Zhu, Q., C. W. Thomson, et al. (2008). "Immunization with adenovirus at the large intestinal mucosa as an effective vaccination strategy against sexually transmitted viral infection." Mucosal Immunol 1(1): 78-88.

Zuiable, A., E. Wiener, et al. (1992). "In vitro effects of ethanol on the phagocytic and microbial killing activities of normal human monocytes and monocyte-derived macrophages." Clin Lab Haematol 14(2): 137-47.